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Multidisciplinary Quality Characterization for the Development of Active and Intelligent Packaging Technologies for Muscle Foods

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Abstract

Fresh food products such as meat and fish are highly susceptible to spoilage. Despite high efforts and advances in food processing and packaging technologies, inevitable microbial activity is the primary reason for their deterioration. Spoilage of muscle foods packaged under modified atmospheres typically manifests itself as changes in the properties of the food product and the surrounding headspace, leading to consumer rejection. Food spoilage is thus a major ecological and economic concern that calls for the development of innovative packaging solutions. These solutions could extend the product's shelf life by targeting the spoilage microbiota (active packaging) or by providing with real-time information about the product's quality status (intelligent packaging). Consequently, significant improvement in food quality and decrease of food waste could be foreseen, ultimately benefitting the whole food supply chain.

This doctoral dissertation contributes to the development of active and intelligent packaging technologies for muscle foods by means of interdisciplinary quality characterization, involving aspects of packaging material development, food spoilage analysis and multivariate statistical analysis. The main purpose of the dissertation was to define the key aspects of the food quality characterization process within the aforementioned context, to develop novel methods to enhance this process and to address specific research questions about muscle food quality. The theoretical framework and current scientific knowledge is thus reviewed with a focus on the properties and spoilage of muscle foods, the use of silver and oxygen absorbers as well as intelligent packaging concepts. The experimental part of the dissertation describes the materials and methods used for assessing the quality status of meat and seafood packaged under modified atmospheres. The doctoral dissertation is based on four original manuscripts P1-P4 where an antimicrobial releasing system (P1), antimicrobial absorbing system (P2) or quality monitoring principles for an intelligent packaging system (P3-P4) were studied.

In the first manuscript (P1), preparation and antimicrobial characterization of silver-containing packaging materials is described. Silver-containing films were produced by coextrusion and liquid flame spraying. Antimicrobial efficiency of the films was examined with bioluminescence imaging as well as with traditional antimicrobial assay. Selected films were used in meat packaging and their impact on the meat microbiota was assessed with chemical, sensory analyses and microbiological analyses, including 16S rRNA sequencing.

In the second manuscript (P2), statistical analysis was used for characterizing the impact of high-O_2 (80/20/0), common anoxic (0/20/80) and enhanced anoxic (0/20/80 + O_2 absorber) atmospheres (O_2/CO_2/N_2 %) on physicochemical properties of pork sirloin.

Changes in headspace gas composition (O_2/CO_2 %), surface pH and color (CIELAB) was monitored as a function of time. Mixed ANOVA was used for determining the effects of storage time, atmosphere and blooming time on the studied variables.

In the third manuscript (P3), spoilage of Atlantic cod (*Gadus morhua*) was examined with microbiological, chemical and sensory analyses. Selected-ion flow-tube mass spectrometry was used for real-time quantification of volatile organic compounds in the package headspace throughout storage time. Cod microbiota was examined with 16S rRNA sequencing.

In the fourth manuscript (P4), multivariate statistical analyses were applied for determining potential spoilage indicators of Atlantic cod and brown shrimp (*Crangon crangon).* Evolution of volatile organic compounds over storage time was explored with hierarchical cluster analysis, principal components analysis and partial least squares regression. Consequently, partial least squares regression was used as a selective tool for identifying most potential spoilage indicators.

Results of the present dissertation provide with new insights into the food quality characterization process as well as into the spoilage of packaged muscle food products. The requirements of packaging technology development and the characteristics of food quality information were identified as the main aspects of the characterization process and their impact on the experimental setup and methodology was examined. Efficiency of antimicrobial packaging solutions was found to be highly dependent on food product properties, antimicrobial activity mechanisms and material preparation techniques. Even though nanoscale silver showed high efficiency against typical spoilage bacteria *in vitro*, they were not effective *in situ* meat. The impact of varying oxygen levels on pork properties was demonstrated, suggesting that anoxic packaging could have benefits in pork packaging. On the other hand, a systematic procedure was developed for identifying and quantifying volatile organic compounds that could be used as food spoilage indicators. Several compounds were identified as potential spoilage indicators for both Atlantic cod and brown shrimp and their critical concentration levels were defined. Overall, the present dissertation highlights the importance of a multidisciplinary approach and novel methods in food quality characterization when aiming at improving food quality, combining different aspects of microbiology, (bio)chemistry, materials science and multivariate statistical analysis.

Tiivistelmä

Tuoreet elintarvikkeet kuten liha ja kala pilaantuvat erittäin herkästi. Elintarviketuotannon ja pakkaustekniikoiden huomattavista pyrkimyksistä ja kehityksestä huolimatta näiden tuotteiden laadun heikkeneminen johtuu etenkin vääjäämättömästä mikrobitoiminnasta. Suojakaasuun pakatun lihan ja kalan pilaantuminen ilmenee tavallisesti muutoksina tuotteen ja sitä ympäröivän ilmatilan ominaisuuksissa, mikä johtaa hylkäämiseen kuluttajan taholta. Elintarvikkeiden pilaantumisen huomattava ekologinen ja taloudellinen vaikutus vaatii siten innovatiivisia pakkausratkaisuja. Näillä ratkaisuilla tuotteen säilyvyyttä voitaisiin pidentää joko kohdistamalla huomio pilaajamikrobikantaan (aktiivinen pakkaaminen) tai tuottamalla reaaliaikaista tietoa tuotteen laadusta (älykäs pakkaaminen). Näin ollen elintarvikkeiden laadussa ja jätteen määrän vähentämisessä voitaisiin saavuttaa merkittäviä parannuksia, joista hyötyy koko tuotantoketju.

Tämä väitöstyö tukee aktiivisten ja älykkäiden pakkaustekniikoiden kehitystä lihalle ja kalalle monitieteisen laatutarkastelun keinoin, sisällyttäen pakkausmateriaalikehityksen, pilaantumisanalyysin ja monimuuttuja-analyysin näkökulmia. Työn päätarkoitus oli määritellä laatutarkasteluprosessin keskeiset näkökulmat edellä mainitussa viitekehyksessä, kehittää uusia menetelmiä tämän prosessin edistämiseksi ja tarkastella erityisiä lihan ja kalan laatuun liittyviä tutkimuskysymyksiä. Työn teoreettinen viitekehys ja tämänhetkinen tietämys esitellään täten keskittyen lihan ja kalan ominaisuuksiin ja pilaantumiseen, hopean ja hapenpoistajien käyttöön sekä erilaisiin älykkäisiin pakkausratkaisuihin. Työn kokeellisessa osassa kuvataan suojakaasuun pakatun lihan ja merenelävien laadun tarkastelussa käytetyt materiaalit ja menetelmät. Väitöstyö perustuu neljään alkuperäiseen käsikirjoitukseen (P1-P4), joissa tutkittiin aineiden vapautumiseen (P1) tai absorboimiseen (P2) perustuvia antimikrobisia systeemejä tai laadun seurannan periaatteita älykästä systeemiä varten (P3-P4).

Ensimmäisessä käsikirjoituksessa (P1) on kuvattu hopeaa sisältävien pakkausmateriaalien valmistus ja antimikrobinen karakterisointi. Hopeafilmit valmistettiin koekstruusiolla ja nesteliekkiruiskutuksella. Filmien antimikrobista tehoa tarkasteltiin bioluminesenssikuvauksella ja perinteisellä menetelmällä. Valittuja filmejä käytettiin lihan pakkaamiseen ja niiden vaikutusta lihan mikrobikantaan tarkasteltiin kemiallisten, aistinvaraisten ja mikrobiologisten analyysien avulla, mukaan lukien 16S rRNA -sekvensoinnilla.

Toisessa käsikirjoituksessa (P2) tarkasteltiin tilastollisella analyysillä korkeahappisen (80/20/0), perinteisen hapettoman (0/20/80) ja parannetun hapettoman (0/20/80 + hapenpoistaja) suojakaasun vaikutusta sian ulkofileen fysikokemiallisiin ominaisuuksiin.

Suojakaasun koostumuksen (O_2/CO_2 %), pinta-pH:n ja värin (CIELAB) muutoksia tarkasteltiin ajan funktiona. Mixed ANOVA -varianssianalyysin avulla määritettiin säilytysajan, suojakaasun ja punastumisajan vaikutusta tarkasteltaviin muuttujiin.

Kolmannessa käsikirjoituksessa (P3) tutkittiin turskan (*Gadus morhua*) pilaantumista mikrobiologisin, kemiallisin ja aistinvaraisin analyysein. Selected-ion flow-tube -massaspektrometriaa käytettiin haihtuvien orgaanisten yhdisteiden pitoisuuksien reaaliaikaiseen määrittämiseen pakkauksen kaasuatmosfääristä koko säilytysaikana. Turskan mikrobikantaa tutkittiin 16S rRNA -sekvensoinnin avulla.

Neljännessä käsikirjoituksessa (P4) monimuuttuja-analyysia hyödynnettiin turskan ja hietakatkaravun (*Crangon crangon*) potentiaalisimpien pilaantumisindikaattoreiden määrittämiseksi. Haihtuvien pilaantumisyhdisteiden kehitystä tarkasteltiin hierarkkisen klusterianalyysin, pääkomponenttianalyysin ja osittaisen pienimmän neliösumman regression avulla. Jälkimmäistä vaihtoehtoa käytettiin selektiivisenä menetelmänä potentiaalisimpien pilaantumisindikaattoreiden tunnistamiseksi.

Väitöstyössä saavutetut tulokset avaavat uusia näkökulmia elintarvikkeiden laadun tarkasteluprosessiin sekä pakattujen liha- ja kalatuotteiden pilaantumiseen. Pakkausteknii-koiden kehittämisen asettamat vaatimukset ja elintarvikkeen laatua koskevan tiedon tyypilliset piirteet tunnistettiin tarkasteluprosessin päänäkökulmiksi ja niiden vaikutusta koeasetelmaan ja metodologiaan tarkasteltiin. Antimikrobisten pakkausratkaisujen tehokkuuden todettiin riippuvan merkittävästi elintarvikkeen ominaisuuksista, antimikrobisista vaikutusmekanismeista ja materiaalinvalmistusmenetelmistä. Vaikka nano-kokoluokan hopean havaittiin olevan huomattavan tehokasta tyypillisiä pilaajabakteereita vastaan *in vitro*, vaikutusta ei havaittu kontaktissa lihaan. Työssä todettiin erilaisten happipitoisuuksien vaikutus sianlihan ominaisuuksiin, ja hapettomilla olosuhteilla havaittiin olevan mahdollisia etuja sianlihan pakkaamisessa. Väitöstyössä kehitettiin myös systemaattinen menetelmä potentiaalisten pilaantumista indikoivien haihtuvien orgaanisten yhdisteiden tunnistamiseksi ja niiden pitoisuuksien määrittämiseksi. Useita yhdisteitä voitiin tunnistaa potentiaalisiksi pilaantumisindikaattoreiksi niin turskan kuin hietakatkaravunkin kohdalla ja niiden kriittiset pitoisuustasot määritettiin. Kokonaisuudessaan väitöstyö korostaa monitieteellisen lähestymistavan ja uusien menetelmien merkitystä laatutarkastelussa kun tavoitteena on elintarvikkeen laadun parantaminen, yhdistäen mikrobiologian, (bio)kemian, materiaaliopin ja monimuuttuja-analyysin eri näkökulmia.

Samenvatting

Verse voedingsproducten zoals vlees en vis zijn zeer gevoelig voor bederf. Ondanks de hoge inspanningen en vooruitgang op het gebied van voedselverwerking en verpakkingstechnologieën, is microbiële activiteit onvermijdelijk en de belangrijkste reden voor bederf. Bederf van vlees en vis verpakt onder gemodificeerde atmosfeer manifesteert zich meestal als veranderingen in de eigenschappen van het voedingsproduct en de omgevende *headspace*, wat leidt tot afwijzing door de consument. Voedselbederf vormt dus een grote ecologische en economische zorg die vraagt om de ontwikkeling van innovatieve verpakkingsoplossingen. Deze oplossingen kunnen de houdbaarheid van het product verlengen door zich te richten op de bederfmicrobiota (actieve verpakking) of door *real-time* informatie te verstrekken over de kwaliteit van het product (intelligente verpakking). Hierdoor kan een aanzienlijke verbetering van de voedselkwaliteit en een afname van voedselverspilling worden verwacht, wat uiteindelijk de hele voedselvoorzieningsketen ten goede komt.

Deze doctoraatsthesis draagt bij aan de ontwikkeling van actieve en intelligente verpakkingstechnologieën voor vlees en vis door middel van interdisciplinaire kwaliteitskarakterisatie, waarbij aspecten van de ontwikkeling van verpakkingsmateriaal, analyse van voedselbederf en multivariate statistische analyse aan bod komen. Het hoofddoel van de thesis was om de belangrijkste aspecten van het kwaliteitskarakterisatieproces te definiëren, om nieuwe methoden te ontwikkelen om dit proces te verbeteren en om specifieke onderzoeksvragen over de kwaliteit van vlees en vis te beantwoorden. Het theoretisch kader en de huidige wetenschappelijke kennis worden zo herzien met een focus op de eigenschappen en bederf van vlees en vis, het gebruik van zilver- en zuurstofabsorbeerders en intelligente verpakkingsoplossingen. Het experimentele deel van de thesis beschrijft de materialen en methoden die worden gebruikt om de kwaliteitsstatus van vlees en vis verpakt onder gemodificeerde atmosfeer te beoordelen. De thesis is gebaseerd op vier originele manuscripten (P1-P4) waarin antimicrobiële verpakkingssystemen gebaseerd op de afgifte (P1) of absorptie (P2) van stoffen of principes van kwaliteitsmonitoring voor een intelligent verpakkingssysteem (P3-P4) werd onderzocht.

In het eerste manuscript (P1) wordt de bereiding en antimicrobiële karakterisering van zilverhoudende verpakkingsmaterialen beschreven. Zilverbevattende films werden geproduceerd door coëxtrusie en *liquid flame spray*. De antimicrobiële efficiëntie van de films werd onderzocht met bioluminescentie beeldvorming evenals met conventionele antimicrobiële methodes. De geselecteerde films werden gebruikt in vleesverpakkingen en hun impact op de vleesmicrobiota werd beoordeeld met behulp van chemische, sensorische en microbiologische analyses, waaronder 16S rRNA sequenering.

In het tweede manuscript (P2), werd statistische analyse gebruikt voor het karakteriseren van de invloed van hoge O_2 (80/20/0), gemeenschappelijke anoxische (0/20/80) en versterkte anoxische (0/20/80 + O_2 absorbeerder) atmosferen (O_2/CO_2/N_2%) op de fysisch-chemische eigenschappen van varkenslende. Veranderingen in de samenstelling van de *headspace* (O_2/CO_2%), oppervlakte-pH en kleur (CIELAB) werden gevolgd als functie van de tijd. Mixed ANOVA werd gebruikt voor het bepalen van de effecten van opslagtijd, atmosfeer en bloomingtijd op de bestudeerde variabelen.

In het derde manuscript (P3) werd bederf van Atlantische kabeljauw (*Gadus morhua*) onderzocht met microbiologische, chemische en sensorische analyses. *Selected-ion flow-tube* massaspectrometrie werd gebruikt voor real-time kwantificering van vluchtige organische stoffen in de hoofdruimte van de verpakking gedurende de opslagtijd. De microbiota van kabeljauw werd onderzocht met 16S rRNA sequenering.

In het vierde manuscript (P4) werden multivariate statistische analyses toegepast voor het bepalen van de meeste potentiële bederfindicatoren van Atlantische kabeljauw en grijze garnalen (*Crangon crangon*). De evolutie van vluchtige organische stoffen gedurende de opslagtijd werd onderzocht met hiërarchische clusteranalyse, principale componentenanalyse en partiële kleinste kwadratenregressie. De laatste methode werd gebruikt als een selectieve methode om de meest potentiële indicatoren te identificiëren.

De resultaten van deze thesis bieden nieuwe inzichten in de kwaliteitskarakterisatie en het bederfproces van verpakte vlees en vis. De vereisten voor de ontwikkeling van verpakkingstechnologieën en de kenmerken van kwaliteitsinformatie werden geïdentificeerd als de belangrijkste aspecten van het karakterisatieproces en hun impact op het onderzoeksopzet en de methodologie werden onderzocht. De efficiëntie van antimicrobiële verpakkingsoplossingen bleek sterk afhankelijk te zijn van de eigenschappen van het voedselproduct, van antimicrobiële activiteitsmechanismen en materiaalbereidingstechnieken. Hoewel nanozilver *in vitro* een hoge efficiëntie vertoonde tegen typische bederfbacteriën, werd dit effect *in situ* in vlees niet waargenomen. De invloed van verschillende zuurstofniveaus op de eigenschappen van het varkensvlees werd aangetoond, wat suggereert dat anoxische verpakkingen voordelen kunnen hebben in de verpakking van varkensvlees. Anderzijds werd een systematische procedure ontwikkeld voor het identificeren en kwantificeren van vluchtige organische stoffen die kan worden gebruikt als indicator voor voedselbederf. Verschillende chemische verbindingen werden geïdentificeerd als potentiële bederfindicatoren voor zowel Atlantische kabeljauw als grijze garnalen en hun kritische concentratieniveaus werden gedefinieerd. Over het geheel genomen benadrukt de huidige thesis het belang van een multidisciplinaire aanpak en nieuwe methoden voor de kwaliteitskarakterisatie, gericht op het verbeteren van de voedselkwaliteit, waarbij verschillende aspecten van microbiologie, (bio)chemie, materiaalwetenschap en multivariate statistische analyse worden gecombineerd.

Preface

This doctoral dissertation work was carried out between 2012-2018 towards a joint PhD degree between Tampere University of Technology (TUT, Finland) and Ghent University (UGent, Belgium). The research was realized within the framework of the research projects Comeat (2742/31/2010, supported by the Finnish Funding Agency for Technology and Innovation (TEKES)) and CheckPack (IWT-SBO-130031, supported by the Flanders Innovation & Entrepreneurship (VLAIO, formerly the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT)). Personal funding from the Doctoral Programme of the President of TUT (01/09/2012-31/08/2016) and Tampereen Teknillisen Yliopiston Tukisäätiö (01/09/2016-31/12/2016) is gratefully acknowledged. I also acknowledge mobility support from a Tampere University Technology mobility grant for doctoral students and a STSM Grant from COST Action FP1104 (supported by European Cooperation in Science and Technology, COST). In addition, I wish to thank the different project partners who donated working materials for the research.

My time as a doctoral student has been full of exploration, endeavor and excitement. I have so much to be grateful for, and so many people who have made all this possible. First of all, I am most grateful to my supervisors – Prof. Jurkka Kuusipalo, Prof. dr. ir. Frank Devlieghere and Prof. dr. ir. Peter Ragaert – for all their advice and trust. Throughout the whole dissertation process, I had the freedom to follow my own way while knowing that I could always trust on their support.

As a joint doctoral student, I had the chance to improve my dissertation manuscript on the basis of excellent advice and criticism provided by my Examination Board members. I wish to thank my whole Board – Prof. Bruno De Meulenaer, Prof. Xavier Gellynck, Dr. Jenneke Heising, Prof. Matti Karp, Dr. Pauli Kuosmanen, Prof. Georgios-Ioannis Nychas, Prof. Vasilis Valdramidis and Prof. Herman Van Langenhove – for their valuable comments.

During these years, I have had the chance to work in cooperation with several academic and industrial partners whose expertise, innovativeness and dedication to research have impressed me over and over again. In particular, I wish to thank everyone involved in the Comeat and CheckPack projects and the coauthors of my publications.

As a joint doctoral student, I have also had the privilege to work in two excellent research groups. I wish to thank the whole staff of Research Group Paper Converting and Packaging Technology (TUT) and the Research Unit Food Microbiology and Food Preservation (UGent) for their help and support throughout the dissertation work. I am especially thankful to my Comeat colleagues MSc. Sanna-Maarit Auvinen and Dr. Kari Kolppo in Finland and to my CheckPack colleagues MSc. Angelos-Gerasimos Ioannidis and Dr. ir.

Mike Vanderroost in Belgium. I also wish to express my sincere thanks to the students who have worked with me during my time as a PhD student.

Last but not least, I wish to thank my family for all the support and encouragement throughout the dissertation work.

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Lotta Kuuliala

Abbreviations and remarks

$AgNO_3$	Silver nitrate
AHL	Acylated homoserine lactone
CAP	Controlled atmosphere packaging
CFU	Colony forming unit
Ch.	Chapter
CIELAB	Commission Internationale de l´Eclairage L*a*b*
CO	Carbon monoxide
CO_2	Carbon dioxide
CSI	Chemical spoilage index
EFSA	European Food Safety Authority
EPA	United States Environmental Protection Agency
ESSO	(Ephemeral) specific spoilage organism
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FMS	Full mass scan
GC-MS	Gas chromatography-mass spectrometry
GRAS	Generally recognized as safe
HCA	Hierarchical cluster analysis
H_2S	Hydrogen sulfide
IAL	Iron agar Lyngby
KMO	Kaiser-Meyer-Olkin (test)
LAB	Lactic acid bacteria
LDPE	Low density polyethylene
LFMFP	Laboratory of Food Microbiology and Food Preservation (Ghent University)
LFS	Liquid flame spraying
LSD	Least significant difference
LOQ	Limit of quantification
MA	Marine agar / Modified atmosphere
MAP	Modified atmosphere packaging
MRS	de Man-Rogosa-Sharpe agar
MIM	Multiple Ion Monitoring
mMRS	Modified MRS
m/z	Mass-to-charge ratio
N_2	Nitrogen
NIPALS	Non-linear iterative partial least squares
NPN	Non-protein nitrogen
O_2	Oxygen
OAV	Odor activity value
OT	(Human) olfactory threshold
OTU	Operational taxonomic unit

PA	Pseudomonas agar
PCA	Principal components analysis
PIQES	Preliminary Identification, Quantification, Exploration and Selection
PLA	Poly(lactic acid)
PLS	Partial least squares
PPS	Physiological peptone solution
PTR-MS	Proton transfer mass spectrometry
PVC	Polyvinyl chloride
RFID	Radio frequency identification
ROS	Reactive oxygen species
SIFT-MS	Selected-ion flow-tube mass spectrometry
SSO	Specific spoilage organism
STAA	Streptomycin thallium acetate actidione agar
TEKES	Finnish Funding Agency for Technology and Innovation
TEM	Transmission electron microscopy
TMA	Trimethylamine
TMAO	Trimethylamine oxide
TPC	Total plate count
TSB	Tryptic soy broth
TTI	Time-temperature indicator
TUT	Tampere University of Technology
TVB-N	Total volatile basic nitrogen
TVC	Total viable count
UH	University of Helsinki
UGent	Ghent University
USDA	United States Department of Agriculture
VIP	Variable importance in projection
VLAIO	Flanders Innovation & Entrepreneurship
VOC	Volatile organic compound
WHC	Water holding capacity

In this dissertation, the following in-text referencing system is used throughout the text:

When referring to multiple sentences, the reference is placed after the period of the last sentence. A comma is also placed after the reference number. [1.]

When referring to a single sentence only, the reference is placed before the period of that particular sentence [1].

List of publications

This dissertation is based on the following original publications, reproduced with kind permission from Elsevier Ltd., and one unpublished manuscript.

Publications

P1 Kuuliala, L., Pippuri, T., Hultman, J., Auvinen, S.-M., Kolppo, K., Nieminen, T., Karp, M., Björkroth, J., Kuusipalo, J. & Jääskeläinen, E. (2015). Preparation and antimicrobial characterization of silver-containing packaging materials for meat. Food Packaging and Shelf Life, 6, 53-60. DOI: https://doi.org/10.1016/j.fpsl.2015.09.004.

P3 Kuuliala, L., Al Hage, Y., Ioannidis, A.-G., Sader, M., Kerckhof, F.-M., Vanderroost, M., Boon, N., De Baets, B., De Meulenaer, B., Ragaert, P. & Devlieghere, F. (2018). Microbiological, chemical and sensory spoilage analysis of raw Atlantic cod (*Gadus morhua*) stored under modified atmospheres. Food Microbiology, 70, 232-244. DOI: https://doi.org/10.1016/j.fm.2017.10.011.

P4 Kuuliala, L., Abatih, E., Ioannidis, A.-G., Vanderroost, M., De Meulenaer, B., Ragaert, P., & Devlieghere, F. (2018). Multivariate statistical analysis for the identification of potential seafood spoilage indicators. Food Control 84, 49-60. DOI: https://doi.org/10.1016/j.foodcont.2017.07.018.

Unpublished manuscript

P2 Kuuliala, L., Ali-Löytty, S., Auvinen, S.-M., Kolppo, K. & Kuusipalo, J. Effect of high, residual and absent oxygen on the development of headspace gas concentrations, surface pH and color in pork sirloin packaged under modified atmospheres.

Author's contribution to original publications

The following description of contributions has been accepted by all coauthors.

Publications

P1 The author designed the experimental setup with the coauthors. The author prepared the packaging materials together with S.-M. Auvinen and assisting persons, carried out the bioluminescence imaging experiments under the guidance of M. Karp, selected the materials for antimicrobial assay and meat storage trials and participated in meat packaging with T. Pippuri and E. Jääskeläinen. T. Pippuri carried out the meat shelf life tests under the guidance of E. Jääskeläinen and J. Hultman carried out 16S rRNA sequencing. The author wrote the manuscript as a corresponding author, and all coauthors commented the manuscript.

P3 The author designed the experimental setup with the coauthors. The experiments were carried out by the author and Y. Al Hage, excluding 16S amplicon sequencing that was carried out by F.-M. Kerckhof (sample preparation by A.-G. Ioannidis). The author wrote the manuscript as a corresponding author, and all coauthors commented the manuscript.

P4 The original idea of the research was the work of the author. The author designed the experimental setup with the coauthors. The author carried out the data collection and statistical analyses. E. Abatih provided consultations for the statistical analyses. The author wrote the manuscript as a corresponding author, and all coauthors commented the manuscript.

Unpublished manuscript

P2 The author planned the experimental setup with S.-M. Auvinen, K. Kolppo and J. Kuusipalo. The author carried out or supervised the data collection. The author planned and carried out the statistical analyses. S. Ali-Löytty provided consultations for the statistical analyses. The author wrote the manuscript as a corresponding author, and all coauthors commented the manuscript.

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1 Introduction

Muscle foods are highly valued food products throughout the world. According to the November 2017 Food Outlook report of the Food and Agriculture Organization of the United Nations (FAO) [1], the global production of bovine, poultry, pig and ovine meat was 67.6, 116.9, 116.1 and 14.4 million tonnes in 2015 and was foreseen to yield 69.5, 118.2, 117.0 and 14.5 million tonnes by the end of the year. In case of fish and fishery products, respective sums were 169.2 million tonnes in 2015 – where 76.6 million tonnes originated from aquacultures – and 174.0 million tonnes in 2017. Most of the produced quantities are consumed in human nutrition: in 2015, personal consumption of meat and fish was 43.3 and 20.2 kg. The high demand and appreciation for muscle foods can also be seen in the global trade: in 2017, world trade of meat and fishery products has been estimated to reach 31.5 and 60.7 million tonnes. [1.]

Unfortunately, muscle foods are also highly perishable food products. According to the framework defined under the Save Food initiative [2], food loss has been defined as decrease in quantity or quality, whereas food waste is a part of food losses and refers to food that 1) has been removed from the supply chain even though it would have been suitable for consumption or 2) has become spoiled or expired. However, several alternative definitions have also been presented in the literature [3]. Food losses occur throughout the production chain, starting from slaughtering or harvesting and ending in household storage and consumption. Consequently, approximately one third of worldwide food production has been estimated to be lost each year. In industrialized countries, more than 40 % of food losses occur at the retail or consumer level while the product may still be fit for consumption. For example, more than 20 % of all produced meat and meat products are lost in Europe and the US; half of this in the consumption stage. On the other hand, even though primary losses cover 9-15 % of total fish catches, a high proportion is also lost at the consumption stage. The total global losses yield approximately 30 %, while even 50 % has been estimated to be lost in the US. [4.] At the retail level, this is primarily due to exceeding the shelf life because of products remaining unsold, inadequate quality control or improper handling [5; 6]. On the other hand, the reasons of household food waste production have been recognized to be highly complex and affected by several factors, such as the attitudes, behavior, values and knowledge of the consumers. At this level, waste production can be due to i.e. excessive or unsuccessful cooking, sensory changes or exceeding the labelled date. [7; 8.]

Shelf life of a food product can be defined as the time during which the product retains its desired and characteristic properties, nutritional value and safety. The limits of shelf life are thus defined by the microbiological safety of the product as well as its microbiological, chemical and sensory quality. [9.] Shelf life of muscle foods depends on several intrinsic and extrinsic factors, such as the initial quality, packaging materials and storage conditions [10]. Eventually, changes in the sensory properties of the food product result in unacceptable quality for human consumption and the product can thus be considered spoiled. These changes can be due to physical damage, chemical reactions or

microbiological activity. [11.] Evaluation of spoilage is thus closely linked to sensory assessment. In addition, different microbiological or chemical indices have been developed for the determination of food quality and spoilage. [12.] However, it should be noted that spoilage is subjectively evaluated by the consumer [13] and that since every food product has its characteristic properties, unacceptability is typically highly product-specific [11].

During the past decades, expanding consumption and trading have provoked growing interest in food safety and quality, nutrition and waste reduction [14]. However, the food supply chain is constantly confronted by factors and phenomena which challenge its ability to respond to these interests, such as the need for minimal processing, convenience, globalization and distribution from centralized processing [15]. Health, ethics, sustainability, variety and value for money are becoming increasingly important for consumers. Simultaneously, a constant shift towards modern retail channels like supermarkets affects the consumption patterns. [14.] Regulatory requirements and demands towards fewer preservatives further increase the complexity of the modern society and its requirements for the food industry [16]. Even though the shelf life of muscle foods can be prolonged to certain extent by monitoring and optimizing different factors that affect their quality, total control cannot be achieved. For example, microbial contamination and growth cannot be entirely prevented even with high hygienic practices. In addition to reducing shelf life, microbial growth may also pose a safety risk for the consumer [17]. These high demands set for the modern food supply chain thus call for novel packaging technologies.

Packaging has a key role in the food supply chain. All stages of the supply chain set different demands that the packaging and its design have to comply with. [18.] Packages need to contain products with different sizes and shapes, protect them against deteriorative environmental effects, promote consumer convenience and act as a marketing tool in communication with the consumer [16]. In order to inhibit or retard microbial growth and deteriorative reactions, modified atmosphere packaging (MAP) is commonly used for perishable food products [19]. Maintaining food safety and quality is of primary importance, which is why novel technologies such as active and intelligent packaging are constantly becoming more important, also in combination with MAP. Active packaging technologies are used for developing an interaction between the package and the product [18]: instead of merely creating an inert barrier against the environment, this kind of packaging has an active function in the preservation of food products [16]. On the other hand, intelligent packaging can be used for monitoring the product or its surroundings [18]. These packaging technologies have thus extended functions over traditional solutions and aim at improved safety and quality of the food product, promotion of consumer health and safety as well as reduction of food waste and packaging material consumption. Ultimately, these technologies aim at packaging solutions that are both efficient and economic, thus benefitting the whole supply chain.

In this dissertation, quality characterization is performed within the context of developing active and intelligent packaging technologies for muscle foods and is defined as a process that aims at providing information about food quality under relevant packaging and storage conditions in order to define,

evaluate or improve the intended packaging concept. The information about quality changes that take place in the food package during storage time is vital for developing novel packaging technologies and thus greatly affects their success. However, information about the features, requirements and limitations that affect the quality characterization process within this particular context is still limited in the scientific literature. Furthermore, because of the complex nature of food spoilage, quality characterization requires extensive data collection. This can be achieved with the help of storage experiments where regular spoilage analyses are carried out for food samples stored under well-defined conditions. However, this kind of data production is typically time-consuming, labor-intensive and often not directly applicable in packaging technology development. Novel methods are thus needed for facilitated, faster and targeted production of food quality information.

The purpose of this dissertation can be summarized as 1) *defining the key aspects of food quality characterization within the context of developing active and intelligent packaging technologies for muscle foods,* 2) *developing novel methods and techniques for improving data collection and analysis,* and 3) *increasing the scientific knowledge regarding specific quality characterization topics.* Simultaneously, this dissertation directly contributes to the development of active and intelligent packaging technologies by means of microbiological, chemical and sensory spoilage analyses, multivariate statistics as well as packaging material development. The dissertation is based on four original research papers (P1-P4) with the focus on three major types of active/intelligent packaging systems:

1) Development of an antimicrobial releasing system (P1)

2) Development of an antimicrobial absorbing system (P2)

3) Development of quality monitoring principles for an intelligent packaging system (P3-P4)

The dissertation consists of five main chapters (Ch.) 2-6 following the introduction (Ch. 1). In Ch. 2, a literature review focusing on the scientific background of the study is given. The detailed aims and framework of the study are defined in Ch. 3 and an overview of the main materials and methods in Ch. 4. Results of the study are presented and discussed in Ch. 5, while Ch. 6 provides a summary of the main findings and discusses their impact and perspectives. A more detailed description of the research can be found in the manuscripts P1-P4 that are provided in the end of the dissertation.

2 Literature review

Meat and seafood are highly susceptible to spoilage due to their intrinsic properties. Muscle foods typically have a high content of nutrients available for microbial consumption, high water activity (a_w) and neutral or slightly acidic pH; these properties are generally favourable for microbial growth [12]. Eventually, spoilage manifests itself as deteriorative changes in microbial growth, production of VOCs, odor, flavor, lipid oxidation, moisture content, color, structure and/or composition [20]. Microbial growth and metabolism are the most prominent causes of food spoilage and may cause slime formation, visible microbial colonies, discoloration, off-odors and off-flavors [11]. Analysis of these changes under specified packaging and storage conditions thus forms the basis of food quality characterization.

Spoilage of muscle foods is a complex process that results in unacceptable quality. Generally, quality can be defined either as functional quality that refers to the desirable properties of the product, or as conformance quality that represents the consumers' expectations [21]. However, even though consumers generally recognize discoloration, slime and off-odors as the main signs of quality deterioration, the perception of quality is subjective and depends on the assessor's background, evaluation capacity, experience and position in the food supply chain, as well as on the extent of changes in the food product [13; 21]. For this reason, food quality characterization frequently needs to address subjective questions in an objective manner.

Different quantitative and qualitative analytical methods can be used for characterizing the changes in the properties of packed food products and/or in the surrounding headspace and consequently for the determination of the quality status on their basis. However, the utilization and development of these methods and the interpretation of the obtained results are greatly complicated by the fact that several factors affect the quality of food products. Furthermore, a specific context – such as packaging technology development – limits the amount of applicable methods.

2.1 Spoilage of meat and fish

2.1.1 Intrinsic properties of meat and fish

2.1.1.1 Composition and structure

The term meat is primarily used for animal muscle tissue, even though organs such as liver and kidneys are also included among edible flesh [22]. According to the European Union (EU) definition [23], the term meat relates to mammalian and avian skeletal muscles with naturally included or adherent tissue that are suitable for human consumption. The primary components of carcass meat are muscle tissue, fat, connective tissue and bone. Muscle tissue consists of muscle fibres where

multinucleate cells are combined to long and thin structures by connective tissue and surrounded by sarcolemmas. Connective tissue primarily consists of collagen and surrounds muscle fibres (endomysium), fibre bundles (perimysium) as well as whole muscles (epimysium). Fat can be located under the skin (subcutaneous), between muscles (intermuscular) or within individual muscles (intramuscular). [21; 22.]

Several terms can be used for referring to fishery resources. Fish belong to the phylum *Chordata* and can be classified into bony fish (superclass *Osteichthyes*) and cartilaginous fish (superclass *Chondrichthyes*), whereas crustaceans belong to the phylum *Arthropoda* and molluscs (i.e. mussels, oysters, squids) to the phylum *Mollusca* [24]. However, the definition of the broader term seafood is not consistent in the literature and may have been restricted to marine species [25]. In this dissertation, the term seafood is used in accordance to the Scientific Opinion of the European Food Safety Authority (EFSA) on seafood health benefits [25] and thus includes both marine and freshwater animals of either wild or farmed origin and excludes aquatic mammals, reptiles, echinoderms, jellyfish and aquatic plants. Composition of fish and mammalian muscles is primarily similar. Lean fish muscle consists of muscle blocks called myotomes that form the characteristic flaky texture of fish and are separated by connective tissue and small blood vessels. The fibres that form the myotomes are shorter than meat muscle fibres. In fresh fish, the muscle is tightly attached to connective tissue along the backbone and the muscle surface is continuously smooth. Fish muscles can be divided into white and red muscles: proportions of these two types depend on the fat content of the fish. Higher proportion of red muscle occurs along with higher fat content. [22; 26.]

The main constituents of meat and fish are water, proteins, lipids and carbohydrates (Table 1).

Table 1. The approximate proportion of water, protein and lipid (%) in meat [27] and seafood [26].

Product	Water (%)	Protein (%)	Lipid (%)
Beef	70-73	20-22	4.8
Lamb	73	20	5-6
Pork	68-70	19-20	9-11
Poultry	73-77	20-23	4.7
Atlantic cod (*Gadus morhua*)	78-83	15-19	0.1-0.9
Haddock (*Gadus aeglefinus*)	79-84	14.6-20.3	0.1-0.6
Hake (*Merluccius merluccius*)	80	17.8-18.6	0.4-1.0
Herring (*Clupea harengus*)	60-80	16-19	0.4-22.0
Mackerel (*Scomber scombrus*)	60-74	16-20	1.0-23.5
Grey shrimp (*Crangon crangon*)	68-70	10.5-23.2	0.9

Meat primarily consists of water (75 %) and proteins (20 %). Lipids such as fats and oils form the majority of the remaining 5 % along with carbohydrates, amino acids, dipeptides and nucleotides. The composition of meat is affected by species, breed, age, sex, hormonal levels, feed and housing. [21.] In fish, the composition is respectively dependent on factors such as species, age, sex, environment, feed, movement and season [26; 28]. In contrast to mammalian muscles, variation in the

fish muscle composition is high because of the high number of different fish species. Typically, living conditions and production methods of fish are also less controlled since the majority of fish is caught by fishing instead of farming. [29.] Water content of fish can be 30 - 90 % and may vary in different body parts, though 70 - 80 % is typically observed depending on the fat content. Water content may be used for estimating fish condition since it generally increases in living fish towards the spawning time. [26.] The total protein content of fresh fish is typically between 15 - 20 % and lipid content up to 25 %. Smaller relative amount of connective tissue (ca. 3 %) contributes to the softness of fish when compared to mammalian muscles (15 %). [22; 26.]

Lipid content is the most varying component in fish muscles [28]. In contrast to meat where separate fat deposits can be found, fish lipids are mostly located between muscle fibres [22]. The proportion of lipid content is dependent on fish size and maturity, water content and seasonal variation. Lipid content can be used for classifying fish into fatty (3 - 25 %; mackerel, herring) and lean (ca. 0.5 %; cod, haddock). In contrast to mammalian lipids where double bonds are not typical, fish lipids consist of highly unsaturated fatty acids. Generally, marine fish contain more polyunsaturated fatty acids than freshwater fish. Since polyunsaturated fatty acids oxidize more readily than saturated ones, fish is usually more prone to the development of rancid off-odors than meat. [22; 28.]

Meat and fish proteins typically consist of carbon (C), hydrogen (H), oxygen (O), nitrogen (N) and sulfur (S). These proteins can be divided into structural, sarcoplasmic and connective tissue proteins. [21; 26.] Structural proteins such as actin and myosin constitute approximately 70 - 80 % of total protein content in fish and ca. 40 % in mammals. Connective tissue proteins such as collagen account for ca. 10 % of fish proteins and 17 % of mammalian proteins. [28.] Degradation of proteins could be used for spoilage analysis since it may occur as a result of microbial growth, enzymatic activity or processing practices and have deteriorative effects on flavor, nutritional quality and commercial value [20]. In fish, the composition and activity of sarcoplasmic proteins remains undisturbed during post-mortem storage, whereas cytoskeletal proteins are degraded by proteolysis [30]. Different approaches for analyzing protein changes have been presented in the scientific literature, including spectroscopic methods [31; 32] and gel electrophoresis [33; 34]. However, monitoring of protein changes during storage has been found challenging because of labor-intensive and technically demanding experimental setups [20].

Even though carbohydrates form a small fraction of muscle tissues, they contribute to microbial growth. In warm-blooded animals, glycogen is used as an energy reservoir via glycolysis. After slaughter, breakdown of glycogen continues even though O_2 is no longer available and leads to the accumulation of lactic acid and pH decline. This process continues until all available glycogen is consumed or glycolytic enzymes are inactivated by low pH. The final pH depends on muscle type, pre-slaughter exercise, stress and temperature. [21; 22.] In contrast to meat, the carbohydrate content of fish is mostly very low (< 0.5-1 %) due to the absence of glycogen. Consequently, lactic acid is only produced in low quantities, which is why the post-mortem pH of fish is relatively high. This increases the susceptibility of fish to spoilage since microbial growth is inhibited by low pH. [22; 35;

36.] Furthermore, since water-soluble carbohydrates and non-protein nitrogen compounds are consumed before excessive proteolysis, the low carbohydrate content of fish promotes the utilization of nitrogenous compounds, thus resulting in earlier generation of off-odors and off-flavors than in case of meat [22].

Both carbohydrates and proteins have high importance for the water-holding capacity (WHC) of meat and fish. After rigor mortis, accumulation of lactic acid and subsequent acidification lead to denaturation of proteins which reduces their WHC. Decrease in WHC leads to water loss referred to as drip. [21.] At the isoelectric point, WHC and protein solubility reach their minimum because of their hydrophilicity. Fish proteins reach this point at approximate pH 4.5 - 5.5 and meat proteins at 5.3 - 5.5. [21; 28.] A poor WHC may have a central impact on meat and fish quality since drip loss deteriorates the product's appearance, leads to weight loss and may reduce its juiciness [21].

2.1.1.2 Non-protein nitrogen fraction

Meat and fish contain a non-protein nitrogen (NPN) fraction that consists of nitrogen-containing compounds with low molecular weight and high solubility in water. Compounds belonging to the NPN fraction commonly contribute to spoilage since they provide with a readily available source of nutrients for microbial growth [35]. These compounds, such as free amino acids, volatile bases, nucleotides and creatine, are present in meat and fish in varying quantities. In addition to animal species, the composition of the NPN fraction is affected by variation between individual animals. In teleosts, approximately 9 - 18 % of total nitrogen is included in the NPN fraction. Creatine is the most abundant NPN compound in fish, whereas the amino acid content depends on the species. Histidine is typically found in dark fish flesh and can be used by microbes in the production of histamine. [37.]

The total volatile basic nitrogen (TVB-N) content of fish has traditionally been used as an indicator of the quality status. The European commission regulation No 2074/2005 [38; 39] has set the maximum limits for the TVB-N content in ice-stored fish products intended for human consumption: 25 mg N/100 g in redfish, 30 mg N/100 g in *Pleuronectidae* excluding halibut and 35 mg N/100 g in Atlantic salmon, *Merlucciidae* and gadoid fish. However, even though these values represent legislational limits, it should be taken into account that different values have been presented in the scientific literature for different species and packaging conditions. For example, TVB-N content of up to 20 mg N/100g where the contribution of trimethylamine (TMA) is up to 3 mg N/100 g has been associated with fresh cod stored under ice [40].

Trimethylamine oxide (TMAO) is one of the most characteristic NPN fraction compounds in marine fish [37]. TMAO contributes to the maintenance of osmotic balance in the fish tissue in correspondence with the environmental conditions [41] and has been identified as a counteracting solute that stabilizes the protein structure and protects it against the deteriorative effects of pressure [42], salts [43], urea [44] and temperature stress [45]. However, the lipid content of fish has been observed to correlate with both depth and TMAO content, suggesting that TMAO could be produced as a by-

product of lipid metabolism and storage [46; 47]. The amount of TMAO in the fish muscle is dependent on several factors, including species, living conditions and season [37].

The reaction products of TMAO are dependent on the storage conditions of fish. Under refrigerated conditions, TMAO is reduced to TMA. [11; 41; 48.] Several spoilage microbes are able to use TMAO as a final electron acceptor during their anaerobic respiration [35]. For example, TMAO reduction mechanism by *Shewanella* spp. has been proposed by Ruiter et al. ([49], cited in [50]):

$$\underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{TMAO}}{(CH_3)_3NO} \rightarrow \underset{\text{Pyruvate}}{CH_3COCOOH} + \underset{\text{TMA}}{(CH_3)_3N} + H_2O \quad (1)$$

$$\underset{\text{Pyruvate}}{CH_3COCOOH} + \underset{\text{TMAO}}{(CH_3)_3NO} \rightarrow \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{TMA}}{(CH_3)_3N} + CO_2 \quad (2)$$

Under chilled or frozen conditions, enzymatic activity becomes more prominent due to reduced microbial activity. Enzymatic activity in fish tissue, such as the catalysing effect of TMAO aldolase, leads to the generation of dimethylamine (DMA) and formaldehyde. [41; 48.] Post-mortem toughening of fish tissue has been associated with cross-linking of muscle proteins in the presence of formaldehyde [41].

TMA is generally present in low quantities in fresh marine fish and is produced as a result of microbial activity. In freshwater fish, the role of TMA has traditionally been considered negligible because of the absence of TMAO. [28.] However, TMAO has been detected in species such as Nile perch and tilapia [51; 52] and has also been identified as a spoilage indicator in certain freshwater fish, though in lower concentrations than in marine fish [48]. Several methods have been developed for the determination of TMA concentration. Dyer's method [53] is based on the colorimetric determination of picrate salt formed as a reaction product of picric acid and free bases of fish. The method has been widely applied and modified [48]. However, overestimation of TMA levels has also been reported due to the reactions between picric acid and other volatile bases such as DMA [54].

Since the aforementioned traditional quantification methods are based on the extraction of volatile bases from the fish muscle, the direct applicability of their results in food quality monitoring is limited. Alternative methods are needed when aiming at the development of non-destructive quality monitoring, such as the determination of TMA concentration in the headspace of the packaged product. For this purpose, chromatographic methods involving gas chromatography [55-57] or liquid chromatography [48; 58] have been utilized. However, additional limitations are also associated with this kind of methods (Ch. 2.1.3).

2.1.1.3 Color

Color is highly important for the perceived quality and consumer acceptance of muscle foods. The importance of color and its stability is especially critical for meat where color is generally used as an indicator of freshness and has been identified as a highly influential determinant of consumer appeal

and purchase decision. [59-63.] Meat color is dependent on the water-soluble heme protein myoglobin which contains eight α-helices that are combined with non-helical sections. An iron ion located in the center of the heme ring bonds to four pyrrole nitrogens, a proximal histidine-93 and a ligand. The ligand and its valency have the highest impact on meat color, even though other heme proteins such as haemoglobin and cytochrome C may also contribute to it. The four main forms of myoglobin are deoxymyoglobin, oxymyoglobin, metmyoglobin and carboxymyoglobin, whose occurrence is dependent on the headspace gas composition. [60.]

Deoxymyoglobin is the dominating form of myoglobin under the absence of oxygen (O_2) and results in purple color when meat is packaged under vacuum or very low O_2 concentrations. In this state, no ligand is present in the heme iron (Fe^{2+}). In the presence of O_2, oxygenation of deoxymyoglobin leads to the formation of oxymyoglobin and causes a bright red color associated with meat packaged under high O_2 concentrations. This is based on O_2 binding to the heme iron as a ligand without changing its valence (Fe^{2+}). The structure and stability of myoglobin changes as a result of interaction between the ligand and a distal histidine. Under very low O_2 partial pressures, oxymyoglobin is eventually converted to deoxymyoglobin by reduction. Both deoxymyoglobin and oxymyoglobin can also become subject to oxidation, which results in the formation of metmyoglobin and brown discoloration. Metmyoglobin is produced under low O_2 concentrations and involves a change in the heme iron valence (Fe^{3+}). [60.] Metmyoglobin can also be formed as a consequence of prolonged exposure to light, heat, freezing or microbial growth [64]. In the presence of O_2, metmyoglobin is present between the superficial oxymyoglobin and inner deoxymyoglobin layers and moves closer to the surface over time. The depth of the oxymyoglobin layer depends on the availability of O_2, meat temperature and pH. Finally, carboxymyoglobin is formed when carbon monoxide (CO) binds to the heme iron as a ligand. [60.] Low concentrations of CO have been used to produce a stable and bright red color in meat [65-73]. In addition to maintaining color stability, CO has been found advantageous in preventing premature browning during cooking [65]. However, concerns about misleading color stability have been expressed. Since CO produces a highly stable red color, the appearance of the packaged product could be associated with freshness even though high microbial levels had been reached. [68; 72.]

Meat color is affected by several intrinsic and extrinsic factors. Breed, diet and living conditions have been observed to affect the color of beef. In pork, effects related to genetics, glycolytic potential, diet, housing, preslaughter stress and slaughtering conditions have been observed. [60.] Variation in pork loin color has also been observed throughout the length of the loin [74]. Color changes that occur when meat is exposed to O_2 are referred to as blooming. This is characterized by the oxygenation of myoglobin and subsequent generation of red color. However, these color changes depend on the myoglobin content, pH, O_2 and reducing equivalents [75]. When meat is exposed to aerobic conditions, the first 30 to 60 minutes characterize the blooming ability [76].

Respectively as in case of meat, the color of seafood is dependent on its myoglobin content [77]. In fish muscles, myoglobin is retained in the intracellular structure and the different muscle fibre types

are generally more distinctively separated than in meat [78]. Myoglobin is characteristic to red fish flesh and may be present in smaller quantities in white flesh. In crustaceans, myoglobin is absent. [77.] This emphasizes the significance of carotenoids for seafood color. Carotenoids are isoprenoids that are produced by photosynthetic plants, algae and bacteria and by certain non-photosynthetic organisms. These compounds can be classified into carotenes and xanthophylls, where only the latter group contains oxygen. [79.] Despite the fact that certain carotenoids are colorless [80], most of them absorb light at the 400-500 nm range and thus exhibit red, orange or yellow color [79]. For this reason, carotenoids contribute e.g. to the characteristic color of salmonid fish and crustaceans [78]. However, the intensification of crustacean color during cooking is due to protein denaturation [77]. In contrast to myoglobin which is an inherent part of muscles, carotenoids are obtained from the diet or other external sources [81].

In addition to visual inspection, color of meat can be instrumentally analyzed. Continuous scales can be used for describing color as a combination of pure red, blue and green. Generally, color is defined as a point in space using three-stimulus values X, Y and Z. In the Commission Internationale de l´Eclairage L*a*b* (CIELAB) color space, three-stimulus values define the coordinates L*, a* and b*. Lightness (L*) is described from 0 (black) to 100 (white), redness (a*) from negative (green) to positive (red) and yellowness (b*) from negative (blue) to positive (yellow). The chromacity coordinates a* and b* can also be used for the calculation of hue angle or chroma. [21]. Depending on the food product and packaging materials, color measurements can be destructive or non-destructive. Non-destructive measurements through the packaging materials allow the follow-up of specific packages over time, thus resulting in less material consumption. However, possible fat or purge may affect the transparency. Direct measurements from the product surface are often preferred, although the loss of desired headspace gas composition requires that the samples are immediately assessed after opening the package. [60.] In order to prevent light from passing through the sample, sufficient thickness is required. High pH enhances the pink color of meat: the darker and more red the meat is, the more intense pink color can be perceived. Decrease in pH towards the isoelectric point of water-binding proteins can be expected to result in increasing amount of free water and subsequently light scattering. On the other hand, low pH supports the dominance of reduced myoglobin. [76.]

Instrumental parameters have also been observed to affect the measured color values. Brewer *et al.* [76] used Minolta (Minolta Camera Co., Ltd., Osaka, Japan) and HunterLab (MiniScan XE, Hunter and Assoc., Reston, VA, USA) colorimeters and illuminants A, C, D_{65} and F to evaluate the color of pork. The observed differences between illuminants and instruments indicate that data comparison may not be valid if different experimental approaches were used [76]. Respective inconsistency between different instruments were observed by Brewer *et al.* [75]. Tapp *et al.* [82] found that Minolta colorimeters (60.0 %) were preferred over Hunter instruments (31.6 %) among 1068 peer-reviewed articles. Illuminant was typically D_{65} (32.3 %) or not reported (48.9 %). Aperture size and observation angle were not reported in the majority of the articles, whereas blooming time of freshly cut muscle was not reported in 36.8 %. It was thus concluded that a standardized set of minimum reportable parameters should be used. [82.]

2.1.2 Microbial contamination and growth

Spoilage processes of meat and fish can be divided into three main groups: microbial growth, autolysis and lipid oxidation [83; 84]. Even though microbial growth and metabolism is the most prominent cause of food spoilage, autolytic and oxidative reactions may contribute to spoilage in certain food products. Autolysis refers to degradative enzymatic reactions that are immediately initiated after death: these reactions are mostly related to fish spoilage and have been considered less relevant in meat [13; 83]. In fish, autolysis dominates the spoilage at 0 °C during the first 4 - 6 days and is affected by the initial quality, temperature, O_2 levels and pH. Progressing autolysis promotes microbial growth by allowing bacteria to enter the tissues from outer surfaces and by providing with nutrients for microbial growth. However, significant muscle degradation is associated with extremely spoiled products. [83.] On the other hand, products having a high fat content readily undergo oxidation and subsequent generation of discoloration and rancidity [37].

Microbial growth is initiated in meat and fish as a result of contamination. Every food product has its characteristic microbiota that changes over time and depends on the raw materials, processing methods and storage conditions [11]. Muscles and internal organs of healthy living animals are normally sterile since microbes are prevented from entering the tissues or their growth is controlled. Sources of meat contamination include skin, hide, fleece, feet as well as the alimentary tract and its contents. A contact between muscles and these contamination sources may occur directly or via cross-contamination on the processing line. Good processing hygiene and washing can be used for minimizing these risks. [21; 22.] However, washing water and aerosols pose additional contamination risks [21].

In addition to aforementioned risks, additional sources of contamination can affect the microbiota of fish and other seafood. Contamination of fish is affected by the living habitat and its characteristics: water temperature, salt content, swimming depth and pollution degree. Contaminating microbes are present on the skin, gills and alimentary tract. [35.] Fishing equipment and procedures may damage these surfaces, allowing microbes to access fish muscles. Possible gutting on-board before cooling eliminates a major source of contamination, though exposes the fish muscle surfaces: a high ratio between surface area and volume increases the spoilage rate. Furthermore, packaging and storage of fish under ice as well as processing in the docks or markets can cause contamination. In order to prevent spoilage of highly perishable species like crustaceans, these are commonly kept alive as long as possible. Contamination can also be reduced by cooking or freezing immediately after catch. [22.]

Initial microbial load of meat and fish may be highly variant. Microbiological quality of meat is affected by the physiological state of muscles at the time of slaughter, slaughtering and processing practices, storage and distribution conditions as well as temperature [13]. Typically, total viable count (TVC) on the carcass surface is up to 3-4 log colony forming units per square centimeter (CFU/cm^2) under high processing hygiene and even over 6 log CFU/cm² under poor hygiene [21].Variation in the initial

microbial levels between animal species is affected by slaughtering and processing methods. Since pig skin is not removed from the carcass, higher microbial counts are typically observed in pork than in beef. In fish, initial microbial counts can be even higher: 2-7 log CFU/cm^2 on skin and 3-9 log CFU/cm^2 in the gills and gut have been reported. The composition of the microbiota living on fish is dependent on the water temperature and salt content: marine fish microbiota is required to tolerate elevated salt contents. [22.]

Progress of microbial growth can be divided into four main phases. Bacteria adapt to their environment during the lag phase and increase exponentially in the log phase. In the stationary phase, growth and death rates become equal due to e.g. depletion of nutrients or production of antimicrobial substances. In the death phase, death rate exceeds the growth rate. [21.] Several intrinsic and extrinsic factors affect this process. Under certain packaging and storage conditions, microbial population meets a selection pressure and microbes that are most adapted towards the particular conditions become dominant. [85.] This depends on the physical and chemical properties of the product, such as pH, buffering capacity, availability of nutrients and water activity. Generally, post-mortem acidification of meat inhibits microbial growth since neutral pH (ca. 7) is optimal for many microbial species. [21] Respectively, high post-mortem pH of fish (> 6.0) enhances microbial growth [35]. High water activity also promotes microbial growth, though typically a_w over 0.91 is required [21]. However, from all possible extrinsic factors, temperature is one of the most influential. Generally, increasing growth rate can be observed with increasing temperatures, whereas chilling delays the onset of log phase and reduces the bacterial growth rate. Furthermore, bacteria can be divided into four major groups on the basis of their optimal growth temperature ranges: psychrophiles generally grow at -8 to 25 °C and psychrotrophs at -2 to 25 °C, mesophiles are favored at 10 to 40 °C, and thermophiles grow at 43 to 66 °C. Even though psychrotrophic bacteria tolerate chilled temperatures, their growth is optimal at higher temperatures. [21.]

The total microbial load is not necessarily a comprehensive indicator of spoilage. For example, sensory rejection of fish has been observed to occur at varying levels between 10^6-10^9 CFU/g [86-90]. Despite the variation in the initial microbiota, relatively few microbes directly contribute to meat and fish quality. Spoilage association has been used to refer to the microbiota present in the food product at the time of spoilage, whereas spoilage organisms are responsible for off-odor and flavor production [35]. More recently, the concept ephemeral or specific spoilage organisms ((E/S)SOs) has been assigned to the part of microbiota that causes spoilage. These microbes typically form a fraction of the initial microbiota. [11; 13.] Over time, SSOs become dominant and lead to changes in the sensory quality. The spoilage domain of SSOs is determined by factors including temperature, gas atmosphere, pH and water activity. [11.] Typically, correlation between SSO growth and freshness is more informative than between TVC and freshness [30].

Microbial interaction has been considered highly significant for spoilage. In addition to the packaging and storage conditions, competition affects the development of spoilage microbiota [85]. Growth of SSOs is affected by the symbiosis or antagonism between different microbial species, competition

for nutrients or production of antimicrobial agents. For example, lactic acid bacteria (LAB) are able to produce lactic acid and thus to inhibit the growth of several other microbial species. Many Gram-negative species have been observed to produce acylated homoserine lactones (AHLs) that are used in quorum sensing. [11.] In addition to SSOs, other microbes that are present in the food or packaging may have an impact on spoilage through interactions, even though their growth has no direct impact on sensory properties. Interaction of microbiota has also been considered as a likely cause of spoilage metabolite production. The concept metabiotic spoilage interaction has been used for describing the exchange of nutrients or metabolites between microbial species that contributes to spoilage. [91; 92.]

2.1.3 Volatile organic compounds

Spoilage of food is commonly characterized by the generation of unacceptable off-odors due to the production of volatile organic compounds (VOCs) during microbial metabolism [11]. A certain food product has a characteristic VOC profile that not only determines its odor but also changes during spoilage [93]. Off-odors are a prominent cause of food spoilage and have thus a high economic impact [94]. Especially in fish, odor is considered to be one of the most important quality parameters [30]. Abnormal odors in the product pass over other perceived quality attributes [21].

Common VOCs produced during food spoilage include acids, alcohols, aldehydes, amines, ethyl esters, ketones and sulfides [11; 95]. These VOCs are produced as nutrients (Table 2) are consumed during both primary and secondary metabolism [96]. The most common precursors can be characterized as 1) compounds of the glycolytic pathway, 2) metabolic compounds or 3) nitrogenous compounds [13].

Table 2. Typical precursors that can be utilized by common spoilage microbes under aerobic[A] and/or anaerobic[B] (limited O_2 and/or high CO_2) conditions: Pseudomonads (P), *Shewanella putrefaciens* (S), *Brochothrix thermosphacta* (B), enterobacteria (E) and lactic acid bacteria (L) [13].

Precursor	Microbes
Amino acids	P^{AB}, S^{AB}, B^{A}, E^{AB}, L^{AB}
Ethanol	S^{A}
Gluconate	P^{AB}, S^{A}
Gluconate-6-phosphate	P^{AB}, S^{A}
Glucose	P^{AB},S^{AB}, B^{AB}, E^{AB}, L^{AB}
Glucose-6-phosphate	P^{AB},S^{AB}, B^{AB}, E^{AB}, L^{AB}
Lactate	P^{A}, S^{A}, E^{A}
Pyruvate	P^{AB}, S^{A}

For example, glucose is the first substrate to be used by the Table 2 bacteria and has been shown to act as a precursor for a high number of different alcohols, acids and ketones [13]. Sulfurous odors are produced as microbes degrade L-cysteine and L-methionine, whereas decarboxylation of free amino acids that are readily available in protein-rich food products produces volatile biogenic amines

[97]. However, it should be noted that the utilization of precursors depends on several factors, such as the composition of the microbiota, packaging and storage conditions as well as the type and availability of other substrates [13]; relations between precursors, microbiota and VOC production have been presented in detail in several reviews [13; 95; 96]. In accordance with these remarks, the development of the VOC profile is a complex event that primarily depends on the microbial species and strain, growth phase, pH, humidity, available nutrients, temperature and atmosphere. In addition to having an indirect effect on the VOC profile via the microbiota, these factors may also directly affect the generation of VOCs. [96.]

Concentrations of VOCs produced as a result of microbial metabolism can be used to indicate microbial growth [96]. Chemical spoilage index (CSI) refers to the metabolites that cause off-odors and subsequently consumer rejection [11]. VOCs have been utilized in different applications, such as air analysis [98-100] and infection diagnostics [101-104]. In food science, determination of VOCs can be used as a fast and effective technology for determining food quality or spoilage [20; 30; 96]. The spoilage potential of an SSO refers to its qualitative ability to produce off-odors, whereas spoilage activity is determined by the quantitative ability to produce spoilage metabolites. SSOs can thus be identified by comparing the spoilage related changes that occur in food products to those caused by microbial isolates. [11.]

Different methods can be used for the identification and/or quantification of VOCs present in the package headspace. Traditionally, chromatographic methods have often been used for concentrating the VOCs prior analysis by dynamic or static headspace methods, distillation or solvent extraction [30]. Gas chromatography-mass spectrometry (GC-MS) has been the most frequently applied approach for the characterization of meat products [105-108] and fish products [57; 89; 109-115]. However, due to the need for destructive sample preparation, GC-MS cannot be applied for direct and real-time analysis of a food package headspace. In contrast, selected-ion flow-tube mass spectrometry (SIFT-MS) can be used for this purpose. SIFT-MS is a quantitative method based on chemical ionization that has gained increasing interest in food science during the recent years. When using this technology, precursor ions are first produced at the ion source. These ions should be highly reactive with trace gases and remain unaffected by the main components of air: H_3O^+, NO^+ and O_2^+ are typically used. Next, precursor ions with a certain mass-to-charge ratio (m/z) are selected with a quadrupole mass filter and injected into helium gas flow. These ions react with trace gases present in a gaseous sample that has been injected into the carrier gas with a certain flow rate. Another quadrupole mass filter is used for detecting and counting the different product ions and the remaining precursor ions. When the count rates of a certain product ion and the corresponding precursor ion as well as the reaction time and rate coefficient of their reaction are known, concentration of the trace gas in the sample can be determined. [116; 117.]

SIFT-MS has several advantages in trace gas analysis. This technology allows fast and accurate real-time quantification even at parts-per-billion (ppb) level. The ability to use several precursor ions

increases the versatility of the method and facilitates the analysis of a complex mixture of compounds, since a certain precursor ion might not react with all present trace gases. SIFT-MS is thus more versatile when compared to proton transfer reaction mass spectrometry (PTR-MS) where H_3O^+ is solely used as a precursor ion. Previously, SIFT-MS has been applied in health sciences [118-122], biological sciences [123-125] and comparison of quantification technologies [126]. In food science, SIFT-MS has been used for the characterization of the VOC profile of different products, including meat and fish [127-131], vegetables and fruit [132-136] and cheese [137-139].

2.2 Modified atmosphere packaging of meat and fish

2.2.1 Principles of MAP

Food products that are stored under air are prone to the growth of aerobic microbes, changes in moisture content and oxidation. In MAP, the composition of the gas atmosphere inside the packaging differs from that of atmospheric air and changes over storage time because of microbial activity and chemical reactions. [19.] This is commonly achieved by removing atmospheric air from the package headspace and replacing it with another gas mixture. Vacuum packaging is a form of MAP where gases are removed from the package before sealing. [64.] In contrast, controlled atmosphere packaging (CAP) maintains the desired gas composition throughout storage [19]. Modified atmospheres (MAs) typically consist of O_2, carbon dioxide (CO_2) and nitrogen (N_2) in different ratios, depending on the packaged food product. Other gases that can be applied in MAP include CO, sulfur dioxide (SO_2) and inert gases such as argon (Ar). [19.] In order to retain the advantageous properties of MAP, packaging materials with sufficient barrier properties against gases and moisture are needed [64].

Carbon dioxide is used in MAP because of its antimicrobial properties. CO_2 can dissolve into water, lipids and some other organic compounds, leading to the generation of carbonic acid (H_2CO_3), acidification and pH decrease. [19.] The efficiency of MAP depends on the amount of dissolved CO_2 in the water phase of the product. Initial concentration of CO_2 in the gas phase and the gas-product ratio have been recognized as the most important factors affecting the dissolving of CO_2, along with the additional contribution of pH, temperature and fat content. [140.] Dissolving of CO_2 increases with decreasing temperature, which is why its antimicrobial effects are most pronounced under 10 °C. However, dissolving can reduce the headspace volume, thus causing package collapse. In order to compensate for this phenomenon, N_2 is often used. N_2 is odourless, tasteless and has a low solubility to food products, and while it does not prevent the growth of anaerobic microbes, it does not support aerobic growth. [19.] In muscle foods, the use of elevated CO_2 concentrations shifts the dominating microbiota from Gram-negative to Gram-positive bacteria that are associated with extended lag phases, reduced growth rates and higher tolerance towards CO_2 [10; 141].

The presence of O_2 promotes several deteriorative reactions in meat and fish. Under aerobic conditions, lipid oxidation can lead to the generation of rancid off-odors and off-flavors. Furthermore, O_2 promotes pigment oxidation and aerobic microbial growth. [70; 142; 143.] Especially in case of red meat products, deteriorative effects of O_2 on color may significantly reduce the shelf life (Ch. 2.1.1.3). Breakdown of proteins may result from their oxidation and cause reduced juiciness, tenderness and nutritional value [144]. However, O_2 has advantages in certain food packaging applications and is commonly used in order to achieve a desirable red color (Ch. 2.1.1.3), prevent TMAO reduction (Ch. 2.1.1.2) and inhibit the growth of certain spoilage organisms (Ch. 2.2.2.1 and 2.2.3.1).

Carbon monoxide has been applied in MAP primarily because of its impact on color (Ch. 2.1.1.3). In addition, CO has been found advantageous in inhibiting oxidized off-flavor, bone darkening, microbial growth and loss of tenderness. However, along with concerns about potential safety risks associated with high color stability (Ch. 2.1.1.3), the hazardous nature of CO has provoked negative consumer attitudes towards its use. [145.] Currently, the use of CO in the meat and seafood industry is prohibited in the EU. In the USA, the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) have approved the use of 0.4 % CO in anaerobic MAP containing 30 % CO_2 [146; 147].

MAP has several advantages over storage under air. MAP can be used for extending the shelf life of packaged food products and to support the maintenance of desired product properties. MAP also provides with solutions for displaying the products in an attractive manner. [64.] However, poor product quality cannot be improved with MAP. High initial quality as well as hygienic handling practices and temperature control are thus required for successful MAP. [19.] It should also be noted that in addition to microbial growth, shelf life is dependent on the sensory properties of the product that should be retained at an acceptable level. The selection of headspace gases and their relative proportions should thus aim at optimizing all factors that affect the shelf life of the packaged food product.

2.2.2 Meat

2.2.2.1 Microbial growth

Under chill temperatures, meat microbiota principally consists of the genera *Acinetobacter*, *Brochothrix*, *Clostridium*, different *Enterobacteriaceae*, *Flavobacterium*, *Moraxella*, *Micrococcus*, *Pseudomonas*, *Psychrobacter*, *Staphylococcus* and LAB. MAP changes the internal conditions of the package and subsequently affects the development of meat microbiota. Headspace gases and their concentrations can be used for creating a selective pressure towards certain microbiota and thus certain SSOs. [85; 95.] Table 3 presents an overview of typical spoilage microbes of raw meat under different packaging and storage conditions.

Table 3. Typical spoilage microbes of raw meat under different atmospheres at 0-4 °C [13].

Microbes	Atmosphere
Brochothrix thermosphacta	Modified atmospheres (< 50 % CO_2 + O_2; > 50 % CO_2 + O_2)
	Vacuum
Lactic acid bacteria	Modified atmospheres (50 % CO_2; 100 % CO_2; < 50 % CO_2 + O_2)
Enterobacteria	50 % CO_2
Pseudomonads	Air
	Vacuum
Shewanella putrefaciens	Vacuum

When stored under air, the *Pseudomonas* genus generally dominates the spoilage of raw refrigerated meat [148-152]. Pseudomonads are cold-tolerant and may form over 90 % of the total microbiota on chilled carcass surfaces [21]. The species *P. fragi*, *P. fluorescens* and *P. ludensis* have been identified as the most important spoilage organisms of meat under aerobic storage [13; 151]. However, some growth of pseudomonads has also been observed under MAs and vacuum [150; 153-155]. Among pseudomonads, *P. fragi* has been most commonly isolated from meat [156] and considered as the dominant species under both aerobic and MAP conditions [85]. After metabolizing readily available glucose and lactate, pseudomonads utilize amino acids and other nitrogenous compounds. Their growth thus typically becomes evident as off-odors and slime after reaching 10^7-10^8 CFU/g. [13.]

Under different MAP conditions, LAB and *B. thermosphacta* have been identified as the main spoilage organisms of refrigerated meat [157]. Under MAs containing high O_2 concentrations, the dominating microbes also include pseudomonads [158] whose growth is inhibited by elevated CO_2 concentrations [159]. Under anaerobic MAs, growth of aerobic microbiota can be inhibited. In beef *longissimus dorsi* stored under vacuum, growth of pseudomonads, *B. thermosphacta* and *Enterobacteriaceae* was inhibited and LAB became dominant [154]. Generally, vacuum packaging favors the growth of CO_2 tolerant microbiota [160]. *B. thermosphacta* is a psychrotrophic bacterium that is able to grow under air, vacuum and MAP [155; 160; 161]. Its growth has also been associated with the spoilage of high pH (> 6.5) meat [21]. Russo *et al*. [162] studied the *in vitro* growth of meat spoilage microbes at 5 °C and found that *B. thermosphacta* growth was reduced in the presence of LAB and dominating in the presence of a mixture of LAB, *Enterobacteriaceae* and *Pseudomonas* spp. Respectively, LAB have been observed to outcompete *B. thermosphacta* in other studies [163; 164]. Growth of *B. thermosphacta* has been associated with cheesy, sour and fermented off-odors [112].

LAB are Gram-positive rods and cocci that belong to various genera, including *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* [165]. LAB are aerotolerant, CO_2-resistant and psychrothrophic, which is why their growth is favored by the packaging and storage conditions that are typically applied for red meat: high O_2, elevated CO_2 and chill temperatures. LAB may thus grow regardless of packaging and storage conditions, yet depending on other microbes. Due to their capability to adapt to various conditions, the natural diversity of

LAB is considered to be wide both on species and strain level. [92.] Growth of LAB is characterized by the formation of lactic acid, CO_2, slime and off-odors as well as decreasing pH [12].

Among LAB, the genera *Carnobacterium*, *Lactobacillus* and *Leuconostoc* have been closely associated with meat spoilage [157; 158; 161]. Lactobacilli and/or leuconostocs, especially *Lactobacillus sakei* and *Leuconostoc gelidum* subs. *gasicomitatum*, have commonly been found in beef or pork packaged under MAs [150; 154; 155; 163; 164; 166; 167]. Leuconostocs have been observed to be able to dominate the meat microbiota both under air [167] and MAP [166]. *L. gelidum* subs. *gasicomitatum* has been recognized as the dominating spoilage microbe in marinated broiler strips and minced meat packaged under MAs [157; 168] and its growth has been associated with the formation of gas and slime, sour or buttery off-odors and green surface discoloration [21; 169-172]. Carnobacteria, mainly *C. divergens* and *C. maltaromaticum*, are characteristic to meat, fish and dairy products stored at chill temperatures and packaged under vacuum or MAs [154; 168; 173; 174].

In addition to the aforementioned microbes, other species may be able to grow on meat. Gram-negative bacteria that may also contribute to meat spoilage under different conditions include *Aeromonas*, *Enterobacter*, *Hafnia*, *Rahnella*, *Shewanella* and *Serratia* [95]. Under MA conditions and vacuum, *Enterobacteriaceae* such as *Serratia liquefaciens* and *Hafnia* spp. can grow at 0-10 °C [161]. Growth of microbes belonging to genera *Serratia* and *Rahnella* has been observed in beef under vacuum [173]. *Rahnella* spp. have also been observed to grow in beef packaged under air or MAP containing 20 % O_2 and 40 % CO_2 [150]. Spoilage caused by enterobacteria is generally characterized by gas, slime and bitter off-odors and off-flavors [12]. The genus *Clostridia* has been associated with excessive gas and exudate formation, putrid off-odors and softening of texture occurring in vacuum packaged meat products, known as blown pack spoilage [175; 176]. The *Shewanella* genus has been associated with the spoilage of vacuum packaged meat [13; 160]. *S. putrefaciens* contributes to spoilage by producing green discoloration and sulfurous off-odors [85]. However, the *Shewanella* genus has been considered to have less importance in meat spoilage than in fish (Ch. 2.2.3.1) because of its pH sensitivity. The postmortem acidification of meat inhibits the growth of *Shewanella* and favors LAB. [165.]

Temperature increase causes a shift in the microbiota of MAP meat from that growing under chill temperatures. Doulgeraki *et al.* [167] found that the microbiota of minced beef packaged under 40 % CO_2 and 30 % O_2 was dominated by *Leuconostoc* spp. at 5 or 10 °C and by *L. sakei* at 0 or 5 °C. Enterobacteria have been observed to grow at different temperatures under respective MAP conditions, which could be due to differences in adaptation at different temperatures and competition between the microbes [177].

Several studies have focused on the comparison of different headspace gas compositions for raw red meat. Sørheim *et al.* [178] studied the effect of (% $CO_2/O_2/N_2$) 100/0/0, 50/0/50, 25/0/75, 25/10/65 and vacuum on microbial growth, color, off-odors and pH of pork loin. The samples were stored for up to 22 days at 1 °C in dark, followed by storage in O_2 permeable materials at 3 °C and illumination.

Highest off-odor generation was associated with loins stored under 25/10/65 or vacuum, whereas highest drip loss was observed under 100 % O_2 and lowest under vacuum. Loins stored under 25/10/65 had most severe discoloration, off-odor generation and highest psychrotrophic counts. Packaging under anaerobic conditions was thus identified as the most favorable approach for pork loin. [178.] However, Zhang and Sundar [63] reported several advantages when pork loin was stored at 4 °C under increasing O_2 concentrations (5-55 %) combined with 20 % CO_2. O_2 content of 45 % was observed to result in highest quality. High O_2 content had an inhibitive effect of total microbial growth and improved the acceptability of the samples. During eight days of storage, TVB-N decreased as a function of increasing O_2 content, which was associated with the inhibitive effect of O_2 on pseudomonads. [63.]

2.2.2.2 Volatile organic compounds

Evolution of the VOC profile of meat and seafood is dependent on the product properties as well as packaging and storage conditions. Since the same microbial genera or species commonly contribute to spoilage in both meat and seafood (Ch. 2.2.2.1 and 2.2.3.1), their spoilage is often characterized by the same VOCs. A major part of studies regarding the VOC generation in muscle foods has focused on seafood where off-odors have high impact on the shelf life. A detailed review of the origin and odor of common spoilage metabolites is thus given in Ch. 2.3.2.2.

The aroma of fresh raw meat has been attributed to several VOC groups. Fatty, cheesy, dairy or gamy odors have been associated with volatile fatty acids and ketones. Fatty and grassy odors can also be due to aldehydes that change from acceptable to unpleasant as their concentrations increase. Sweet and fruity odors can be caused by alcohols and esters, whereas aromatic compounds, sulfuric compounds and terpenes have their characteristic odors. During storage, the odor profile changes along with microbial growth. [95.] Characteristic VOCs produced during meat spoilage have been reviewed by Casaburi *et al.* [95].

VOCs associated with beef spoilage have been characterized in previous studies. Martin *et al.* [179] stored beef patties under high O_2 (80 % O_2, 20 % CO_2) or low CO atmospheres (0.4 % CO, 30 % CO_2, 69.6 % N_2) at 22 °C. Temperature abuse lead to the rapid development of 20 VOCs. Even though the same VOCs were detected under both packaging conditions, differences were found in their production rate and concentrations. [179.] Respectively, Jääskeläinen *et al.* [105] studied the spoilage of beef packaged under high O_2 (80 % O_2, 20 % CO_2) or vacuum at 6 °C and found that although many compounds were produced under both packaging conditions, the diversity and concentration levels of VOCs were higher under high O_2, which highlighted the undesirable effects of O_2 in meat packaging. Acetic acid, acetoin, diacetyl and ethanol were detected under both conditions. Acetoin, diacetyl, hexanoic acid, nonanal, oct-1-en-3-ol and oct-2-en-1-ol showed statistically significant ($p < 0.01$) correlation with packaging conditions. Under high O_2, buttery off-odor was associated with the growth of leuconostocs and subsequent production of acetoin and diacetyl, whereas oct-1-en-3-ol, hexanal and nonanal were attributed to chemical reactions. [105.]

A limited number of studies have been published about VOC production in pork. Acetoin and diacetyl have commonly been identified as important pork spoilage metabolites [180]. Nieminen *et al.* [180] studied the VOCs of pork loin and collar packaged under high O_2 (60 % O_2, 22-25 % CO_2, rest N_2) at 4 ± 1 °C. They unexpectedly detected high numbers of *Photobacterium phosphoreum* which likely contributed to the VOC profile. From a total of 48 detected compounds, 10 compounds were found to increase during storage. However, concentrations of 1-hexanol, 1-pentanol, 1-octen-3-ol, 2-pentanone, hexanal and styrene also increased in sterilized pork, indicating that they were not completely of microbial origin. Increase in their concentrations was associated with lipid oxidation. On the contrary, concentrations of 3-methyl-1-butanol, acetoin and diacetyl could be associated with microbial growth and sensory defects. Under the tested conditions, diacetyl was also identified as the most potential growth indicator of aerobic bacteria, LAB and *B. thermosphacta* in pork. [180.]

2.2.2.3 Color

Studies focusing on the effects of headspace gas composition on meat color have highlighted the role of O_2; however, the focus of these studies has frequently been limited to beef. Jakobsen and Bertelsen [181] evaluated the effect of 20-80 % O_2 along with CO_2 on the color of beef *Longissimus dorsi* and suggested that ca. 55 % is needed to avoid discoloration. Martínez *et al.* [182] packaged pork sausages under vacuum, O_2-permeable overwrap or MAs (% O_2/CO_2/N_2) 0/20/80, 0/20/80 + O_2 absorber, 20/20/60, 40/20/40, 60/20/20 and 80/20/0 and stored the samples at (2 ± 1) °C for 20 days. Discoloration was observed under all conditions containing O_2, even though highest redness (a*) was detected until day 8 of storage with the highest O_2 concentration. Moderate red and stable color was obtained with 0.3 - 0.7 % residual O_2 and especially in its absence (0 - 0.1 %). [182.] Esmer *et al.* [183] studied the effect of various O_2 and CO_2 concentrations on the color of minced beef at 4 °C for up to 14 days and found that even though increasing CO_2 had no effect on lightness, both a* and b* decreased. When combined with 30 % CO_2, no difference in redness was observed at 50 and 70 % O_2, whereas discoloration occurred at 30 % O_2 [183].

Evaluation of the impact of different MAs on meat lightness has shown some contradictory results. Under 100 % CO_2, lightness of pork has been observed to increase [184], decrease [185] or remain constant [71; 186] during refrigerated storage. Differences in residual O_2 levels have been suggested to contribute to the inconsistence in these results [185]. Differences in the evolution of lightness have also been observed between different muscle types [187].

Under high O_2 concentrations, meat is generally redder than under anoxic conditions [71; 187], though decrease in redness has commonly been observed over time under high O_2 [71; 72; 185]. In order to improve color stability and to avoid deteriorative effects, low concentrations of O_2 have been used in MAP. However, residual O_2 concentrations can have negative effects on meat color and blooming ability [60; 184; 188; 189]. Sørheim *et al.* [184] found that under CO_2- atmospheres containing residual O_2 (0.5 or 1 %), lower redness and higher yellowness was obtained in pork when

compared to completely anaerobic conditions. According to Mancini and Hunt [60], residual O_2 concentrations should be less than 0.05 % for beef and less than 1 % for pork. When ultra-low O_2 concentrations are used, the low levels should be maintained throughout storage in order to preserve the blooming ability [60]. With O_2 absorbers (Ch. 2.3.1.3), improved color has been achieved [184; 186].

Blooming has been studied under different packaging and storage conditions. Several studies have stated that blooming has no effect on lightness (L*) [75; 76; 190] or meat packaged under high O_2 [187]. Generally, the presence of O_2 promotes blooming and increases redness [76]; however, a low O_2 content has been found to prevent blooming [188; 189]. Brewer *et al.* [76] evaluated the effect of pH, muscle and blooming time on instrumentally and visually determined color of pork and observed variation between different muscles, which was attributed to differences in pigment concentration, muscle fibres and pH. No statistically significant ($p < 0.05$) difference was observed between the blooming rates of different muscles [76].

Consumer acceptance of meat is highly dependent on meat color. Buys [59] studied the acceptance of bulk packaged pork stored under MAs (% O_2/CO_2/N_2) 80/20/0, 25/50/25 and 0/100/0 + O_2 absorber at 0 °C and displayed in polyvinyl chloride (PVC) overwrap for up to 4 days. Pork chops packaged under 100 % CO_2 were acceptable throughout storage and display, respectively as chops packaged under O_2 enriched atmospheres [59]. Aaslyng *et al.* [191] compared the preferences of Swedish, Norwegian and Danish consumers for beef steaks packaged (% CO_2/O_2/N_2) either under high O_2 (20/80/0) or anaerobic MAP (30/0/69.6 + 0.4 % CO or 30/0/70). Steaks stored under anaerobic conditions were preferred in all three countries [191].

2.2.3 Fish

2.2.3.1 Microbial growth

The principal composition of the initial microbiota is closely alike in fish and meat (Ch. 2.2.2.1). The dominating microbiota of fish consists of microbes typically found in the aquatic environment [97]. Differences may be observed between marine and freshwater fish as well as between temperate and tropical water fish [35]. Generally, total microbial load of 10^7 CFU/g has been considered as an acceptance limit for raw fish [192]. Microbiota of temperate water fish typically consists of bacteria belonging to genera *Acinetobacter*, *Aeromonadaceae*, *Flavobacterium*, *Moraxella*, *Pseudomonas*, *Shewanella* and *Vibrionaceae*. These psychrothrophic Gram-negative bacteria can grow at low temperatures (0 °C), though their optimal growth temperature is around 25 °C. In addition to these genera, certain Gram-positive organisms such as *Bacillus*, *Corynebacterium*, *Clostridium*, *Lactobacillus* and *Micrococcus* can grow in fish. [35.] Dominance of Gram-positive bacteria has also been observed in some tropical fish [97].

The typical fish spoilage microbiota (Table 3) resembles that of meat (Table 4; Ch. 2.2.2.1); however, its composition and development depends on the habitat of the fish.

Table 4. Typical spoilage microbes of raw fish stored under different atmospheres at 0-4 °C [11; 35].

Microbes	Atmosphere
Lactic acid bacteria	Vacuum
	Modified atmospheres with CO_2
Photobacterium phosphoreum	Modified atmospheres with CO_2
Pseudomonads	Air
Shewanella spp.	Air

Aerobically stored unpreserved fish is spoiled by Gram-negative fermentative rod-shaped bacteria. such as *Vibrionaceae*. At chill temperatures, the dominating microbiota primarily consists of *Pseudomonas* spp. and *Shewanella* spp.. [11; 35.] Their growth is favored over LAB by the high postmortem pH and low carbohydrate content of fish [165]. *Shewanella* spp. have been identified as SSOs of both temperate and tropical marine fish stored aerobically under ice [35; 70]. These facultative anaerobic bacteria are more characteristic to fish than meat (Ch. 2.2.2.1). However, even though *S. putrefaciens* produces intensive off-odors and has thus a high spoilage potential (Ch. 2.2.3.2), high levels (10^8 CFU/g) are needed before off-odors can be detected [193]. On the other hand, *Pseudomonas* spp. have been identified as SSOs of fish stored aerobically under ice, regardless of its living conditions [35].

Under MAs, fish microbiota differs from that growing under air. High CO_2 concentrations can be used for inhibiting the growth of both *Shewanella* and *Pseudomonas*, limiting their levels to 10^5-10^6 CFU/g [35]. For inhibiting the growth of *Shewanella* spp., a combination of 60-70 % CO_2 with 30-40 % O_2 has been recommended [194]. Respectively, *S. putrefaciens* has been identified to dominate in cod fillets packaged under vacuum [195] and to have no relevance in cod packaged under MAs [10; 193]. Synergy between the effects of CO_2 and O_2 on *S. putrefaciens* was observed by Boskou and Debevere [194] at 50 % CO_2 combined with 10 % O_2 and by López-Caballero *et al.* [196] especially at 40 % CO_2 and 60 % O_2.

The effect of CO_2 on fish shelf life is limited due to the growth of CO_2-tolerant *P. phosphoreum* that has been frequently identified as an SSO of marine fish under MAs [10; 11; 35; 40; 86; 193; 197]. Its spoilage domain has been defined to range from 0 % to 50 - 100 % CO_2 [10]. Even though O_2 has inhibitory effect on the growth of *P. phosphoreum*, this bacterium has also been found able to dominate under air [198] or O_2-containing MAs [37; 86]. Dalgaard *et al*. [86] observed that *P. phosphoreum* often dominated in Icelandic and Danish marine fish, had a slower growth rate in Greek marine fish and did not grow in freshwater fish species. This was suggested to be due to its heat lability and NaCl dependency. Furthermore, even though *P. phosphoreum* reached even over 10^7 CFU/g under air, it was not able to dominate the microbiota under these conditions due to the rapid growth of other microbes. [86.]

The role of LAB in seafood spoilage has been highlighted by Leroi [165], suggesting that LAB have been disregarded because of fish properties that favor the growth of other microbiota: high pH, low temperature, high content of nitrogenous compounds and low content of carbohydrates. Under air

and vacuum, *Pseudomonas* and *Shewanella* are able to outcompete LAB at chill temperatures. However, CO_2-containing MAs inhibit the growth of these bacteria and favor LAB along with *P. phosphoreum*. Consequently, LAB have been recognized as SSOs in both marine and freshwater fish packaged under CO_2 or vacuum. [165.] Furthermore, mild processing technologies and use of ingredients in fish products enhance the growth of LAB while inhibiting other microbes.

The spoilage processes of fish and crustaceans are highly similar; however, shrimp and other crustaceans are especially vulnerable and rapidly spoiling seafood species. Shrimp contain high amounts of free amino acids and other non-nitrogenous compounds that promote microbial growth [199]. Under chill temperatures and air storage, the spoilage microbiota has been found to contain species such as Aeromonas spp., *Acinetobacter* spp., *B. thermosphacta*, *Carnobacterium* spp., *Enterobacteriaceae*, *Flavobacterium* spp., *Neisseria* spp., *Pseudomonas* spp., *Serratia* spp., *Shewanella* spp., *Vagococcus* spp. and/or *Vibrio* spp. [200-202]. Under MAP, different LAB have commonly been found [203-205], also together with *B. thermosphacta* [206]. The dominant microbiota of preservative-free cooked and peeled brown shrimp (*Crangon crangon*) has been characterized in multiple studies. Noseda et al. [131] evaluated the impact of several aerobic and anaerobic MAP conditions and suggested that *P. phosphoreum* likely contributed to spoilage. Broekaert *et al.* [205] found that the genera *Pseudoalteromonas* and *Psychrobacter* dominated the microbiota under air storage in ice or at 7.5 °C. Calliauw *et al.* [203] identified *Aliivibrio* spp., *Arthrobacter bergerei*, *B. thermosphacta*, *Carnobacterium* spp., *Psychrobacter* spp., *S. putrefaciens* and *Vagococcus salmoninarum* to be dominating at 4 °C under 40 % CO_2 and 60 % N_2.

The effect of different packaging and storage conditions of marine fish has been examined in several studies, many of them focusing on Atlantic cod. Debevere and Boskou [40] stored cod fillets under MAs (% $CO_2/O_2/N_2$) 60/10/30, 60/20/20, 60/30/10 and 60/40/0 at 6 °C and observed some inhibition of TMA production under increased O_2 concentrations. Sivertsvik [207] stored pre-rigor filleted farmed Atlantic cod under various MAs at 0 °C and found that the optimal gas composition in terms of TVB, TMA, odors, exudates and microbial growth was 63 % O_2 and 37 % CO_2.

2.2.3.2 Volatile organic compounds

Fish VOCs can be divided into three categories based on their association with freshness, microbiological spoilage or oxidation. Different C_6 - C_9 alcohols and carbonyl compounds have been observed to contribute to the planty, mushroom or cucumber-like odor of fresh fish. [30.] Lipid oxidation may result in the production of aldehydes especially in fatty fish. Compounds such as hexanal, hepta-2,4-dienal and deca-2,4,7-trienal are associated with oxidized and rancid odors. [30; 208.]

Several VOCs have been identified as potential indicators of microbial growth in fish. An overview of fish volatiles produced during storage in previous studies is given in Table A1 (Appendix 1). However, it should be noted that in a majority of studies, samples have been stored under air and/or analyzed with destructive techniques. VOCs that have been most frequently detected under MAP

conditions include 2- or 3-methylbutanal, 2,3-butanediol, 3-methyl-1-butanol, acetic acid, acetoin, ethanol, ethyl acetate, different sulfides and TMA.

Alcohols and aldehydes have commonly been detected during seafood spoilage (Table A1, Appendix 1). Consumption of valine and leucine has been associated with the production of different alcohols and aldehydes, and ethanol is produced from carbohydrate resources. Ethanol, 2,3-butanediol and 3-methyl-1-butanol have frequently been simultaneously detected and often accompanied by 2- or 3-methylbutanal. Duflos *et al.* [55] found that these three alcohols and TMA were produced in highest quantities in whiting, mackerel and cod stored under ice and air. Ólafsdóttir *et al.* [198] suggested that alcohols could be used for detecting early spoilage in Atlantic cod fillets packaged under ice. Mikš-Krajnik *et al.* [89] associated the formation of 3-methyl-1-butanol, 2-methylbutanal and 3-methylbutanal with *Pseudomonas* spp. in raw salmon stored under air at 4, 10 or 21 °C. Respectively, Miller *et al*. [209] found pseudomonads to be able to produce alcohols and aldehydes. Production of 2,3-butanediol has also been observed in salmon inoculated with *Aeromonas spp.*, *Enterobacteriaceae* or *S. putrefaciens* and stored under vacuum at 6 °C [210]. The odor of 2,3-butanediol is fruity or buttery, whereas 3-methyl-1-butanol is described as fruity, pungent and ethereal [211]. Aldehydes have been associated with mild and sweet odors [198].

Certain bacteria such as *P. phosphoreum* and LAB may produce a wide array of VOCs. Acetoin, which is formed by the reduction of 2,3-butanedione and can be further reduced to 2,3-butanediol [212], has been associated with anaerobic bacteria such as *P. phosphoreum,* LAB and *B. thermosphacta* [198; 213]. Production of acetic acid has been observed in the presence of *B. thermosphacta*, LAB, *P. phosphoreum* and *S. putrefaciens* [210; 214]. On the other hand, formation of esters resulting in sweet and fruity off-odors has been associated with pseudomonads [198; 209]. Spoilage bacteria that can produce ethyl acetate include *Lactobacillus* spp. [210] and *P. fragi* [209]. Acetic acid can be described as pungent, acidic and cheesy, acetoin as sweet and buttery, and ethyl acetate as fruity and ethereal [211].

Production of sulfuric compounds has been associated with many bacteria characteristic to fish spoilage such as *S. putrefaciens,* pseudomonads, certain LAB and different *Vibrionaceae* [35; 209; 210]. *Shewanella* spp. has been observed to produce hydrogen sulfide (H_2S), dimethyl sulfide and methyl mercaptan [70; 196]. On the other hand, *P. phosphoreum* and pseudomonads do not produce H_2S in significant quantities [35]. Analogously, only low concentrations of H_2S and methyl mercaptan were detected in Atlantic cod packaged under MAs [215]. No increase in dimethyl sulfide concentration was found by Duflos *et al.* [55] in aerobically stored cod, mackerel or whiting.

TMA, one of the most typical VOCs associated with marine fish spoilage, has a “fishy” or “ammonia-like” odor and is especially characteristic to gadoid fish such as cod and whiting [11; 198]. Several bacterial species have been identified as TMA producers, including *Aeromonas* spp., psychrotolerant *Enteriobacteriaceae*, *P. phosphoreum*, *Shewanella* spp. and *Vibrio* spp. [11; 97; 210]. Due to the low TMAO content and absence of *P. phosphoreum* and *Shewanella* spp. (Ch. 2.1.1.2. and 2.2.3.1),

TMA is generally not produced in freshwater fish [35]. Production of TMA is associated with *Shewanella* spp. under aerobic storage and with *P. phosphoreum* under MAs [97; 193; 215]. A single *P. phosphoreum* cell can reduce 10-100 times more TMAO than *S. putrefaciens* [207]. MAP inhibits the TMA production of *Shewanella* spp. since the reductase enzyme is directly inhibited by O_2 and indirectly via pH decrease by CO_2 [216]. Inhibitory concentrations of 10 % O_2 and 30 - 50 % CO_2 have been reported for *S. putrefaciens* and 30 - 40 % O_2 and 60 - 70 % CO_2 for another *Shewanella* species at 7 °C [194; 217]. High TMA levels have been detected in Atlantic cod under MAs [10].

In addition to the aforementioned VOCs, production of several other compounds has been related to certain microbes when isolates or inoculated fish samples have been tested [112; 210; 218-220]. Even though these experiments give a direct indication of the VOC production capability of different microbial species, the effect of competition and microbial interaction on the VOC profile cannot be assessed. In naturally contaminated samples, interactions between microbes and/or VOCs contribute to the overall perceived odor. The impact of MAs on microbial metabolism may also alter the VOC profile. Pin et al. [221] studied the metabolism of *B. thermosphacta* under different MA conditions by analysing the consumption of glucose and production of several VOCs at 5 °C. A shift from anaerobic to aerobic metabolism was observed when O_2 levels were set higher than 10.17 + 0.6717*CO_2. Under anaerobic conditions, lactic acid and ethanol were produced from glucose. Under aerobic conditions, compounds such as acetoin, acetic acid and 3-methyl1-butanol were produced. Both acetic acid and ethanol were also likely used as precursors for other compounds and did thus not accumulate during the stationary growth phase. [221.]

2.3 Active and intelligent packaging

2.3.1 Active packaging

2.3.1.1 Principles and design

During the European FAIR-project CT 98-4710 Actipack (1999-2001), active packaging was defined to change the condition of the packaged product in order to extend its shelf life or to improve its safety or sensory properties, simultaneously maintaining its quality. This requires interaction between the packaging and the food product or headspace [222]. Active packaging can be divided into three main categories on the basis of their operation: absorbers or scavengers, releasing systems and other systems [18].

Antimicrobial packaging is a form of active packaging that acts against microbes present in the food product or packaging materials in order to inhibit, limit or retard their growth. This may involve inactivation of microbes, reduction of their growth rate or maximum population, or extension of their lag phase. [17.] Five main categories of antimicrobial packaging can be defined: sachets or pads with absorption or emission capacity, incorporation of antimicrobial compounds into or onto polymers,

antimicrobial coatings, immobilization of antimicrobial compounds to polymers and inherently antimicrobial polymers [15].

Design of an antimicrobial packaging system depends on the packaging, product and possible headspace (Fig. 1).

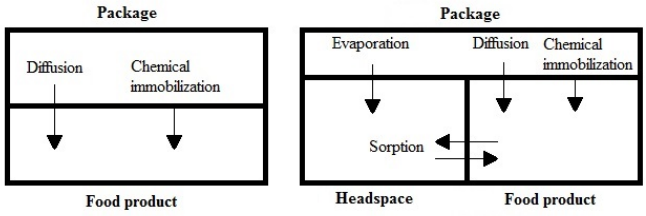

Fig. 1. Mechanisms of antimicrobial activity in food packaging (according to [223]).

When antimicrobial agents are directly introduced to food products, neutralization or diffusion limits their efficiency. Prolonged exposure and sufficient concentrations thus call for advanced packaging technologies. Depending on the intended mechanism (Fig. 1), antimicrobial agents need to be or be able to come into direct contact with microbes present on the food product surfaces since agents that are trapped in polymeric matrices are generally not readily available, which limits their costs-efficiency. Improved efficiency can be achieved by using antimicrobial agents as additives in extrusion processes and by incorporating them in the food contact layer. In multilayer films, layer structure or thickness can be used for controlling the release rate. However, high processing temperatures may limit the range of potential agents. The remaining antimicrobial capacity after packaging material preparation is referred to as residual antimicrobial activity. It should also be noted that incorporation of antimicrobial agents may affect the properties of packaging materials and that material performance should not be allowed to decrease as a consequence. [17; 223.]

Antimicrobial activity depends on mass transfer between package, food product and possible headspace. Migration mechanisms depend on the type of packaging and their rate is generally reduced as a function of decreasing temperature. In headspace-free systems, incorporation of antimicrobial agents in packaging materials allows the diffusion between the materials and food and partition at the interface. In the presence of a headspace, evaporation of volatile antimicrobial agents is possible. Antimicrobial agents may also be immobilized onto packaging material surfaces, allowing activity without diffusion or sorption. Furthermore, antimicrobial activity depends on the properties of the packaged food product, such as the initial microbiota, pH and water activity. [17; 223.]

Extrusion coating and coextrusion are commonly used in incorporating antimicrobial agents as additives in polymers. During extrusion coating, a moving substrate such as paper, board, foil or film is covered with a continuously flowing polymer melt. An extruder typically consists of three zones: feed, compression and meter zones. Polymers that are usually introduced as pellets in the extruder are

conveyed in the feed zone and compressed and plasticated in the compression zone. Temperature and homogenization of the polymer melt are adjusted in the meter zone. The melt is forced with a constant rate through a die which determines its shape and cross-section. [224-226.] In coextrusion, two or multiple polymer layers are combined into one layer or film. The layer structure can be produced by using two or three extruders in different combinations. Layer thickness can be adjusted with extruder screw speed and total thickness with the die. [227.]

The use of active packaging is subject to international and national legislation. In the EU, all food contact materials except edible packaging materials and antiques currently fall under Regulation (EC) No 1935/2004 [228]. In this Regulation, active food contact materials are defined to release components into or absorb substances from foodstuffs. Materials that release active agents may change the composition or properties of food according to relevant legislation. [228.] Substances that can be used in plastic food contact materials are defined in the Commission Regulation (EU) No 10/2011 [229] and in the provisional list [230]. Additional legislation concerning active and intelligent food contact materials is defined in the Commission Regulation (EC) No 450/2009 [231]. According to this Regulation, an active substance that is intended to migrate into food must be in compliance with regulations concerning food additives. The substance is considered to be a food additive even if it is brought into the packaging by immobilisation and is thus not intended to migrate. [231.]

2.3.1.2 Silver

Silver has been used as an antimicrobial agent for thousands of years in different applications and has the highest antimicrobial activity among all heavy metal ions [232]. It has long-term activity, low volatility and low toxicity towards eukaryotic cells [233]. Silver can be introduced in the packaging system as ionic or metallic silver, clusters, complexes or salts. The antimicrobial activity is dependent on the form and biological availability and is typically attributed to silver ions (Ag^+) [232; 234]. Several activity mechanisms have been proposed, including binding and precipitation to cell walls and membranes, accumulation inside microbial cells, binding to cellular proteins, catalyzation of oxidative destruction, inhibition of metabolism, and binding to DNA [232]. The activity of silver ions is dependent on the compounds in the environment and simultaneously occurring redox processes. Different anions, proteins and other compounds can be used for controlling the quantity of available ionic silver, whereas generation of reactive oxygen species (ROS) may lead to reactions with silver and possible synergic effects. These processes are affected by light, temperature and O_2. [234.]

Effects of silver ions on bacterial cells have been evaluated in several studies. Generally, susceptibility of microbes is dependent on the cell wall structure. Gram-positive microbes have higher peptidoglycan content and thus thicker cell walls than Gram-negative ones; the thicker cell wall has been observed to give enhanced protection against the penetration of silver ions into the cytoplasm [235]. Feng *et al*. [235] observed that in the presence of silver ions, the cytoplasm membranes of *Escherichia coli* and *Staphylococcus aureus* cells were damaged and the DNA was condensed, preventing its replication and thus cell multiplication. They also suggested that silver inhibits bacterial activity by

binding to the thiol groups of proteins and preventing their enzymatic activity [235]. The stable S-Ag bond has been reported to inhibit the hydrogen transfer system. Furthermore, in the presence of aqueous media, O_2 that has been adsorbed onto silver may react with the thiol hydrogen, leading to the formation of R-S-S-R bonds and thus disruption of electron transfer and respiration. [236.] Liau *et al.* [237] demonstrated that the activity of silver against sensitive *P. aeruginosa* was neutralized in the presence of amino acids containing thiol groups, whereas no effects were observed with most of the tested amino acids containing disulfide bonds or sulfur-free amino acids. Holt and Bard [238] found that up to 10 µM silver nitrate ($AgNO_3$) inhibited respiratory chain enzymes of *E. coli*, especially in the presence of high potassium concentrations. Glucose was found to reduce the effects of silver ions, which was associated with the formation of silver-glucose complexes or the capacity of *E. coli* to efflux silver [238].

In order to allow controlled and continuous release of silver, different carrier systems and matrices have been developed. Silver has been incorporated in clinoptilolite [239], glass [240], nano-SiO_2 [241] and zeolites [242-244]. These systems are usually based on cation exchange. For example, silver-substituted zeolites are hydrated crystalline aluminosilicate minerals with a negatively charged three-dimensional structure. Cations Na^+, K^+ and Ca^{2+} balance the structure and are exchangeable with other cations in the environment. [242.] Among antimicrobials incorporated in food contact polymers in Japan, silver zeolites have been the most common [233]. Pehlivan *et al.* [245] studied the effect of different silver zeolite concentrations on the properties of polypropylene films. Optimal zeolite concentration was found to be 2 - 4 % with 4.36 mg Ag^+/g zeolite. Higher zeolite concentration lead to brittleness and higher silver concentration to discoloration. Susceptibility to thermal degradation was also found to increase in the presence of zeolite. [245.] Dogan *et al.* [246] found that aluminum foil coated with a vinyl acetate lacquer solution containing 1 - 3 % zeolite carrying 0.9 % silver had antimicrobial effect on *E. coli*. Fernandez *et al.* [233] found that silver zeolite-poly(lactic acid) (PLA) films prepared by solvent casting had higher silver migration and antimicrobial effect against *E. coli* and *S. aureus* than films prepared by melt mixing. The rough surface and less dense structure of cast films allowed improved silver release [233]. Boschetto *et al*. [247] found that low-density polyethylene (LDPE) films with 5 % silver-exchanged zeolite Y containing 5 % silver had antimicrobial effect against *E. coli*. Films prepared by wet casting had higher performance compared to hot casting, which was suggested to be due to high processing temperatures or non-uniform silver distribution associated with hot casting [247].

Silver nanoparticles have been found highly effective against a broad range of micro-organisms. Their activity is affected by their size, surface area, surface charge and geometry [234]. Lok *et al*. [248] found that nanoscale silver caused accumulation of envelope proteins into the cytoplasm of *E. coli*, which was suggested to be due to the destabilization of outer membrane, collapse of proton motive force and reduction of adenosine triphosphate (ATP) levels. Even though both nanoscale silver and silver ions acted against the membranes, respective effects were observed at nanomolar level of nanoscale silver and micromolar level of silver ions [248]. On the other hand, Lalueza et al. [234] compared the effects of various silver-containing materials on *S. aureus.* Antimicrobial activity

was observed to increase as the amount of available silver ions increased. Silver nitrate exhibited greatest activity at a specific total silver content, whereas silver-exchanged zeolite had moderate effect and 100 nm no effect on *S. aureus*. These results indicated that the availability of silver was lower when zeolites or nanoparticles were used. The ability to produce silver ions was suggested to be of higher importance for antimicrobial activity than the concentration of silver that is in contact with microbes. [234.]

In addition to *in vitro* assays, silver has been applied in food packaging systems; however, relatively few studies including a food component are still available in the scientific literature. Enhanced release of silver ions has been observed in bacterial broth compared with water [244]. The higher concentration of cations in broth allows improved cation exchange and leads to increasing concentrations of free silver ions. Activity of silver is also generally higher in broth than in real foods. [249.] Lee *et al.* [249] studied the effect of wrapping papers containing silver zeolite (2 or 4 %) on raw beef, pork and turkey inoculated with *Pseudomonas putida* and stored for 4 days at 4 or 10 °C. Papers with 2 % silver zeolite had no significant effect, whereas 4 % silver zeolite inhibited *P. putida* growth at 10 °C (days 3 and 4). Lower protein content of beef was suggested to lead to higher population reduction when compared to the other tested meat types. [249.]

The toxicity of silver in the human body and in the environment has been arousing concern since migration has been considered essential for its antimicrobial activity [250]. Even though different symptoms arising from exposure to silver have been documented, discoloration of skin (argyria) has been considered as the major concern [251]. In order to examine and evaluate the health and safety impacts of silver, toxicity [252-255] and migration [256-258] tests should be carried out. Generally, the dissolution rate determines the concentration of available silver and is affected by particle size and crystallinity, surface structure, temperature and molecules present in the environment [250]. Kittler *et al.* [250] studied the toxicity of silver nanoparticles against human mesenchymal stem cells and observed that the release of silver ions increased as a function of time, leading to increasing toxicity. Up to 90 % of nanoparticle weight was able to dissolve into an aqueous solution [250].

The use of nanoparticles in food packaging requires a thorough risk assessment. According to the EFSA guidance regarding nanoscience and nanotechnologies in the food and feed chain [259], the use of engineered nanomaterials requires both physico-chemical and hazard characterization. The physico-chemical properties should be characterized in order to determine whether the nanomaterials are representative and relevant in terms of the intended exposure and in order to allow the comparison between different products, manufacturers or tests. Identification and characterization of potential hazards arising from the properties of the materials should include the *in vitro* evaluation of absorption, distribution, genotoxicity, excretion and metabolism, as well as a repeated-dose 90-day oral toxicity tests for rodents. The aforementioned tests should also be accompanied with an uncertainty analysis.

The use of silver in food packaging applications needs to be in compliance with the legislation concerning active packaging (Ch. 2.3.1.1). Currently, several silver-based materials have been approved for food contact by FDA and the United States Environmental Protection Agency (EPA). EFSA has given positive scientific opinions on certain silver-containing materials intended to be used in food contact and a general migration limit of 0.05 mg Ag/kg food [260]. Silver-based antimicrobial agents have been commercialized under trade names such as AgION, Alphasan, Irgaguard, Novaron, Sanitized and Zeomic. However, even though silver is currently allowed as a food additive (E174) for exterior decorative purposes in Annex II of the Regulation (EC) No. 1333/2008 [261], EFSA has indicated the need for re-evaluating its safety [262]. Evaluation on the basis of currently available information was considered insufficient because of the lack of data regarding i.e. the toxicity, particle size distribution and potential silver ion release of E174. Hence, enhancing the E174 characterization with the mean particle size, particle size distribution and nanoparticle percentage was recommended. The characterization methods should comply with the EFSA guidelines [259]; for example, scanning electron microscopy (SEM) or transmission electron microscopy (TEM) could be used.

2.3.1.3 Oxygen absorbers

Oxygen absorbers or scavengers can be used for removing O_2 from a closed package. Packaging of O_2-sensitive food products under low-O_2 MAP or vacuum may not be sufficient to completely avoid the deteriorative effects of O_2 [263-265]. MAP cannot be used for eliminating O_2 that is trapped in the food product or permeate through the packaging materials during storage [265]. Residual O_2 concentrations of up to 0.5-2 % are associated with MAs containing CO_2 and N_2. In the presence of light, these concentration levels contribute to discoloration and off-odor development. [266.]

Absorption of O_2 can be based on several technologies. Iron oxidation has traditionally been the most widely applied technology [265]:

$$Fe \rightarrow Fe^{2+} + 2e^- \quad (3)$$

$$½\ O_2 + H_2O + 2e^- \rightarrow 2\ OH^- \quad (4)$$

$$Fe^{2+} + 2\ OH^- \rightarrow Fe(OH)_2 \quad (5)$$

$$Fe(OH)_2 + ¼\ O_2 + ½\ H_2O \rightarrow Fe(OH)_3 \quad (6)$$

Removal of 300 ml O_2 with 1 g iron has been considered as a rule of thumb [267]. Commercial applications based on iron absorption include Ageless® (Mitsubishi Gas Chemical Co., Japan) and ATCO® (Standa Industries, France) sachets. Another technology is based on photosensitive dye oxidation [268]:

$$photon + dye \rightarrow dye^* \quad (7)$$

$$dye^* + O_2 \rightarrow dye + O_2^* \quad (8)$$

$$O_2^* + acceptor \rightarrow acceptor\ oxide \quad (9)$$

$$O_2^* \rightarrow O_2 \quad (10)$$

where * denotes an excited state. When an ethyl cellulose film containing photosensitive dye and a singlet O_2 acceptor is exposed to light with a certain wavelength, O_2 molecules become sensitized by the dye molecules and react with the acceptors [265]. Thirdly, O_2 is consumed during the oxidation of ascorbic acid ($C_6H_8O_6$) to dehydroascorbic acid ($C_6H_6O_6$) [269]:

$$C_6H_8O_6 + 2\ Cu^{2+} \rightarrow C_6H_6O_6 + 2\ Cu^{+} + 2\ H^{+} \quad (11)$$

$$2\ Cu^{+} + 2\ O_2 \rightarrow 2\ Cu^{2+} + 2\ O_2^{-} \quad (12)$$

$$2\ O_2^{-} + 2\ H^{+} + Cu^{2+} \rightarrow O_2 + H_2O_2 + Cu^{2+} \quad (13)$$

$$H_2O_2 + Cu^{2+} + C_6H_8O_6 \rightarrow Cu^{2+} + C_6H_6O_6 + 2H_2O \quad (14)$$

Oxygen absorbers are typically introduced in the package as separate sachets. These sachets may simultaneously emit CO_2, which compensates for the reduction of O_2 partial pressure and thus prevents package collapse. Alternatively, absorbers can be incorporated in the packaging materials. This approach may be based on using absorbent polymers as packaging materials, dispersing the absorbent in the polymer or developing absorber sheets, cards or adhesive labels. [265.] These solutions can prevent accidental consumption of absorbing agents, reduce negative consumer responses and enhance cost-efficiency when compared to sachet-based solutions [270]. Sufficient O_2 absorbing capacity can be confirmed by calculating the needed capacity on the basis of the packaging material permeability and initial O_2 concentration at the moment of closing the package. Using an absorber with higher absorbing capacity than the calculated capacity allows sufficient removal of O_2 throughout storage time. [265.]

In the scientific literature, O_2 absorber technologies have been utilized with several food products, including fruit and vegetables [271-274], bakery products [275], meat [276; 277] and fish [278; 279]. Commercial applications have been marketed under different trade names, such as Ageless®, ATCO®, OMAC®,OxyGuard® and Shelfplus® O_2 [280].

2.3.2 Intelligent packaging

2.3.2.1 Principles

According to Yam *et al.* [16], intelligent packaging can be defined as a system that detects, senses, records, communicates or applies another intelligent function in order to enhance the safety and shelf life determination, improve quality, communicate information about the product or warn about problems. While a traditional food packaging can express the origin, composition and theoretical expiry date, intelligent packaging solutions can also inform about microbial growth, headspace gas composition or temperature conditions [280]. Information can thus be obtained about the quality of the food product or package integrity. This allows quality monitoring throughout the food supply chain and detection of defects or abuse, which supports the safety and efficiency of the supply chain and reduces food and packaging material waste. [281.] These technologies can also significantly improve traceability [280]. Intelligent packaging of food is an increasing trend and has been discussed in several reviews [16; 264; 280-282]. Even though intelligent packaging can be used to improve the

food supply chain, these technologies are not targeted to affect the properties of the packaged product or its environment, unlike active packaging. According to Vanderroost *et al.* [281], smart packaging solutions combine active and intelligent packaging in a synergistic manner in order to both monitor the changes and act on their basis, meaning that intelligent and smart packaging cannot be considered as synonyms although this has often been done in the literature.

The three main types of intelligent packaging systems are indicators, sensors and radio frequency identification (RFID) tags. Indicators are used for detecting the presence or absence of a substance or reactions between substances, based on a change in indicator properties. Sensors are capable of producing a continuous signal; typically, a receptor transforms physical or chemical information into energy that is further converted to an informative signal by a transducer. On the other hand, RFID tags carry electronic information associated with the asset that they are attached to and can be accessed by a reader. [264.]

Information obtained with indicators is commonly qualitative or semi-quantitative and can be assessed as changes in color or in comparison with a standard. Indicators can be used for assessing package integrity, product freshness or temperature history. Integrity indicators are commonly based on the detection of excess O_2 under low-O_2 MAP. [264.] Time-temperature indicators (TTIs) express an irreversible, temperature-dependent change in mechanical, chemical, electrochemical, microbiological or enzymatic properties of the product as a visible response [283]. TTIs thus inform about the temperature history of the package either during the whole storage time or after exceeding a defined threshold. On the other hand, freshness indicators respond to microbiological or chemical changes in the product, for example by reacting with spoilage metabolites. Since the generation of metabolites depends on the food product, packaging and storage conditions as well as spoilage microbiota, indicators can be targeted against defined marker metabolites. These may be organic acids, amines, ethanol, sulfuric compounds or CO_2. [264.]

In food technology, sensor applications allow non-destructive, fast and efficient quality analysis. Sensors can be used to detect changes in temperature, pH, humidity, light exposure or chemical reactions. Chemical sensors respond to the presence, composition, concentration or activity of their target analytes. In particular, monitoring of VOCs or gas molecules related to spoilage has been considered interesting under MAP conditions. However, various challenges are associates with sensor development. Small size and sufficient sensitivity, robustness and costs-efficiency are essential for facilitated and efficient use of these technologies. The sensor applications must also comply with the current legislation and food safety. [281.]

2.3.2.2 Applications for meat and fish

TTIs have been used for monitoring the temperature history of meat and fish. These have commonly been based on irreversible color changes that are due to photochromic reactions [284], polymerization reactions [90] or pH decrease caused by enzymatic reactions [285; 286]. Microbial growth and

subsequent pH decrease have also been used as an initiator of color changes [287-290]. Furthermore, different TTI types have been compared [291-293].

Non-destructive monitoring of fish quality has commonly focused on the production of spoilage metabolites, especially those belonging to the NPN fraction. Heising *et al.* [294] monitored the freshness of Atlantic cod by measuring ammonia in the aqueous phase of the package with an ammonium ion selective electrode. Subsequently, Heising *et al.* [295] developed a mathematical model to predict the freshness of Atlantic cod on the basis of TMA content in the aqueous phase. The model parameters were either based on physical and chemical principles or experimentally estimated on the basis of non-destructive sensor measurements and destructive TMA analyses [295].

Electronic noses commonly utilize sensors in order to imitate olfactometry testing [296] and have been applied for different meat and fish products [297-301]. Ólafsdóttir *et al.* [302] used an electronic nose containing electrochemical gas sensors to monitor the VOC evolution of capelin and found that the results were in accordance with traditional TVB-N measurements. Di Natale *et al.* [303] compared the performance of two electronic noses in evaluating the freshness of Atlantic cod: most accurate results were obtained by combining the two technologies.

Even though the number of commercially available freshness indicators is currently limited, different applications have been presented in the literature [280]. Studies regarding meat and fish quality monitoring have generally focused on the produced VOCs. Spoilage has been monitored by colorimetric detection of pH changes that occur as a response to increasing volatile amine concentrations [304-307]. Boscher *et al.* [308] developed a metalloporphyrin-based coating for the colorimetric detection of volatile amines produced in fish. Respectively, biosensors aiming at the detection of spoilage metabolites have been developed for amines [309], glucose [310] and xanthine [311; 312].

3 Aims of the study

As shown in Ch. 1-2, the complexity, inevitability and high societal impact of food spoilage calls for active and intelligent packaging technologies. The research problems and knowledge gaps related to the scope of this dissertation can thus be summarized as follows:

- Despite the high importance of the food quality characterization process in the development of active and intelligent packaging technologies, the central features, requirements and challenges that affect the setup and performance of this process need to be defined
- Traditional methods and techniques used for quality characterization are not always applicable or feasible for producing information for the development of said packaging technologies
- Information about several specific topics within the context of active and intelligent packaging is limited in the scientific literature, including:
    - Silver as an antimicrobial agent in muscle food packaging
    - Comparison of high vs. residual vs. absent O_2 levels on pork color
    - The identity and critical headspace concentrations of VOCs that could be used as spoilage indicators in intelligent packaging solutions

Hence, the main purpose of the present dissertation was to examine and develop the food quality characterization process as presented in Ch. 1. Specifically, this was aimed at by examining the properties and spoilage of meat and seafood packaged under different MA conditions and by directly contributing to the development of active and intelligent packaging technologies for these food products. Research activities for this dissertation were realized within the framework of two projects: COMEAT (2742/31/2010, 2010-2014) supported by the Finnish Funding Agency for Technology and Innovation (TEKES) and CheckPack (IWT-SBO-130031, 2013-2017) supported by the Flanders Innovation & Entrepreneurship (VLAIO).

Generally, successful active packaging technologies should have a sufficient and prolonged favorable impact on the quality of the packaged product. The COMEAT project addressed these needs by aiming at the development of packaging technologies for limiting the growth of LAB that are major contributors of meat spoilage when stored under anaerobic and/or CO_2-rich conditions. Within the framework of this project, the aim of this dissertation research was to develop and examine active packaging technologies that could be beneficial for the quality of meat products packaged under MAs. These technologies were used for introducing antimicrobial activity in the food package and for analyzing the effects of these technologies to the microbiota and/or physicochemical properties of raw meat. On the other hand, development of intelligent packaging technologies requires that relations are established between the food quality status and measurable variables, such as concentrations of spoilage-related metabolites. The CheckPack project aimed at the development of an optical sensor that could be used for fast, non-invasive and real-time monitoring of food quality status on the basis of VOCs produced in the package headspace. Within this framework, the aim of this

dissertation research was to examine the spoilage of seafood packaged under MAs and to both identify and quantify VOCs that could be used as spoilage indicators, thus allowing quality monitoring during storage time.

The dissertation addresses the following main research question (M1) and the associated subquestions (S1-S3):

M1 *What are the key aspects of quality characterization within the context of developing active and intelligent packaging technologies for muscle foods?*

S1 *What is the impact of silver-containing packaging films prepared by coextrusion or liquid flame spray (LFS) on the quality and spoilage of vacuum packaged pork? What are the critical factors determining their antimicrobial efficacy?*

S2 *How do different O_2 levels (high-residual-absent) affect the physicochemical properties (headspace gas composition, surface pH and color) of pork packaged under MAs?*

S3 *Which VOCs (identity and quantity) are potential spoilage indicators of Atlantic cod (*Gadus morhua*) and brown shrimp (*Crangon crangon*) stored under MAs? Which criteria can be used for their identification?*

The questions were addressed in the dissertation and the four research papers P1-P4 as described below.

The first research paper (P1) describes the preparation and antimicrobial characterization of silver-containing packaging films. Polymeric silver-containing films were prepared by coextrusion and LFS. Antimicrobial properties of the films were assessed both *in vitro* against different bacteria and in contact with raw pork.

In the second research paper (P2), statistical analysis was applied for examining the impact of different MAs on physicochemical properties of raw pork. The effects of high-O_2 MAP, common anoxic MAP and anoxic MAP enhanced with an O_2 absorber on headspace gases, surface pH and color were evaluated by mixed analysis of variance (ANOVA).

In the third research paper (P3), spoilage of raw Atlantic cod stored under different MAs and air was characterized by microbiological, chemical and sensory analyses in order to identify and quantify VOCs related to spoilage. This was carried out by studying the relations between microbial growth, sensory quality and VOC production.

The fourth research paper (P4) aimed at enhancing the detection of potential spoilage indicators among the VOCs produced in Atlantic cod or brown shrimp stored under MAs. Multivariate statistical analyses were used for characterizing the VOC profile during storage both as exploratory and selective techniques.

On the whole, the present dissertation aimed at defining the central aspects that should be considered when producing information about food quality for the development of active and intelligent packaging technologies for muscle foods. Different aspects arising from the main requirements and characteristics of the food quality and packaging technology development were identified. Throughout the dissertation work, experimental and statistical methods were combined to produce data that responded to the needs of packaging technology development.

4 Materials and methods

In this chapter, a summary of the materials and methods applied in the study is presented. Detailed information about materials and methods can be found in the original research papers P1-P4.

4.1 Preparation of antimicrobial packaging materials

4.1.1 Coextrusion

Coextrusion was used for producing silver-containing packaging films (P1). The films were produced in a continuous roll-to-roll process on the Paper Converting and Packaging Technology pilot line at TUT. Silver-containing masterbatches were dry blended at 1 % (Ag-TiO_2) or at 2-3 % (Irgaguard B5120, BASF, Ludwigshafen, Germany) concentration with LDPE (CA7230, Borealis, Wien, Austria) and introduced as a thin top layer on pure LDPE. The Ag-TiO_2 masterbatch was prepared by melt mixing LDPE and silver substituted titanium dioxide (SG TP8, Silvergreen Oy Ltd., Helsinki, Finland) in Brabender DSE25 twin screw extruder (Duisburg, Germany). Very low adhesion between the films and the substrate (food-grade paperboard, Stora Enso Oyj, Imatra, Finland) allowed removing the substrate without film damage. Screw or line speed was adjusted to produce total coating weight of approximately 20 g/m^2. Control films (LDPE) were analogously produced. The coextrusion films were denoted as Irgaguard 2 % or 3 %.

4.1.2 Liquid flame spraying

LFS was used for coating LDPE films (Ch. 4.1.1) with metallic silver particles (P1). LDPE sheets (27.5 x 19.5 cm) were coated on a laboratory scale conveyor line [313]. Silver nitrate (Sigma-Aldrich, Germany) in ion exchanged water was used as a precursor at 500 mg/ml (big particle size) or 125 mg/ml (small particle size) concentration. Each substrate sheet was coated 1, 2 or 4 times with precursor feed rate 2 ml/min, burner distance 20 cm, line speed 50 m/min and gas flow rate 40/20 lpm (H_2/O_2). The LFS films were denoted as B1, B2, B4, S1, S2 and S4, where the letter indicates the particle size (B: big, S: small) and the number the amount of coating times.

4.1.3 Transmission electron microscopy

During LFS (P1), a transmission electron microscopy (TEM) grid (S160-3, Agar Scientific) centered to a sample holder and alumina stick was wiped perpendicularly through the flame to collect the nanoparticle deposit. JEOL JEM-2010 instrument was used at 200 kV acceleration voltage for TEM imaging.

4.2 In vitro antimicrobial assays

4.2.1 Bioluminescence imaging

Bioluminescence imaging (BLI) was used for characterizing the antimicrobial activity of silver-containing packaging materials (P1) against *E. coli* K12 carrying a plasmid pCGSL-1 [314]. Luminescence of initial *E. coli* cultures was examined with Xenogen IVIS 200 Optical Imaging System (Caliperl Life Sciences, USA)) after overnight culturing on antibiotic Luria-Bertani (L-) agar. Light-emitting colonies were incubated overnight in L-broth containing ampicillin (200 µg/ml) and luminescence was examined with Plate Chameleon™ multilabel counter 1.001 (Hidex Ltd., Turku, Finland). Cultures with highest luminescence (over 10^5 counts) were selected for further analysis. Round packaging film samples (diameter 14 mm) were placed active surface up onto six-well plates containing 2 mL L-agar and covered with a mixture of 1 ml of soft L-agar, 500 µl of luminescent culture and 200 µg/ml of ampicillin. Images were taken with a CCD camera at one hour interval for 16 hours with 5 s exposure time. Living Image® 3.1 program (Caliperl Life Sciences, USA) was used for image analysis and the color scale was interpreted according to Männistö *et al.* [315].

4.2.2 Antimicrobial assay against meat spoilage bacteria

Antimicrobial assay against typical meat spoilage bacteria was based on ASTM E2180-07 (2012) (P1). *Leuconostoc gelidum* subsp. *gasicomitatum* (LMG 18811^T), *Lactobacillus sakei* (23K), *Lactococcus piscium* (MKFS47), *Carnobacterium divergens* (DSMZ 20623^T), *Brochothrix thermosphacta* (DSM20171^T) and *Hafnia alvei* (DSM30163) were cultured for 24 h at 25 °C. Oxoid (Basingstoke, UK) growth media GM17 (*L. piscium* and *C. divergens*), de Man-Rogosa-Sharpe (MRS; *L. gelidum* subs. *gasicomitatum* and *L. sakei*) or Tryptic Soy Broth (TSB; *B. thermosphacta* and *H. alvei*) were used. A mixture of bacterial culture (1 ml) and 99 ml NaCl-water agar (0.85 % NaCl + 0.3 % agar, 0.5 % glucose for *L. gelidum* subs. *gasicomitatum* and *L. piscium*) was prepared and pipetted (0.5 ml) on 2 x 2 cm packaging film samples. The samples were incubated at 25 °C for 24 ± 2 h; anaerobic jars with CO_2 enriched atmosphere (AnaeroGen, Oxoid) were used for LAB. The agars were mixed with 1:10 peptone saline and 5 % horse serum [316] and homogenized for 1 min without sonication. Appropriate serial dilutions were prepared, plated on Tryptic Soy Agar (TSA; *L. piscium*, *C. divergens*, *B. thermosphacta* and *H. alvei*) or MRS (L. *gelidum* subs. *gasicomitatum* and L. *sakei*) and incubated at 25 °C for 5 days, LAB in anaerobic jars.

4.3 Packaging and storage

4.3.1 Pork under vacuum

Pork sirloin, slaughtered up to 24 h prior analyses, was used in the study (P1). Meat was cut to ca. 20 g pieces, wrapped in active film (Ch. 4.1.1 - 4.1.2) and packaged at UH under vacuum (Multivac A 300/168, Sepp. 160 Haggenmuller KG, Wolfertschwenden, Germany) in a high barrier film (90 µm thickness; O_2 transmission rate 1 $cm^3\ m^{-2}\ 24\ h^{-1}\ atm^{-1}$ at 23 °C and 75 % relative humidity; Finnvacum, Östersundom, Finland). The samples were stored at + 6 °C in dark for up to 28 days. Three replicate packages were analyzed per sampling session on a regular basis.

4.3.2 Pork under MAP

Meat obtained from a packing plant was used in the study (P2). Mixed breed pigs were slaughtered at up to 7 months age and 80 - 90 kg carcass weight. Boneless pork loin (310 ± 10 g) was packaged as four stacked cutlets (thickness 1.5-2 cm) at 1:2 product-headspace ratio using trays (O_2 transmission rate 90 cc/m^2/24h, 23 °C, 50 % R.H., measured by MOCON Ox-Tran 2/21 MH, Mocon, Inc., Minneapolis, MN, USA) and top film (O_2 transmission rate 30 cc/m^2/24h, respectively). Two independent experiments A/B and C/D with different MAs (O_2/CO_2/N_2 %) were carried out to compare high-O_2 MAP (80/20/0; A) and common anoxic MAP (0/20/80; B) as well as common anoxic MAP (0/20/80; C) and enhanced anoxic MAP (0/20/80 with an ATCO™ FT-210 O_2 absorber; D). All packages were prepared on day 0, transported to TUT on day 1 at max. 4 °C and stored at 4 ± 0.5 °C in dark prior analyses. Four randomly selected packages per condition were analyzed on a regular basis until day 17.

4.3.3 Atlantic cod

Atlantic cod (min. body weight ca. 4.5 kg) used in the study (P3-P4) was caught in the north Atlantic sea, gutted, filleted and stored under ice without preservatives. The fish was delivered to LFMFP in polystyrene boxes under ice. Fillet portions (217 ± 5 g) were packed under gas-product ratio 2:1 using multilayer packaging trays (PP/EVOH/PP, O_2 transmission rate 0.03 cm^3/tray*24h at 23 °C and 50 % R.H.) and top film (PA/EVOH/PA/PP, O_2 transmission rate 6.57 cm^3/m^2*24h*bar at 23 °C, 50 % R.H. and 1 atm). Independent fish batches were used for each of the five storage experiments (Table 5). Sample-free packages with corresponding gas atmospheres (blanks) were respectively prepared. The samples were stored at (4.0 ± 0.7) or (8.0 ± 0.4) °C and three randomly selected packages were analyzed on each sampling day.

Table 5. Packaging and storage conditions used for Atlantic cod (modified from P3).

	H4	H8	L4	L8	Air
$CO_2/O_2/N_2$ (%)	60/40/0	60/40/0	60/5/35	60/5/35	air
Temperature (°C)	4	8	4	8	4
Days of analysis	0,4,5,6,7,8,11,13	0,3,4,5,6,7	0,4,5,6,7,11,13	0,3,4,5,6,7	0,1,2,3

After sampling, remaining sample material was packaged under vacuum in high barrier film bags (O_2 transmission rate < 2.7 cm^3/m^2*24h*bar at 23 °C and 0 % R.H.) and stored at -32 °C for less than 120 days (sensory evaluation, Ch. 4.7) or one year (sequencing, Ch. 4.8).

4.3.4 Brown shrimp

Brown shrimp used in the study (P4) was caught in the north Atlantic Sea, sorted according to size and washed before cooking according to normal Belgian fishing practices. No additives or preservatives were added during processing. The cooked shrimp was cooled, stored overnight in plastic bags under ice, brought onshore the following morning, transported to LFMFP and hand peeled while being kept on ice. Portions of 150 ± 2 g were packaged at 2:1 headspace-product ratio in multilayer packaging materials (Ch. 4.3.3). Two independent shrimp batches were packaged under MAs ($CO_2/O_2/N_2$ %) 50/0/50 or 30/0/70 and stored at (4.0 ± 0.7) °C for up to 12 days. Three randomly selected packages were analyzed on each sampling day. After sampling, the remaining shrimp was packaged under vacuum using high barrier film bags (Ch. 4.3.3) and stored at -32 °C for no longer than 70 days.

4.4 Microbiological analysis

Growth media used for the microbial enumerations are presented in Table 6.

Table 6. Growth media used for the enumeration of pork (P1), cod (P3) or shrimp (P4) microbiota.

Growth medium	Distributor
Plate count agar (PCA)	Oxoid (Basingstoke, UK)
Marine agar (MA)	Difco (Le Point de Claix, France)
de Man-Rogosa-Sharpe agar (MRS)	Oxoid
Modified MRS	Prepared at LFMFP
Iron Agar Lyngby (IAL)	Oxoid
Pseudomonas agar (PA1)	Merck (Darmstadt, Germany)
Pseudomonas agar (PA2)	Oxoid
Streptomycin sulfate thallium acetate actidione agar (STAA)	Oxoid
Violet red bile glucose agar (VRBGA)	LabM (Bury, UK)

From each individual piece of pork (P1), 10 g was aseptically weighed, homogenized (1:10) with 0.1 % peptone saline in a stomacher blender (Seward, Worthing, UK) and appropriate decimal dilutions were prepared. Aerobic bacteria were incubated on PCA, LAB on MRS in anaerobic jars, pseudomonads on PA1, *B. thermosphacta* on STAA and Enterobacteria on VRBGA. Plates were incubated at 25 °C for 2 (STAA, VRBGA) or 5 days (PCA, MRS) or at 37 °C for 2 days (PA1).

From each individual seafood portion (P3-P4), 30 ± 0.1 g was aseptically weighed, homogenized (1:10) with 0.1 % peptone saline in a stomacher blender (LED Techno, Heusden-Zolder, Belgium) and appropriate decimal dilutions were prepared. Total plate counts (TPC) were determined on MA, LAB on MRS or mMRS, H_2S producers on IAL, pseudomonads on PA2 and *B. thermosphacta* on STAA. Plates were incubated at 22 °C for 2 (PA and STAA), 3 (MRS and IAL) or 5 days (MA).

4.5 Physicochemical properties

4.5.1 Headspace gases

Headspace gas composition (% O2/CO2) was determined with CheckPoint O2/CO2 gas analyzer (PBI Dansensor A/S, Ringsted, Denmark; P2) or CheckMate 9000 (Dansensor; P3) from the package headspace. One (seafood; P3) or three (pork; P2) consecutive gas samples were analyzed from each package.

4.5.2 pH

pH of pork packaged under vacuum (P1) was determined with pH meter (Inolab 720, WTW, Weilheim, Germany) from the first dilution of the homogenate in peptone saline. Surface pH of pork packaged under MAP (P2) was determined with HI1413B surface pH electrode and HI7662 temperature electrode connected with HI2210 pH meter (Hanna Instruments, Woonsocket, RI, USA) from six spots of the topmost stacked cutlet immediately after opening the package. pH of cod (P3) was determined from the fillet portions by a pH electrode (Lab® 427, Mettler Toledo GmbH, Schwerzenbach, Switzerland) connected with a pH meter (SevenEasy, Mettler Toledo GmbH) within 30 minutes after opening.

4.5.3 Color

Color of pork (P2) was determined three consecutive times from the topmost cutlet before and after 20 min blooming time under room temperature and illumination using a portable chromameter (CR-210, Konica Minolta, Osaka, Japan), the CIELAB system, illuminant C and observation angle 2°. Color of cod (P3) was determined through a small Petri dish (diameter 230 mm) with a Spectrophotometer CM 2500d (Konica Minolta Sensing Inc., New Jersey, USA) and SpectraMagic™ NX software using the CIELAB system, illuminant D65 and observation angle 10°.

4.6 Volatile organic compounds

Literature survey and preliminary experiments were used for selecting seafood VOCs to be quantified with a selected-ion flow-tube mass spectrometer (Voice 200, Syft Technologies™, Christchurch, New Zealand) using Multiple Ion Monitoring (MIM) mode (P3-P4; Table 7). The package headspace was sampled twice with a constant flow rate during 60 seconds. Package collapse was avoided by using a needle inlet connecting the headspace to atmospheric air. Blanks (n=9-14) were randomly analyzed throughout storage time for each atmosphere, respectively.

Table 7. VOCs quantified during storage of Atlantic cod (C) or brown shrimp (S), mass to charge ratios (m/z), branching ratios (b) and reaction rate coefficients (k) (modified from P3-P4).

VOC	Precursor	m/z	b (%)	k	Product ion	Seafood
Acids						
Acetic acid	H_3O^+	61	100	2.6 E -09	$CH_3COOH_2^+$	C
	NO^+	90	100	9.0 E -10	$NO^+.CH_3COOH$	C, S
	NO^+	108		9.0 E -10	$NO^+.CH_3COOH.H_2O$	S
	O_2^+	60	50	2.3 E -09	CH_3COOH^+	C
Alcohols						
2,3-butanediol	H_3O^+	91	100	3.0 E -09	C4H10O2+.H+	S
	NO^+	89	100	2.3 E -09	$C_4H_9O_2^+$	C, S
Ethanol	H_3O^+	47	100	2.7 E -09	$C_2H_7O^+$	C, S
	H_3O^+	65			$C_2H_7O+.H_2O$	C,S
	H_3O^+	83			$C_2H_7O+.(H_2O)_2$	C,S
3-methyl-1-butanol	H_3O^+	71	100	2.8 E -09	$C_5H_{11}^+$	C,S
	NO^+	87	85	2.3 E -09	$C_5H_{11}O^+$	C,S
Isobutyl alcohol	H_3O^+	57	100	2.7 E -09	$C_4H_9^+$	
	NO^+	73	95	2.4 E -09	$C_4H_9O^+$	C,S
	O_2^+	33	50	2.5 E -09	CH_5O^+	C,S
2-propanol	H_3O^+	43	80	2.7 E -09	$C_3H_7^+$	S
Aldehydes						
2-methylpropanal	O_2^+	72	70	3.0 E -09	$C_4H_8O^+$	C
3-methylbutanal	NO^+	85	100	2.4 E -09	$C_5H_9O^+$	C
Ketones						
Acetone	H_3O^+	59	100	3.9 E -09	C_3H_7O+	C, S
	NO^+	88	100	1.2 E -09	$NO^+.C_3H_6O$	C, S
Acetoin	O_2^+	88	20	2.5 E -09	$C_4H_8O_2^+$	C, S
Butanone	NO^+	102	100	2.8 E -09	$NO^+.C_4H_8O$	S
2-pentanone	H_3O^+	87	100	3.9 E -09	$C_5H_{11}O^+$	S
	H_3O^+	105		3.9 E -09	$C_5H_{11}O^+.H_2O$	S
	NO^+	116	100	3.1 E -09	$NO^+.C_5H_{10}O^+$	C, S
Sulfur compounds						
Carbon disulfide	O_2^+	76	100	7.0 E -10	CS_2^+	S
Dimethyl sulfide	H_3O^+	63	100	2.5 E -09	$(CH_3)_2S.H^+$	C

	NO^+	62	100	2.2 E -09	$(CH_3)_2S^+$	C, S
Dimethyl disulfide	H_3O^+	95	100	2.6 E -09	$(CH_3)_2S_2.H^+$	C, S
	NO^+	94	100	2.4 E -09	$(CH_3)_2S_2^+$	C, S
	O_2^+	94	80	2.3 E -09	$(CH_3)_2S_2^+$	C
Dimethyl trisulfide	H_3O^+	127	100	2.8 E -09	$C_2H_6S_3H^+$	C
	NO^+	126	100	1.9 E -09	$C_2H_6S_3^+$	C
Hydrogen sulfide	H_3O^+	35	100	1.6 E -09	H_3S^+	C, S
	H_3O^+	53		1.6 E -09	$H_3S^+.H_2O$	S
	O_2^+	34	100	1.4 E -09	H_2S^+	C, S
Methyl mercaptan	H_3O^+	49	100	1.8 E -09	$CH_4S.H^+$	C, S
	H_3O^+	67		1.8 E -09	$CH_4S.H^+.H_2O$	S
Esters						
Ethyl acetate	NO^+	118	90	2.1 E -09	$NO^+.CH_3COOC_2H_5$	C, S
	O_2^+	31	20	2.4 E -09	CH_3O^+	S
Ethyl propanoate	H_3O^+	103	95	2.9 E -09	$C_2H_5COOC_2H_5.H^+$	C
	NO^+	132	60	2.5 E -09	$NO^+.C_2H_5COOC_2H_5$	C
Amines						
Ammonia	H_3O^+	18	100	2.6 E -09	NH_4^+	C, S
	H_3O^+	36		2.6 E -09	$NH_4^+.H_2O$	S
	O_2^+	17	100	2.4 E -09	NH_3^+	C, S
Dimethylamine	H_3O^+	46	100	2.1 E -09	$(CH_3)_2N.H^+$	C, S
Trimethylamine	H_3O^+	58	10	2.0 E -09	$C_3H_8N^+$	S
	H_3O^+	60	90	2.0 E -09	$(CH_3)_3N.H^+$	C, S
	NO^+	59	100	1.6 E -09	$(CH_3)_3N^+$	C
Other						
Ethylene oxide	NO^+	74	100	1.0 E -10	$C_2H_4O.NO^+$	S

Concentrations were averaged over data points per scan and relative standard deviations ($SD_{\%}$) of each VOC during the scan were calculated:

$$SD_{\%} = SD_m/x_m * 100\ \% \quad (15)$$

where x_m is the average and SD_m the standard deviation of a single SIFT-MS measurement. VOCs with over 25 % average $SD_{\%}$ during storage within a certain packaging condition were excluded from further analyses.

In P4, VOC concentrations that were applied for statistical analyses (Ch. 4.9) were the mean values of the measured concentrations. In P3, a limit of quantification (LOQ) was calculated for each VOC:

$$LOQ = x_{bl} + 6*SD_{bl} \quad (16)$$

where x_{bl} is the average and SD_{bl} the standard deviation of the blanks [317]. From each measured concentration exceeding the LOQ, background (average of the blanks) was reduced.

4.7 Sensory evaluation

4.7.1 Test preparations

Pork (P1) was evaluated by a trained panel of 5 persons at UH. The panelists had received a training for the evaluation of raw meat, including a practical training for defect detection using standard solutions. Samples were equilibrated to room temperature and fresh pork from the same batch was used as a reference.

Seafood (P3-P4) was evaluated by a panel consisting of up to 14 laboratory staff members (8-12 on a given evaluation session) at UGent who had experience in seafood sensory evaluation and did not have defects in olfactory capacity. Due to the subjective nature of the tests, the seafood panelists were not trained like the pork panelists. Frozen seafood samples were cut to 5.0 ± 0.1 g portions and thawed at 2 °C overnight prior analyses in odorless, transparent plastic cups (diameter 67 mm; AVA, Temse, Belgium) closed with lids (AVA). One out of three daily replicates (A-C) was randomly selected for an evaluation session. The samples were presented to the panel at 4 °C labelled with three-digit random codes and fresh seafood from the same batch was used as a reference.

4.7.2 Ranking tests

Four seafood samples (P3-P4) were simultaneously presented to the panel and ranked from least fresh (1) to most fresh (4). A second test was performed for the critical days identified by the first test if applicable. The collected data was subjected to Friedman and least significant difference (LSD) tests (Excel 2013 for Windows).

4.7.3 Acceptance tests

Acceptance of pork (P1) and seafood (P3-P4) was evaluated on a five-point scale; different terminology was used when aiming at defect analysis (pork) or quality stage characterization (seafood). Acceptance of pork (odor and appearance) was evaluated on a numerical scale (1-5, where 1 = severe defect, spoiled, 2 = clear defect, spoiled, 3 = mild defect, satisfactory, 4 = good, 5 = excellent). Median of 2 or below was used for indicating spoilage. Acceptance of seafood was evaluated on a verbal scale (very good, good, satisfactory, marginal, spoiled); numbers were excluded in order to avoid assuming equal distances between two consecutive terms. Marginal or spoiled were considered as rejection.

4.8 16S rRNA sequencing

4.8.1 Pork

16S amplicon sequencing was performed for triplicate pork samples stored for 10, 17 or 28 days (P1). DNA was extracted from the meat samples using a FastPrep procedure. After extraction, the V1-V3 area was PCR-amplified with primers 8f and 518r [318] using a PCR program with denaturation at 98 ºC for 30 s, 20 cycles at 98 ºC for 10 s, 65 ºC for 30 s, 72 ºC for 10 s, followed by 72 ºC for 5 min and cooling down to 4 ºC. The products were purified and a second PCR was carried out where sample specific barcodes (8 bp) and sequencing adapters were added to the amplified fragments. Prior to purification of a specific sample, three replicate reactions were done and the products were pooled. Purification and sequencing was carried out with Roche 454 Titanium FLX protocol.

QIIME v. 1.7 [319] was used for sequence analysis. Sequence reads were filtered for quality and assigned to samples based on the sample specific barcode. Reads with length less than 200 bp, ambiqous bases, quality score below q30 or mismatches in the primer sequence were omitted from further analysis. Operational taxonomic units (OTUs) were picked with the uclust [320] algorithm with 97 % similarity. Representative sequence read from each OTU were assigned to taxonomy with BLAST [321] against the Greengenes database (Version 13.8.2014) [322].

4.8.2 Atlantic cod

Fish samples were selected for 16S amplicon sequencing so that early, intermediate and late stages of each storage experiment were represented (P3). One out of three daily replicates was selected for the analysis. DNA was extracted from the fish samples using a FastPrep procedure. Library preparation and sequencing was carried out according to De Vrieze *et al*. [323].

The sequencing data was processed according to the guidelines developed by P. Schloss [324; 325] using the mothur software package v. 1.38.0 [326]. Contigs with lengths outside of the 2.5 - 97.5 % quantiles or sequences with ambiguous base calls were removed and the remaining unique sequences were aligned to the mother-reconstructed SILVA Seed alignment v. 123 [327]. Unique sequences were pre-clustered within a distance of 1/100 nucleotides. Chimeras were screened with UCHIME [328] and sequences were classified using RDP v. 14 [329] and Wang's algorithm.

After removing non-bacterial sequences, OTUs were clustered using average linkage and 97 % sequence identity. Single-read OTUs were discarded from further analyses. Rarefaction curves and different richness, density and evenness estimators were produced for alpha diversity analysis.

4.9 Statistical analysis

4.9.1 Analysis of variance

ANOVA was used for characterizing the development of meat microbiota in contact with silver-containing packaging materials (P1). One-way ANOVA and Tukey post hoc test (SPSS Statistics v. 22 for Windows) were used for analyzing significant differences ($p > 0.05$) in the composition of microbiota between meat samples from different packages at a given time point (day 10, 17 or 28).

Mixed ANOVA was used for analyzing the evolution of the studied physicochemical variables in pork sirloin packages (P2). All analyses were carried out separately for the two storage experiments (A/B and C/D) with R 3.3.1 [330], using individual packages as samples. Prior ANOVA, homogeneity of variances and normality of residuals was examined. Two-way mixed ANOVA and Tukey post hoc test were used for analyzing significant differences ($p < 0.05$) in headspace gas concentrations and pH between means (n=4 per time point and atmosphere):

O_2, CO_2 or pH ~ Atmosphere*Storage time, random=~1|SampleID (17)

where atmosphere and storage time were treated as between samples factors and sample ID as a within samples factor. The main effects and interaction of atmosphere and storage time was examined. Respectively, three-way mixed ANOVA and Tukey post-hoc test were used for analyzing significant differences ($p < 0.05$) in color:

L*, a* or b* ~ Atmosphere*Storage time*Blooming time, random=~1|SampleID (18)

where blooming time (0 or 20 min) was treated as a within samples factor and other factors as in Eq. (17). In case of a non-significant interaction, post hoc tests were carried out for significant lower-order interactions or main effects.

4.9.2 Hierarchical cluster analysis

Agglomerative hierarchical cluster analysis (HCA) was used in the exploratory analysis of the VOC profile during storage of brown shrimp (P4). Daily replicates A-C were treated as samples and VOCs as variables. Prior analyses, VOC data was converted to logarithmic values and standardized to z-scores. Euclidean distance and average linkage were used for constructing heat maps and dendrograms with R 3.3.1.

4.9.3 Principal components analysis

Principal components analysis (PCA) was used in the exploratory analysis of VOC evolution during storage of brown shrimp (P4). Logarithmic and standardized VOCs were used for producing PCA

biplots. Suitability for data reduction was tested with Bartlett's sphericity test and sampling adequacy with Kaiser-Meyer-Olkin (KMO) test [331] using R 3.3.1.

4.9.4 Partial least squares regression

Partial least squares regression (PLS) was used in the exploratory analysis of VOCs produced during storage of brown shrimp as well as for the selection of most potential spoilage indicators of Atlantic cod and brown shrimp (P4). JMP v. 12, the non-linear iterative partial least squares (NIPALS) algorithm and leave-one-out cross validation was used for producing PLS biplots where logarithmic and standardized VOCs were used as predictor variables and time, TPC or sensory rejection % as the response variable. For each variable, variable importance in projection (VIP) values and regression coefficients were determined. VOCs with positive correlation with the response variable, VIP > 1 and a positive regression coefficient were considered to be potential spoilage indicators.

5 Results and discussion

In this chapter, main results of the dissertation are presented and discussed. Detailed description of the results and discussion is presented in the original research papers P1-P4.

5.1 Spoilage of pork

5.1.1 Effect of silver

Antimicrobial activity of silver is dependent on its concentration and availability (P1). BLI results (Fig. 2) showed that increasing concentration of nano-scale silver lead to improved antimicrobial efficiency against bioluminescent *E. coli*.

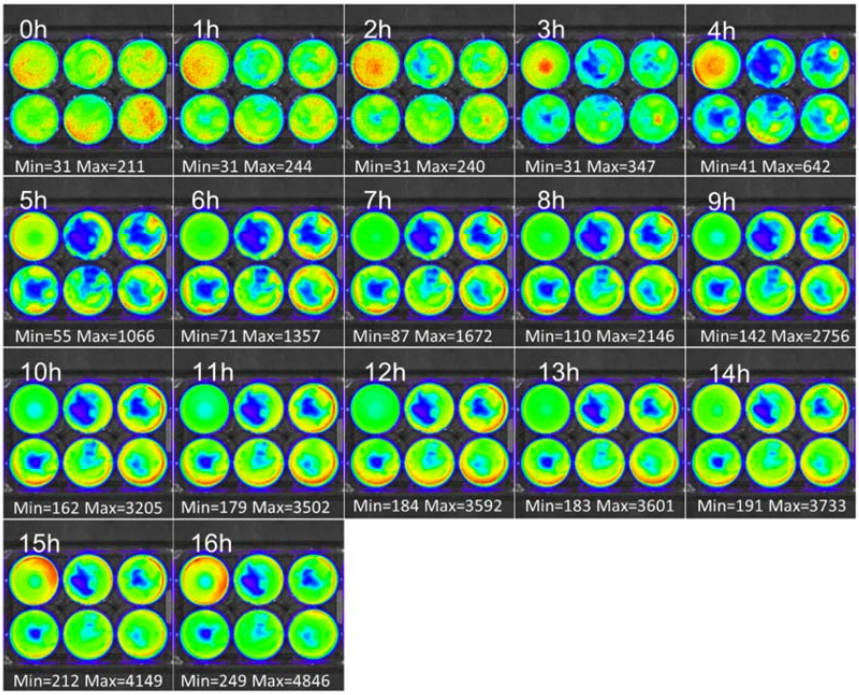

Fig. 2. Bioluminescence of E. coli K12 carrying a plasmid pCGSL-1 in the presence of silver-containing packaging films prepared by LFS. Plate top row: control, B4, S4; bottom row : B2, S2, B1; the letter indicates the particle size (B: big, S: small) and the number the amount of coating times. Each image (0-16 h) has individual color scale expressed as minimum and maximum counts. (P1)

Blue inhibition zones appeared on and around the packaging films samples containing nanosilver coating, suggesting that the coating was highly inhibitive against *E. coli* and that inhibition was enhanced by increasing LFS coating times. Inhibition occurred within the first five hours after exposure to nano-scale silver, whereas packaging films prepared by coextrusion had no impact on microbial growth throughout the study. These results highlight the importance of the availability of active silver. Silver was most likely captured within the polymer matrix during coextrusion, preventing its migration and thus limiting its availability in comparison with LFS films where silver was applied as a loose coating and was thus able to diffuse to the growth medium surroundings. Furthermore, concentration of silver was greatly lower in coextruded films since it was introduced in carrier systems containing low levels of silver. As a conclusion, the superior antimicrobial efficiency of LFS films over coextruded films could be associated with higher silver concentration, improved surface contact with microbes and higher diffusion capacity. Despite the use of two different silver concentrations during LFS (Ch. 4.1.2), no clear differences between the antimicrobial efficacy of materials denoted with B (big) and S (small) particle size were observed. This could be due to the overlap in particle diameter distribution; even though diameters exceeding 10 nm were typically found in B and rarely in S, diameters between 3-15 nm were observed in both material types (P1). Given that a small size and high surface-to-volume ratio have been suggested to increase the antimicrobial activity of silver nanoparticles [332; 333], further studies about their impact on packaging material performance could be beneficial.

Nano-scale silver was effective against several meat spoilage bacteria *in vitro* (P1). In comparison with silver-free control film, nanosilver was highly effective against *B. thermosphacta*, *H. alvei* and *L. piscium.* Especially Gram-negative microbes were found to be susceptible to silver, in accordance with previous studies [244; 334]. Furthermore, some reduction of *L. sakei* levels and growth inhibition of *C. divergens* was observed. However, none of the tested films affected microbial growth in meat packaging (Fig. 3) and sensory acceptability remained throughout storage under all packaging conditions. Sequencing results indicated that the composition of meat microbiota was highly similar under all packaging conditions and was dominated by lactobacilli, lactococci and leuconostocs. The results thus indicate that respective bacteria that were susceptible to nano-scale silver *in vitro* were not affected *in situ* meat.

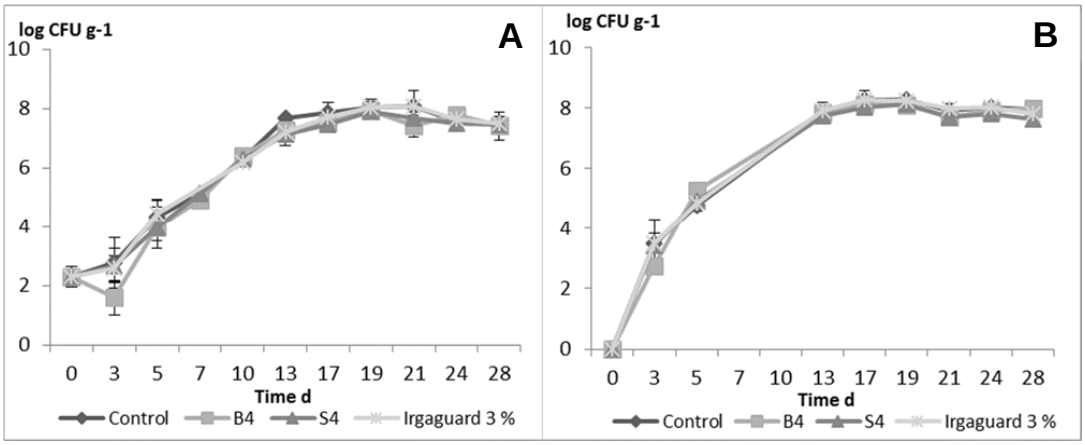

Fig. 3. Total plate counts (A) and lactic acid bacteria (B) in pork samples in contact with silver-containing films prepared by coextrusion (Irgaguard 3 %) or LFS (B4 and S4) (modified from P1). In LFS films, the letter indicates the particle size (B: big, S: small) and the number the coating times.

Antimicrobial efficiency of silver was thus likely lost because of meat properties. (P1) Previously, this phenomenon has commonly been observed during storage of different food products [15; 249; 335-337] and has been associated with the inactivation of silver in the presence of food compounds, reduction of silver ions to elemental silver or insufficient surface contact between silver and bacteria [335; 337]. In meat, reactions between thiol groups and silver have been highlighted [335]. Furthermore, it is possible that in the present study (P1), the *in vitro* efficiency of LFS materials was due to an initial burst effect, given that silver was applied as a loose coating on polymer surfaces. Since prolonged exposure to antimicrobial agents can be considered a more potential approach (Ch.2.3.1.1), extended silver releasing capacity should be emphasized in future studies. However, migration tests should be included to confirm the suggested reasons of activity/inactivity.

Bhakta *et al.* [338] reported that certain LAB may tolerate high levels of heavy metals. This was demonstrated in the present dissertation (P1) as *L. gelidum* subs. *gasicomitatum* was not affected by any of the tested films *in vitro* or in contact with meat. This suggests that silver-based packaging materials could support its growth by inhibiting the growth of more susceptible bacteria (P1). Since *L. gelidum* subs. *gasicomitatum* is a major spoilage bacterium of meat stored under MAs [157; 169; 339], silver resistance could further promote its impact on meat spoilage (P1).

5.1.2 Effect of different oxygen concentrations

The main and interactive effects of atmosphere, storage time and blooming time on several physicochemical variables of pork sirloin packaged under different O_2 concentrations were determined (P2). Three or two-way interactions of atmosphere, storage time and blooming time were commonly found significant (Table 8).

Table 8. Statistical significance (p-values) of modified atmosphere (MA) time (T), blooming (B) and their interactions on the studied variables (P2). Independent statistical analysis was carried out for the two independent storage experiments A/B and C/D, where the letters denote the atmospheres (O_2/CO_2/N_2 %) 80/20/0 (A), 0/20/80 (B or C) and 0/20/80 + O_2 absorber (D).

	O_2		**CO_2**		**pH**		**L***		**a***		**b***	
	A/B	C/D	A/B	C/D	A/B	C/D	A/B	C/D	A/B	C/D	A/B	C/D
MA	<0.0001	<0.0001	<0.0001	<0.0001	0.0259	0.9518	0.4274	0.0496	<0.0001	0.0001	<0.0001	<0.0001
T	0.0032	0.0032	<0.0001	0.0002	<0.0001	<0.0001	0.2709	0.2689	<0.0001	0.1398	0.0199	0.0147
B	-	-	-	-	-	-	0.6088	0.0514	<0.0001	<0.0001	<0.0001	<0.0001
MA x T	0.0050	0.0123	0.0027	0.0055	0.3225	0.2483	0.5211	0.3505	0.0017	0.1491	0.0003	0.2412
MA x B	-	-	-	-	-	-	0.3807	0.0970	<0.0001	0.1210	<0.0001	<0.0001
T x B	-	-	-	-	-	-	0.0018	0.5434	<0.0001	0.7261	0.0012	0.1040
MA x T x B	-	-	-	-	-	-	0.6017	0.6359	<0.0001	0.4490	0.0070	0.1600

However, significant effect could have been due to only a single pairwise difference among a large number of means, which may have been coincidental. Tukey post hoc tests were thus used for determining significant differences between pairs of means. (P2)

During the expected shelf life (ca. 9 days) and even beyond it, most of the studied physicochemical variables remained relatively stable under all studied atmospheres. By the tenth day of storage, significant increase was only observed in yellowness under common anoxic conditions (B) and decrease in CO_2 concentration under enhanced anoxic conditions (D). This limits the potential of the studied variables in meat quality analysis during shelf life. (P2) Quality indicators should be able to give information about the status of meat throughout storage and allow the recognition of expired meat. In the present dissertation (P2), storage time was extended over the expected shelf life in order to examine changes occurring during the shelf life in contrast to progressing spoilage. This allowed the separation of samples from the early and late days of the entire storage time on the basis of gas concentrations. Eventually, slight increase in CO_2 concentration was observed under most of the tested MAs, accompanied with a decrease in O_2 concentration under the high-O_2 atmosphere (A). This was likely affected by an increase in microbial levels and consequent CO_2 production [340]. However, on a given day of storage, differences in gas composition between samples stored under different MAs could often be associated with the selected packaging conditions instead of spoilage-related phenomena. Slight decrease in surface pH was only observed over time when averaged over the levels of atmosphere. (P2) In previous studies, respectively, pH has been stable [185] or decreased along with increasing microbial growth [341; 342]. Especially LAB can grow under modified atmospheres and produce CO_2 and lactic acid during their metabolism [343], thus leading to pH decrease.

Lightness (L*) had no significance in meat quality analysis under the tested storage conditions since it remained relatively constant over the entire storage time and was not affected by blooming under any of the tested atmospheres. Furthermore, significant differences were not observed between samples stored under different atmospheres on a given day of storage. (P2) Analogously, few

changes have been observed over storage time in previous studies. The observed changes have been associated with muscle type [187], decreasing pH [76] or residual O_2 [185]. In this dissertation (P2), no indication of such effects were observed.

Color of meat is dependent on the O_2 concentration in the package headspace. Redness of meat is determined by the levels of oxymyoglobin which is the dominating form of myoglobin in the presence of high O_2 concentrations. [70; 142; 143.] In the present dissertation (P2), decrease in redness under high-O_2 conditions (A) over storage time was thus probably due to decreasing O_2 concentration in accordance with previous studies [71; 72; 185], whereas the low availability of O_2 likely prevented any changes under anoxic conditions (B-D) (Tables 9-10). On the contrary, yellowness remained stable under atmosphere A and expressed some increase under atmospheres B-D (Tables 9-10; P2). Respective results have been previously obtained under anaerobic storage [186; 187], even though in the presence of residual O_2, decrease in redness over storage time has also been reported [184].

Commonly, meat stored under aerobic atmospheres has been observed to be more red and yellow than under anoxic atmospheres [69; 71]. In the present dissertation (P2), this was examined by comparing the pre-blooming color of samples stored under different O_2 concentrations. Even though meat packaged under high-O_2 conditions (A) was more yellow than under common anoxic conditions (B), differences in redness were negligible (P2).

Table 9. Redness (a*) and yellowness (b*) under atmospheres (%O_2/CO_2/N_2) A (80/20/0) and B (80/20/0) before and after blooming (20 min). Significant difference ($p < 0.05$) between time points is indicated by different superscripts a-g within a column. At a given time point and stage of blooming, significant difference between atmospheres is indicated by *. At a certain time point and atmosphere, significant difference between stages of blooming is indicated by S. (P2)

Day	a*				b*			
	A		B		A		B	
	Before	After	Before	After	Before	After	Before	After
1	18.58 ± 1.13^{a}	18.56 ± 1.05^{a}	14.04 ± 0.80^{S}	15.26 ± 0.93^{S}	$10.80 \pm 0.32^{*}$	$10.80 \pm 0.23^{*}$	$4.18 \pm 0.44^{a*S}$	$7.23 \pm 0.36^{a*S}$
3	17.19 ± 2.31^{ab}	17.30 ± 2.28^{ab}	13.96 ± 2.40^{S}	15.14 ± 2.45^{S}	$10.87 \pm 0.49^{*}$	$10.98 \pm 0.49^{*}$	$5.45 \pm 1.41^{ab*S}$	$8.07 \pm 0.84^{ab*S}$
6	$16.87 \pm 0.51^{ab*}$	16.86 ± 0.58^{ab}	$12.09 \pm 0.92^{*S}$	12.93 ± 1.14^{S}	$10.58 \pm 0.51^{*}$	10.53 ± 0.52	$7.10 \pm 0.83^{bc*S}$	8.50 ± 0.57^{abS}
8	15.55 ± 1.18^{abc}	15.51 ± 1.16^{abc}	11.50 ± 1.00^{S}	12.37 ± 1.15^{S}	$10.32 \pm 0.57^{*}$	10.27 ± 0.60	$7.71 \pm 0.90^{bc*}$	8.75 ± 0.36^{ab}
10	14.57 ± 1.40^{abc}	14.70 ± 1.53^{abc}	10.62 ± 0.74^{S}	11.22 ± 1.09^{S}	10.35 ± 0.42	10.43 ± 0.59	9.05 ± 0.84^{c}	9.81 ± 0.62^{b}
13	13.11 ± 1.30^{bc}	13.24 ± 1.46^{bc}	12.66 ± 2.99^{S}	13.31 ± 3.20^{S}	$10.48 \pm 0.24^{*}$	10.78 ± 0.19	$6.36 \pm 2.54^{ab*S}$	8.57 ± 1.17^{abS}
15	11.71 ± 1.30^{c}	11.84 ± 1.38^{c}	12.12 ± 1.79	12.62 ± 2.22	10.13 ± 0.68	10.26 ± 0.53	7.65 ± 2.07^{bcS}	9.06 ± 1.12^{abS}
17	12.00 ± 0.53^{c}	12.02 ± 0.53^{c}	13.65 ± 2.19	13.29 ± 2.02	9.97 ± 0.40	10.05 ± 0.40	7.46 ± 1.47^{bcS}	9.44 ± 0.80^{abS}

Table 10. Redness (a*) and yellowness (b*) under atmospheres (%O2/CO2/N2) C (0/20/80) and D (0/20/80 + O_2 absorber) before and after blooming (20 min). Atmosphere x storage time x blooming time interaction was non-significant ($p > 0.05$). (P2)

Day	a*				b*			
	C		D		C		D	
	Before	After	Before	After	Before	After	Before	After
1	13.23 ± 0.50	14.69 ± 0.48	16.14 ± 0.91	17.16 ± 0.88	5.16 ± 1.04	7.56 ± 0.58	3.20 ± 0.31	6.75 ± 0.39
3	14.51 ± 1.08	15.24 ± 1.69	14.94 ± 0.66	16.06 ± 0.55	5.11 ± 0.85	7.38 ± 0.68	3.57 ± 0.43	7.42 ± 0.59
6	13.54 ± 2.29	13.87 ± 2.97	15.46 ± 1.14	16.02 ± 0.76	5.87 ± 2.57	7.78 ± 0.97	3.86 ± 0.56	7.15 ± 0.35
8	12.02 ± 1.00	12.02 ± 0.98	14.03 ± 0.62	13.94 ± 2.26	7.66 ± 1.85	8.55 ± 1.24	3.18 ± 0.66	6.25 ± 0.61
10	11.27 ± 2.40	11.81 ± 3.36	15.03 ± 0.24	15.68 ± 0.47	7.92 ± 1.12	8.62 ± 0.35	3.83 ± 1.12	6.88 ± 0.68
13	13.80 ± 1.58	13.39 ± 2.35	13.63 ± 1.77	15.33 ± 1.88	6.39 ± 1.85	8.09 ± 0.79	4.79 ± 3.01	7.76 ± 1.77
15	12.37 ± 2.92	12.72 ± 2.97	14.97 ± 0.73	15.55 ± 0.75	6.98 ± 2.03	8.36 ± 1.42	4.92 ± 1.58	8.10 ± 1.16
17	15.22 ± 1.71	15.22 ± 2.44	14.26 ± 0.45	14.63 ± 0.12	6.99 ± 1.23	9.00 ± 0.39	5.22 ± 2.06	8.25 ± 1.48

Blooming occurs when meat that has been stored under low O_2 concentrations is exposed to air. In the present dissertation (P2), blooming time had significant impact only under anoxic conditions (B-D) and lead to increase in both redness and yellowness under atmosphere B until late days of storage. Under atmospheres C-D, respective color changes could be observed after averaging over the levels of time and/or atmosphere. Since color differences between samples from different atmospheres were negligible especially after blooming, this suggests that the common drawback of anoxic meat packaging – discoloration – could have less relevance in light-colored pork. (P2) Since meat color is highly influential to consumer appeal and purchase decision [59; 61-63], this could support the application of anoxic packaging for pork and thus reduce the deterioration caused by O_2 (P2).

Oxygen absorbers can be used for the removal of low O_2 levels from the package headspace. Interference of residual O_2 on meat color and/or blooming has often been observed [60; 184; 188; 189]. In contrast, the use of O_2 absorbers in anoxic packaging has been associated with increase in redness and decrease in yellowness when compared to absorber-free packaging systems [184]. This is in correspondence with the results obtained in the present dissertation (P2) when averaged over the levels of storage time and blooming time (redness) or storage time (yellowness), even though no significant color difference between samples packaged under the two anoxic conditions (C-D) was observed on a given day of storage. These results thus suggest that the blooming ability could be disturbed by residual O_2 and that the use of O_2 absorbers could be beneficial for consumer perception of meat quality. In the present dissertation, O_2 absorption was based on iron powder oxidation and lead to the elimination of residual O_2 (< 1.2 %) during the entire storage time. (P2) Some additional decrease in CO_2 concentration was likely due to reactions between CO_2 and iron [344].

The impact of natural variation between samples was likely to interfere with the analysis of significant differences between means. In the present dissertation, new replicate packages were analyzed daily because of the applied destructive analyses. In order to reduce the impact of variation between replicates, the experimental setup would benefit from repeated analysis of the same samples during storage where applicable. (P2)

5.2 Spoilage of seafood

5.2.1 Composition and growth of Atlantic cod microbiota

The applied atmosphere and temperature affected the microbial growth in Atlantic cod stored at 4 or 8 °C (Fig. 4). The impact of temperature could be observed as accelerated growth at 8 °C when compared to 4 °C. LAB (Fig. 4c) were able to grow under all tested conditions and were not affected by different O_2 concentrations before complete depletion from the package headspace under low-O_2 conditions (L4 and L8). LAB grew to high numbers especially at 8 °C. (P3) Even though MRS has been reported to suppress the growth of certain LAB [165], highly similar results were enumerated on MRS and acetate-free mMRS (P3). Higher levels of H_2S producers were observed under air when compared to MAP (Fig. 4d), in accordance with the TPC results (Fig. 4a). Furthermore, the growth of H_2S producers was favored by low-O_2 over high-O_2 MAP; these results are in line with López-Caballero *et al.* [196], suggesting that O_2 and CO_2 had synergistic inhibitive effect on the H_2S producing microbiota. Growth of pseudomonads was inhibited under MAs, which was most likely due to the inhibitive effect of elevated CO_2 concentrations (Fig. 4e). Pseudomonads have been identified as an SSO of aerobically stored fish [35]; respectively, highest enumerations under air were observed on PA in the present dissertation. Finally, the growth of *B. thermosphacta* was not affected by O_2 concentration and yielded up to 7 log CFU/g (Fig. 4f). *B. thermosphacta* was thus not the most abundant microbe under any of the tested conditions. (P3)

Initial TPC of 5.35 ± 0.18, 5.88 ± 0.19, 5.88 ± 0.11, 6.61 ± 0.13 and 5.94 ± 0.27 log CFU/g was enumerated on MA under conditions H4, H8, L4, L8 and Air, respectively (Fig. 4a). Under both MAP conditions, TPC exceeded 7 log CFU/g on day 4 at 4 °C and on day 2 at 8 °C. Under air (4 °C), this occurred on day 2 of storage. After day 0, TPC was constantly 0.5-1 log higher on MA (Fig. 4a) when compared to IAL (Fig. 4b). This suggests that the composition and/or incubation conditions of MA was more favorable for the growth of cod microbiota. (P3) Broekaert *et al.* [345] studied the growth of typical spoilage microbes isolated from marine fish and found that most microbes were able to grow on MA, including *P. phosphoreum* that is generally recognized as an SSO of marine fish [11; 86; 165; 346]. According to the oligotyping results (Fig. 5), the *Photobacterium* genus was typically a small contributor in the initial microbiota and had the highest relative abundance at later stages of storage under all tested conditions.

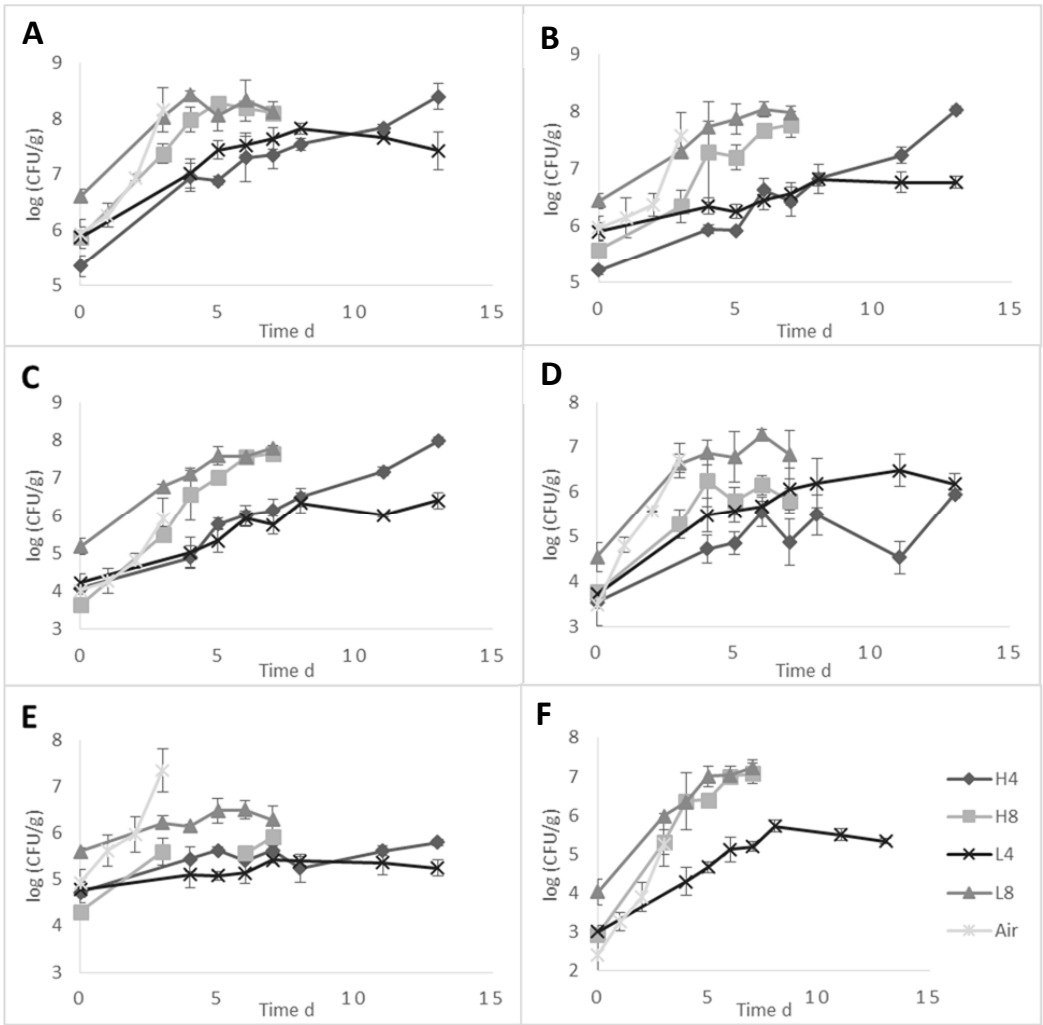

Fig. 4. Microbial growth in Atlantic cod fillet portions stored under atmospheres H4 (60 % CO_2 /40 & O_2/0 % N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C): total viable psychrotrophic bacteria enumerated on Marine Agar (A) or Iron Agar Lyngby (B), lactic acid bacteria (C), H_2S producers (D), pseudomonads (E) and *Brochothrix thermosphacta* (F). (modified from P3)

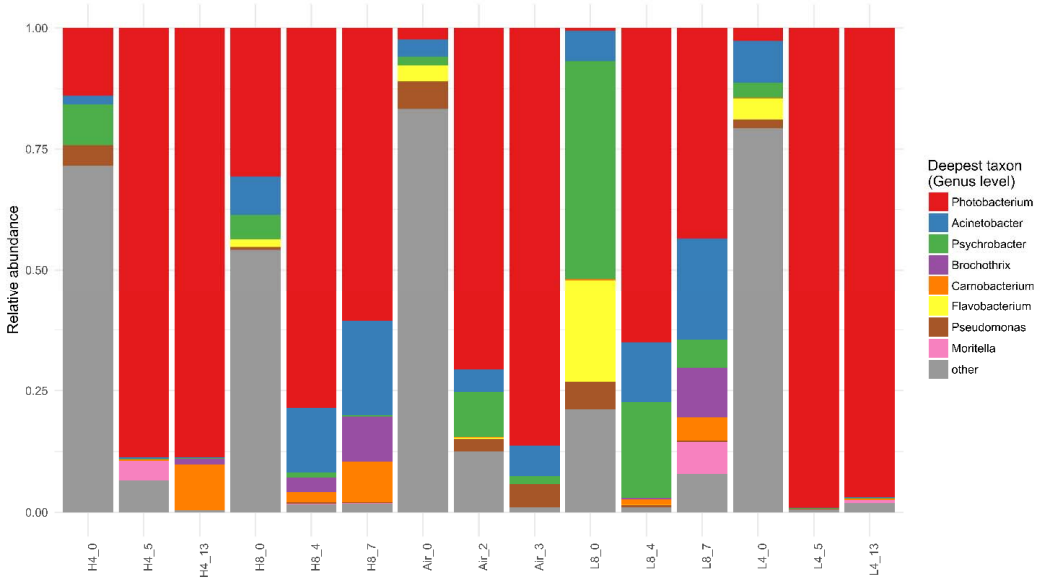

Fig. 5. Composition of Atlantic cod microbiota stored under conditions H4 (60 % CO_2 /40 & O_2/0 % N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C). The sample code denotes the condition and day of storage. (P3)

The difference in TPC enumerated on MA and IAL could thus be due to the heat sensitivity of *P. phosphoreum* and high temperatures (ca. 50 °C) associated with the preparation of IAL pour plates (P3).

The composition of cod microbiota was highly diverse in the beginning of storage (Fig. 5). In total, 503 OTUs were retained at the 97 % sequence identity threshold after data processing and the number of reads was between 215-96819 per sample. The community diversity was highest on day 0 under all tested conditions. The most abundant genera were *Acinetobacter*, *Flavobacterium*, *Photobacterium*, *Pseudomonas* and *Psychrobacter*. Over time, *Photobacterium* became the most abundant genus under all tested conditions. At 4 °C, *Photobacterium* was dominant until the end of storage, whereas some decrease in the relative abundance of *Photobacterium* was observed between days 4 and 7 at 8 °C. (P3) However, in order to confirm *P. phosphoreum* as an SSO of Atlantic cod under the studied conditions, the VOC production capacity of microbial isolates should be tested.

5.2.2 VOCs as spoilage indicators

Identification of spoilage indicators is crucial for efficient quality monitoring of muscle foods. VOCs that could be considered as potential spoilage indicators should be produced by the SSOs, increase during storage time and correlate with microbial growth and changes in sensory properties [347]. Development of the VOC profile is a complex event involving a high amount of variables and a wide

variety of intrinsic and extrinsic factors. In the present dissertation, VOCs were quantified with SIFT-MS throughout storage time under different atmosphere/temperature conditions (P3-P4) and subjected to multivariate statistical analyses (P4).

Clustering of VOCs could be visualized as heat maps (P4) that characterized the evolution of VOCs during storage time and similarities in the VOC profiles of different samples, depending on the applied data transformations. Non-transformed data could be used for classifying the VOCs according to their concentration ranges since many VOCs were produced in highly different quantities. On the other hand, logarithmic conversion and standardization (z-scores) of VOCs allowed the characterization of VOC evolution irrespectively of the differences in concentration magnitudes. Clustering of samples was visualized as dendrograms (P4) on the basis of the concentration ranges or evolution of their VOC profiles. Three main patterns of VOCs could be identified in good correspondence with Küntzel *et al.* [348]: those that were increasing throughout storage time (e.g. ethanol, 2,3-butanediol), those that reached a peak during intermediate storage and decreased thereafter (ethyl acetate, ethylene oxide, trimethylamine) and those that showed no clear trend (butanone). Most of the VOCs could be classified into the first group, suggesting that they could be potential spoilage indicators (P4). According to Küntzel *et al.* [348], however, VOCs belonging to the second group could reflect a change in microbial metabolism and should thus also be further considered. In the present dissertation (P4), HCA was found to be a versatile method for characterizing the VOC profile in terms of concentrations as well as evolution, both having relevance in food spoilage analysis. On the other hand, results of the exploratory PCA (P4) were in correspondence with HCA: the same main VOC and sample groups could be distinguished. Most of the VOCs were characteristic to samples from late days of storage and were thus considered as potential spoilage indicators. (P4)

PLS was applied as an exploratory method for the characterization of brown shrimp data. VOCs that had a positive correlation with the response variable, VIP > 1 and a positive regression coefficient were considered as most potential predictors (Fig. 6).

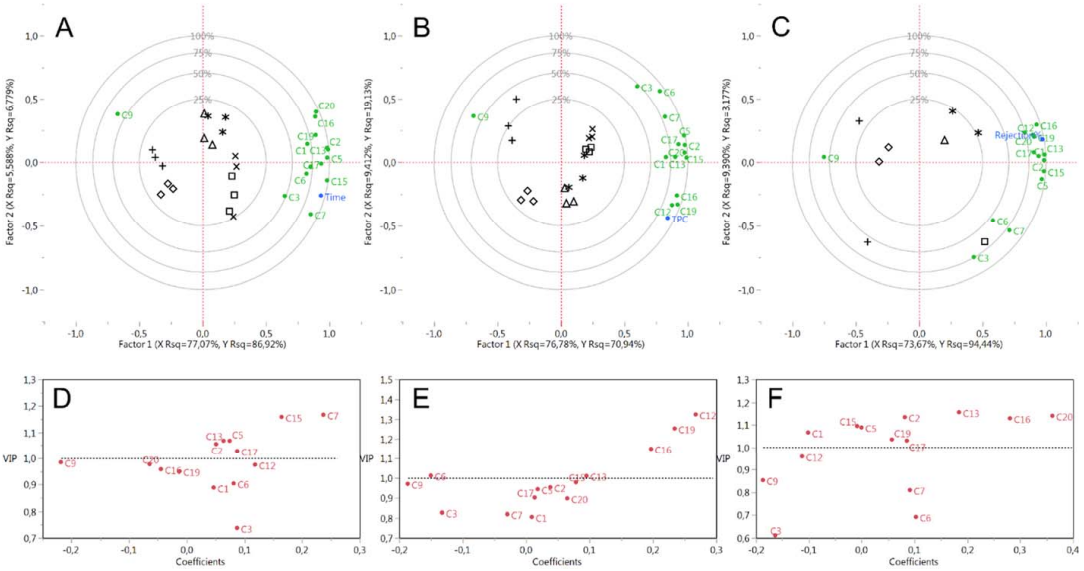

Fig. 6. Partial least squares (PLS) biplots (A-C) and VIP vs. regression coefficient plots (D-F) of brown shrimp: time (A and D), TPC (B and E) or rejection % (C-F) was used as a response variables. Samples are given as scores (day 0: +; day 3: ◊; day 5: Δ; day 7:*; day 10: x; day 12: □) and VOCs as correlation loadings. VOC codes C1-C20 are given in P4. (P4)

The three selection criteria were used in combination in order to optimize the selection of most potential VOCs. (P4) Even though VIP > 1 has generally been used as a cut-off limit for highly influential predictor variables [301; 349], it points out those predictors that are most influential to the model, irrespectively of their correlation with the response. This could lead to the selection of VOCs that decrease over time and have thus little relevance in spoilage analysis, such as butanone (Fig. 6). On the other hand, correlating predictor and response variables do not necessarily have a direct relation. Since several VOCs are likely produced during microbial metabolism, VOCs produced during storage often show multicollinearity and may also be dependent on one another via consumption or degradation. Correlation does not take into account the possible dependencies between VOCs, whereas regression coefficient can be used for analyzing the independent effect of a predictor on its response. From a total of 20 VOCs, six, four and seven VOCs out of 20 quantified compounds fulfilled the three selection criteria when time, TPC and sensory rejection were used as response variables. Since microbial growth reached the stationary and death phase during storage, relatively few VOCs had strong correlations with it. Analysis of VOCs should thus be limited to the log phase of microbial growth. (P4)

On the basis of exploratory analyses, PLS was found advantageous in the selection of potential spoilage indicators and was thus applied for data collected during storage of Atlantic cod and brown shrimp (P3-P4). Many VOCs that fulfilled the three selection criteria were common to different storage conditions and seafood products (Table 11).

Table 11. Potential spoilage indicators of Atlantic cod (C) and brown shrimp (S) stored under different atmospheres (% $CO_2/O_2/N_2$); TPC or rejection % as response variables (modified from P4).

	TPC							**Rejection %**	
	C 4 °C 60/40/0	C 8 °C 60/40/0	C 4 °C 60/5/35	C 8 °C 60/5/35	C 4 °C Air	S 4 °C 50/0/50	S 4 °C 30/0/70	C	S
Acetic acid	x		x		0	x	0	0	0
Ethanol		x							x
2,3-butanediol	x	x	x	x	x	x	0	x	0
2-propanol	-	-	-	-	-			-	
3-methyl-1-butanol	x	x	x	x	x	0	0	x	0
Isobutyl alcohol	x	x		x			0	x	0
2-methylpropanal						-	-		-
3-methylbutanal						-	-		-
Acetone	x				x				
Acetoin	0	0	0	0	0	0	0	0	0
Butanone	-	-	-	-	-			-	
2-pentanone	0		0	0	0	0	0	0	0
Hydrogen sulfide	0	0	0	0	0	0	0	0	0
Methyl mercaptan	0	0	0		0	x		0	
Carbon disulfide	-	-	-	-	-	x		-	
Dimethyl sulfide			x	x	x	x	x		x
Dimethyl disulfide	0	0	0	0	0	0	0	0	0
Dimethyl trisulfide						-	-		-
Ethyl acetate	x	x	x	x	x	x	x	x	x
Ethyl propanoate	0	0	0	0	0	-	-	0	-
Ammonia						x			
Dimethyl amine	0	0	0	0	0	0	0	0	0
Trimethyl amine	x	x	x	x	x	x	x	x	x
Ethylene oxide	-	-	-	-	-			-	

x: fulfilled the selection criteria (VIP < 1, regression coefficient > 0, positive correlation with response)
-: not included in the SIFT-MS analysis
0: relative standard deviation > 25 %

VOCs that were most frequently identified as potential spoilage indicators were 2,3-butanediol, dimethyl sulfide, ethyl acetate, 3-methyl-1-butanol, isobutyl alcohol and TMA (P4). These VOCs have been recognized to be produced during microbial metabolism [30; 95] and have frequently been detected during storage of various seafood products (Appendix 1).

Evolution of the aforementioned potential spoilage indicators in Atlantic cod is presented in Figs. 7-8.

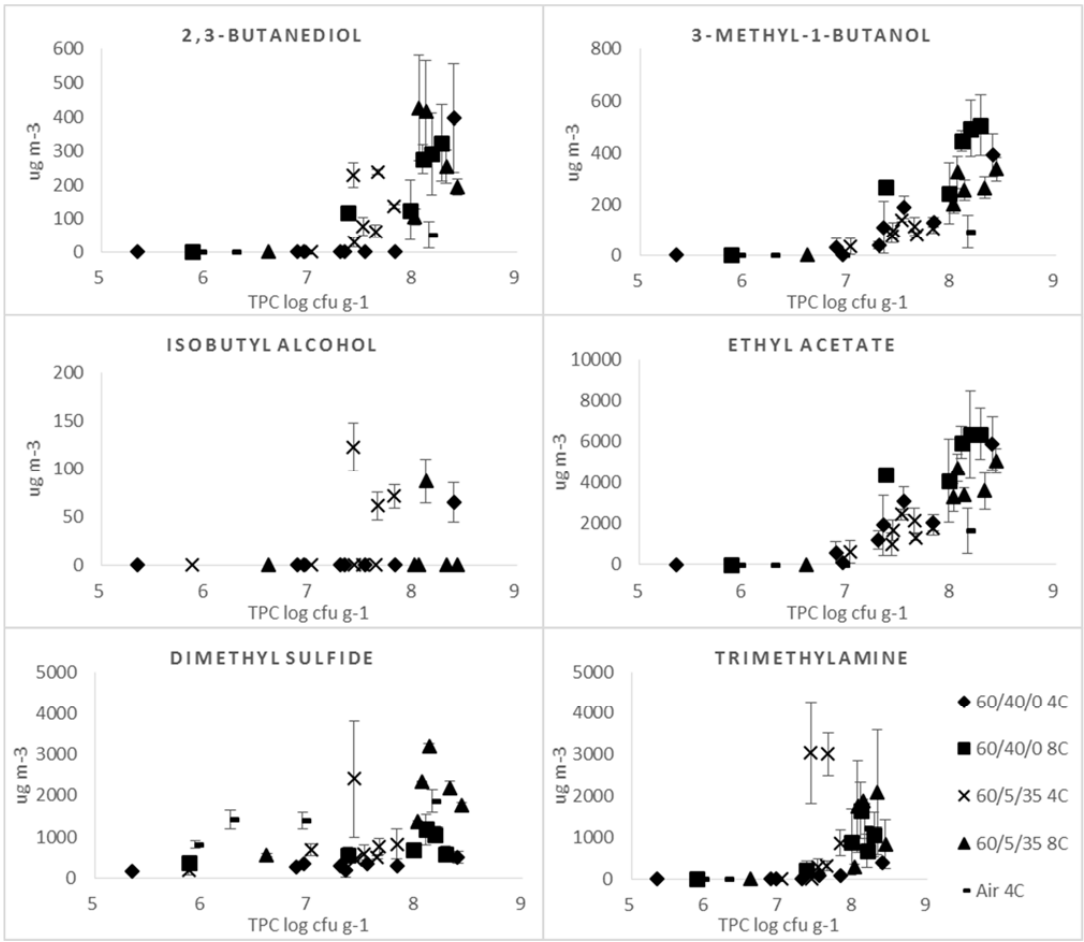

Fig. 7. Production of selected VOCs as a function of total psychrotrophic counts (TPC enumerated on MA) in Atlantic cod packaged under different atmospheres (%CO_2/O_2/N_2) (modified from P3). Concentrations below LOQ were marked as 0 without error bars.

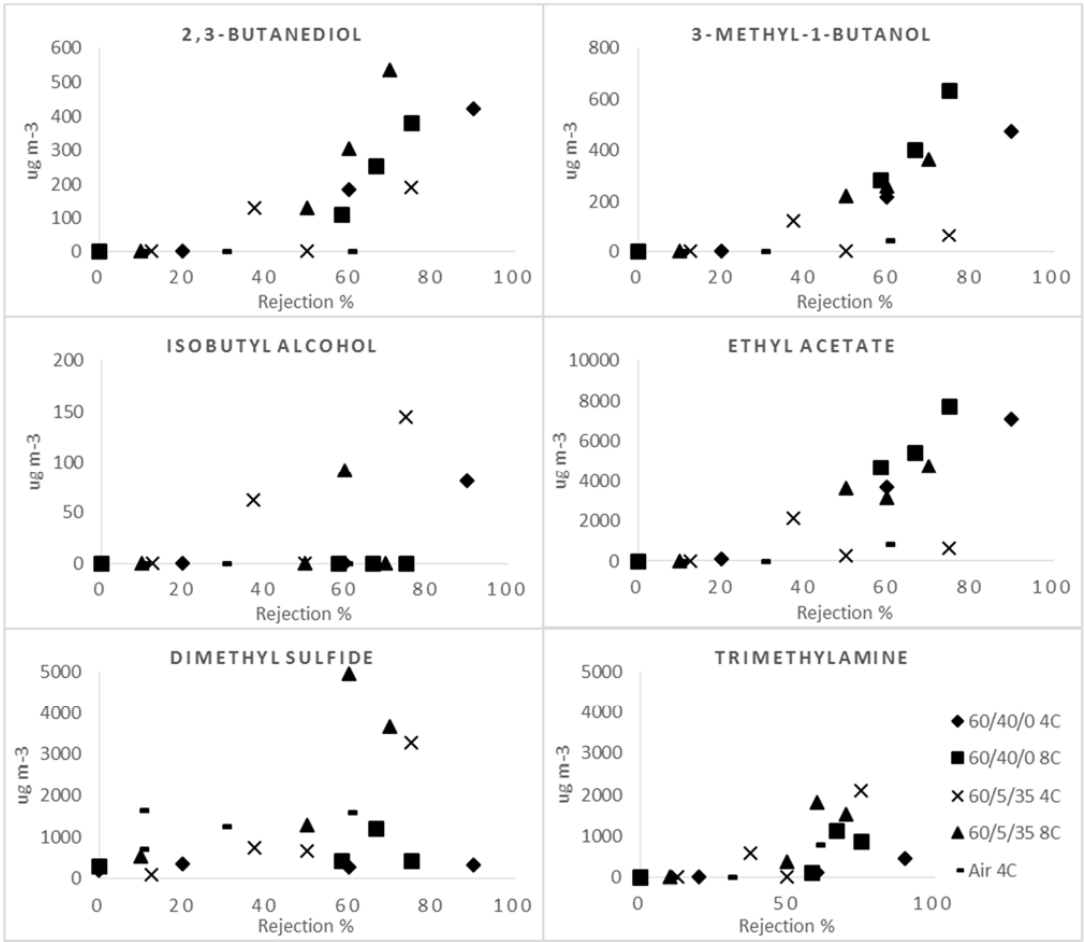

Fig. 8. Production of selected VOCs as a function of sensory rejection (%) in Atlantic cod packaged under different atmospheres (%CO_2/O_2/N_2) (modified from P3).

These VOCs exceeded the LOQ at least under certain storage conditions and increased as a function of microbial growth and sensory rejection. Both 2,3-butanediol and 3-methyl-1-butanol increased up to ca. 500 µg/m^3 under MA conditions, whereas isobutyl alcohol remained below 150 µg/m^3 throughout storage and was characteristic to low O_2 conditions. Ethyl acetate was similarly produced under all tested conditions, whereas higher concentrations of dimethyl sulfide and TMA were typically observed at a certain TPC under low O_2 conditions when compared to atmospheres containing 40 % O_2. Increase in ethanol concentrations was also typically observed. Under MA conditions, ethanol reached the highest concentrations when compared to the other quantified VOCs and exceeded 10^4 µg/m^3 by the end of storage. Because of increasing concentrations detected in the blanks during storage, ethanol rarely exceeded LOQ. Ethanol could thus be considered as an additional candidate among possible spoilage indicators of Atlantic cod. (P3)

Production of VOCs has previously been associated with relatively high microbial levels [198]. In the present dissertation (P3), respectively, the onset of exponential VOC increase could be observed as TPC (MA) had reached 7.0 log CFU/g (Fig. 7). At this moment, ca. 25 % rejection was typically obtained. At 50 % rejection, TPC was typically ca. 7.5 log CFU/g. These results suggest that increasing production of VOCs and subsequent sensory rejection occur at relatively high microbial levels. The high abundance of the *Photobacterium* genus detected in Atlantic cod samples (Fig. 5) suggests that bacteria belonging to this genus contribute to the production of VOCs. (P3) Previously, production of several VOCs also observed in the present dissertation has been associated with the growth of *P. phosphoreum* (Ch. 2.2.3.2).

The impact of a VOC on the overall perceived odor and acceptability of fish depends on its concentration as well as its human olfactory threshold (OT) which is the lowest concentration perceivable by the human olfactory system. OTs can be used for defining odor activity values (OAVs) which represent the concentration of a VOC divided by its OT: VOCs that have OAV below one could be considered to have a low impact on the overall odor of the food product [350]. Different VOCs may have highly different OTs (Table 12).

Table 12. Human olfactory thresholds (OTs) for selected VOCs [351].

VOC	OT µg m-3
Acetic acid	363
Acetone	34673
Ammonia	4073
Dimethyl disulfide	48
Dimethyl sulfide	6
Ethanol	54954
Ethyl acetate	9772
Hydrogen sulfide	27
Methyl mercaptan	2
TMA	6

VOCs having high OTs such as alcohols are only perceivable at high quantities; on the other hand, amine and sulfuric compounds may be detected at very low concentrations. VOCs that have low OTs may thus have high impact on the perceived odor even if they were present in low quantities. However, even though the values determined by Devos et al. [351] have frequently been cited in food-related studies, it should be taken into account that different values may have been reported in other studies (e.g. [352]). Several factors that may affect the determination have been identified, including the experimental setup and panel composition [353] as well as the degree of satiety [354]. Generally, it should be emphasized that the reported OTs have been defined for single compounds in the absence of other VOCs and that the values do not indicate the acceptability of the respective VOCs. Instead, the OTs and acceptability of VOCs likely depend on the VOC profile as a whole (P3);

however, few studies have considered OTs in food-related mixtures [355; 356]. For these reasons, the practical value of the currently available OT/OAV values should be considered with caution.

6 General discussion, conclusions and perspectives

6.1 Food quality analysis

Development of active and intelligent packaging technologies calls for extensive data collection, focusing on the loss of food quality as a function of storage time. Since packaging technologies and quality monitoring need to be adapted to several intrinsic and extrinsic factors arising from the properties of the packaged product and its characteristic spoilage processes, data collection needs to occur under well-defined conditions. Throughout the course of this dissertation research, properties and quality of muscle foods packaged under different MAs were thus analyzed by means of storage experiments that required time-consuming and labor-intensive setups. Application of rapid and convenient analytical technologies was thus considered highly important. In this dissertation, novel and innovative technologies were used e.g. for the characterization of antimicrobial packaging materials prior *in situ* testing (BLI; Ch. 5.2.1, P1) and for the quantification of spoilage metabolites (SIFT-MS; Ch. 4.2.6, P3-P4). These analytical approaches allowed fast, convenient and accurate insight into the studied phenomena, thus advancing the development of both experimental setups and food packaging technologies.

In studies involving packaged food products, destructive analytical methods increase the amount of sample consumption and subsequently research expenses. Moreover, natural variation between samples typically increases the variation in the measured variables. In the present dissertation, this was especially evident in the analysis of pork color (Ch. 5.1.2, P2) and seafood VOCs (Ch. 5.1.4, P3-P4). However, the applicability of data originating from different individual samples was ensured by controlling and optimizing following factors:

- *Homogeneity/uniformity of samples*: similar processing history, dimensions, properties and initial quality
- *Amount of replicates*: at least three meat/seafood samples were examined on each day of a storage experiment
- *Data usage*: statistical analyses were performed on the basis of values representing independent food packages/samples rather than replicate averages

In addition to taking respective factors into account in future studies, the development and utilization of experimental setups involving repeated analyses from the same samples could be considered advantageous. However, it should be noted that the feasibility of this approach depends on the applied measurement technique (Table 13).

Table 13. The three main types of quality analyses for packed food products and their feasibility for performing repeated measurements.

Type	Description	Example	Repeated analyses?
Non-invasive	Package remains intact	Visual inspection Color analysis through the packaging Sensor readout	Yes
Invasive	Sampling of food product or headspace; package remains closed after measurements	Headspace gas composition analysis SIFT-MS Characterization of dissolved gases	Possibly
Destructive	Package is opened; end of storage	Microbiological analysis pH analysis Color analysis Chemical composition analysis	No

It should be noted that certain methods may partially fall under several categories. For example, characterization of CO_2 solubility in packed food products by a volumetric method involves the determination of the total volume and CO_2 concentration in the package headspace [357; 358]; the former analysis does not directly affect the food product as long as the package is not damaged by immersion under water, whereas the latter analysis is invasive. Overall, invasive and destructive analyses alter the package headspace and/or food product, which is why repetitive analysis will be interfered (e.g. headspace gas composition) or is not possible (e.g. meat blooming). In order to ensure that repeated measurements will be representative, focus should be on the development of non-invasive techniques or restoration of the pre-analysis conditions after invasive measurements. For example, repeated VOC quantification from the same package requires that the headspace composition remains as similar as possible after measurements. This could be achieved by increasing the headspace-to volume ratio and/or replacing the sampled volume with a respective gas composition.

Since food spoilage is not only highly product-specific but also subjectively evaluated (Ch. 2), it is of primary importance to highlight the role of sensory evaluation in food quality characterization. In this dissertation, sensory evaluation was used for establishing the link between the perceived quality and other studied variables (Ch. 5.1.1; Ch. 5.2.2; P1; P3-P4). In particular, this approach was considered beneficial in the identification of seafood spoilage indicators (Ch. 5.2.2; Ch. 6.3.2; P3-P4). However, it should be noted that the evaluation setup and panel composition have a key role in the applicability of the results. For this reason, the impact of subjective preferences and expectations in quality assessment and the suitability of the panel for the particular evaluation task should be considered. In the present dissertation, this was controlled by using trained panelists (P1) or experienced panelists who consumed seafood on a regular basis (P3-P4).

Data analysis plays a central role in food quality research. As previously stated, data collection typically requires time and effort, in particular since each food product-packaging combination needs to be separately analyzed. As a consequence, food quality research typically produces relatively small multivariate datasets where complex impacts and interactions can be expected. However, applying

the outcomes of data collection for the development of active and intelligent packaging technologies requires a comprehensive understanding of the meaning and relevance of the obtained results. Efficient data analysis thus calls for appropriate statistical methods. In this dissertation, extensive statistics was applied for characterizing the impact of different storage conditions on the physicochemical properties of pork and for the identification of most potential seafood spoilage indicators (Ch. 4.9, P4). With the help of these approaches, interpretation of the produced datasets was considerably facilitated and a deeper understanding of spoilage processes was obtained. In future studies, further development of analytical methods that enhance the characterization and use of small multivariate datasets would be highly beneficial. Optimally, these methods could also help reducing the workload needed for data collection, for example by means of predictive modelling.

6.2 Development of antimicrobial packaging technologies

Interest in antimicrobial food packaging is constantly increasing and several potential approaches have been previously developed. These packaging solutions could provide with means to extend the shelf life of perishable food products, thus significantly reducing food waste and packaging material consumption. However, the success of antimicrobial films depends on the availability and/or release rate of active substances that come into contact with the food microbiota. As shown in Ch. 2.3.1.1, lack of controlled release has been identified as a major challenge when developing this kind of packaging solutions. In this dissertation research, respectively, activity of silver-containing packaging films was found to be dependent on the availability of active silver on the packaging material surface (Ch. 5.1.1, P1). Films prepared by LFS were found to express rapid and high antimicrobial activity against a wide variety of microbes *in vitro*, including typical meat spoilage bacteria. However, films prepared by coextrusion showed no antimicrobial effect, which was most likely due to the film structure that prevented silver migration. In this case, applicability of coextrusion was thus limited both in terms of antimicrobial activity and cost-efficiency. This indicates that incorporating antimicrobial agents inside polymeric structures may disable their functionality, which leads to unnecessary material costs that could be avoided by enhancing packaging material design. In further applications, availability of active agents on the packaging film surface should thus be ensured. Possible solutions could arise from reduced layer thickness and tailored surfaces and/or coatings.

Concentrations of active agents that are introduced in packaging materials need to be optimized for each packaging application. For example, even though elevating the concentration of available antimicrobial agent incorporated in packaging films may improve its efficiency, the levels have to be in correspondence with the current legislation and to be safe for consumers, while they should also not allow a decrease in the performance of the packaging materials or unfavorable changes in the properties of the food product (Ch. 2.3.1.1). Furthermore, costs-efficiency of food packaging technologies should be in line with the benefits of the approach [359]. Since the production and recycling costs of

antimicrobial packaging are likely to surpass the costs of respective traditional approaches, development of antimicrobial solutions could be of particular importance for highly valuable and yet highly perishable food products, such as delicacy meat and fish.

In addition to the aforementioned packaging material properties, success of antimicrobial packaging films also depends on the unique properties of each food product and its microbiota. In the present dissertation, none of the tested silver-containing films affected meat microbiota even though respective bacterial species were inhibited *in vitro* (Ch. 5.1.1; P1). This was likely due to the properties of meat since close contact between active agents and meat was ensured by vacuum packaging. However, since silver was initially present in high quantities as a coating on LFS films, the observed inefficiency in meat packaging could also be due to a lack of controlled release throughout storage. In future approaches, migration capacity of active agents and their possible reactions with food constituents should thus be monitored. It should be further noted that *L. gelidum* subs. *gasicomitatum*, a characteristic SSO of meat packaged under MAs, was not affected by any of the tested packaging films. Spoilage phenomena associated with this microbial species could thus be promoted in the presence of silver-containing packaging films.

In this dissertation research, development of antimicrobial packaging films focused on the use of silver in different forms. As shown in Ch. 2.3.1.2 and P1, silver has several advantages in food packaging applications and could be considered as a promising candidate for demonstrating antimicrobial activity against meat spoilage microbiota in real packaging applications. However, due to the current legislative status of silver-based packaging materials (Ch. 2.3.1.2), silver-based packaging solutions are still scarcely commercially available in the EU. Furthermore, high material costs, need for efficient recycling and safety concerns limit the potential of silver-based antimicrobial packaging. In accordance with the needs of both consumers and the industry (Ch. 1), interest in natural antimicrobials is constantly growing. In future applications, use of antimicrobials that are of biological origin and generally recognized as safe (GRAS) could be emphasized, provided that sufficiently high antimicrobial effect can also be demonstrated in real food applications. For example, plant extracts [360-362] and nisin [173; 363; 364] have been considered as potential candidates for active packaging solutions. However, some properties may limit the applicability of this kind of compounds as packaging material constituents; for example, common challenges of plant essential oils include volatility and thermal sensitivity [365] as well as an undesirable smell [366].

In the present dissertation, antimicrobial activity was also introduced to meat packages containing modified gaseous atmospheres. The antimicrobial impact of O_2 absorbers is based on the elimination of O_2 that supports the growth of aerobic microbiota (Ch. 2.3.1.3). In this dissertation (Ch. 5.1.2, P2), color of pork was found to be affected by high, residual or absent O_2. During the expected shelf life, differences in color under these conditions were few, especially after blooming. Traditionally, advantages of anoxic packaging have been overshadowed by its negative impacts on meat color (Ch. 2.2.2.3). The results of the present dissertation thus suggest that anoxic packaging could be beneficial for pork. Some additional advantage could be achieved by the removal of residual O_2. However,

in further studies, the impact of anoxic conditions and/or residual O_2 on the microbiological quality of meat should be examined. In order to evaluate the impact of commercial storage conditions, further testing under illumination would also be beneficial.

Even though application of O_2 absorbers as separate sachets inside food packages is a convenient and cost-effective solution, this approach has some technical limitations. In order to effectively absorb O_2, absorbers need to be in proper contact with the atmosphere. In the present dissertation (P2), some samples were omitted from the statistical analyses because of the sachet position in the package; generally, sachets that are misplaced on the production line, for example below the meat product, have limited absorbing capacity. Furthermore, the use of separate sachets may lead to concerns about consumers becoming exposed to the active agents through leakage or unintentional and/or accidental consumption of the sachet contents [367]. Incorporation of O_2 absorbing agents as an inherent part of packaging materials could provide with enhanced and more secure solutions.

6.3 VOCs in food quality monitoring

As discussed throughout the present dissertation, spoilage of muscle foods is a complex event and typically characterized by the development of off-odors. Monitoring of VOCs has several advantages over other quality and spoilage analyses. Traditional methods used for the evaluation of freshness are commonly time and resource consuming, invasive and destructive. These methods lack the advantage of analyzing the same samples repeatedly and require more samples, which causes additional variation between data points (Ch. 6.1).

6.3.1 SIFT-MS: technical considerations

As discussed in Ch. 2.1.3, SIFT-MS has several advantages in food quality monitoring. Also in this dissertation research (Ch. 4.6, P3-P4), SIFT-MS allowed rapid, convenient, non-destructive and efficient real-time measurements from the actual food package headspace. After preliminary experiments, SIFT-MS could be used for estimating food quality immediately on the day of analysis, which facilitated subsequent microbiological analysis.

Limitations related to SIFT-MS should be considered at each stage of data collection:

1. Before the measurements

Firstly, when constructing the scan method (see example in Ch. 4.6, Table 7), the total number of VOCs and the duration of the scan should be optimized in accordance with the sample properties and the aims of the analysis. Even though all relevant VOCs that are present in significant quantities and increase in the package headspace as a function of time should be included, the total amount of VOCs should be limited since each additional VOC increases the length of a single scan cycle

and thus reduces the amount of obtained data points. On the other hand, increasing the amount of data points by extending the scan length involves a risk of sample dilution and/or depletion.

Secondly, when analyzing several closely related compounds, mass overlaps (conflicts) should be considered. These conflicts arise from the fact that as a result of reactions with a certain precursor ion, several VOCs may produce product ions with the same m/z ratio [116]. Separation of these VOCs is thus not possible via the particular m/z; instead, their separation might require the use of product ions with low branching ratios, or in certain cases may not be possible with SIFT-MS. Furthermore, it should be noted that the presence of a compound that is not included in the scan method may lead to hidden conflicts between a quantified VOC. Selection of VOCs to be quantified should thus be based on preliminary experiments, comparison with other techniques (e.g. GC-MS) and/or literature study. However, the possibility to use different precursor ions in SIFT-MS enhances the quantification of individual VOCs.

Thirdly, it should be noted that the high water content of fresh food products is highly influential to their properties and spoilage processes (Ch. 2.1.1.1; Ch. 2.1.2). Consequently, relative humidity inside the package is typically high. When designing the experimental setup for SIFT-MS analysis, possible formation of secondary product ions due to the reactions between moisture and primary product ions should be taken into account. Furthermore, the impact of humidity on the functionality of instruments and devices used for VOC detection should be carefully considered.

2. During the measurements

The impact of VOCs on the precursor ion levels should considered during the scan. In this dissertation, lowest measured concentrations were between 1-50 µg m^{-3} and typically did not exceed 10^4 µg m^{-3} throughout storage (Ch. 5.2.2, P3). However, certain VOCs such as ethanol could exceed these ranges in late stages of storage. Generally, monitoring of precursor ion levels can be recommended since a reduction in these levels may have a significant impact on the measured VOC concentrations. This may occur especially if VOCs are present in high levels in the food package headspace. In future studies, sample dilution or flow rate restriction could be beneficial if extremely high VOC production is observed.

It should also be noted that the quantification of certain VOCs may also be affected by their chemical properties. For example, in the present study (P3), gradual increase in TMA concentrations could be often observed during the scan duration. This was probably due to the accumulation of TMA in the instrument and led to increase in relative standard deviations and LOQs. In order to reduce the risk of accumulation, samples with lowest expected concentrations should be measured first. In case of high concentrations, flushing with N_2 between samples could be beneficial. Furthermore, in order to collect representative data and to avoid interfering the headspace, refrigerated food samples should be maintained at the desired storage temperature throughout measurements.

3. After the measurements

Prior advanced statistical analysis, the quality of the collected data and the impact of measurement inaccuracy should be carefully considered. In the present dissertation (Ch. 5.2.2; P3-P4), SIFT-MS data was characterized by determining the $SD_{\%}$ (P3-P4) and LOQ (P3) values for each individual VOC. Given that sufficiently accurate quantification of VOCs is needed for determining the critical concentration thresholds for food quality monitoring, this approach was considered beneficial in preliminary screening of most potential spoilage indicators.

4. Complementary and alternative methods

Finally, it should be considered that the quantification of certain VOCs by SIFT-MS may be challenging or impossible. For example, quantification of compounds that are present in the headspace below ppb range will most likely be unsuccessful because of relatively high LOQs. However, the relevance of these compounds in food quality monitoring is respectively limited. In general, the use of complementary or alternative mass spectrometry techniques (GC-MS, PTR-MS) can be considered advantageous when these challenges occur.

When operating in the MIM mode, the identity and amount of VOCs needs to be defined in advance. Quantification of the full VOC profile is possible in the FMS mode; however, this approach does not directly allow the determination of VOC concentrations. Development of methods for improving the interpretation of the FMS data could thus be beneficial when aiming at accelerated and facilitated preliminary screening of the full VOC profile.

6.3.2 Identification and quantification of potential spoilage indicators

The composition and evolution of the VOC profile are dependent on the applied packaging and storage conditions. As shown in Ch. 5.2.2, three main VOC types could be identified in the headspace of Atlantic cod and brown shrimp over storage time. Multivariate statistical analysis was found essential for analyzing the produced datasets and allowed the identification of several potential spoilage indicators. However, the use of VOCs as quality indices poses several challenges that require careful consideration.

Determination of potential food spoilage indicators requires a well-defined and systematic approach that combines advanced experimental techniques and data analysis. In the present dissertation, the following three-stage identification procedure (PIQES) was utilized and developed:

1) **P**reliminary **I**dentification: screening and selection of possible spoilage indicators
2) **Q**uantification: monitoring of selected VOCs
3) **E**xploration and **S**election: determination of most potential spoilage indicators by multivariate statistical analysis

In the first stage, a group of possible spoilage indicators was selected on the basis of literature review and preliminary experiments, consequently followed by the construction of a SIFT-MS method. Due

to the high amount of possible indicators and challenges related to their quantification by SIFT-MS (Ch. 6.3.1), this stage was found to be the most time-consuming and labor-intensive when compared to the latter two. In future studies, further development of the PIQES procedure should thus give emphasis on initial screening. For example, the use of SIFT-MS in full mass scan (FMS) mode could provide with a fast and convenient method for preliminary characterization of the full VOC profile. However, this approach requires automated data processing and a thorough understanding of the reactions leading to the generation of product ions. Furthermore, due to the high amount of quantifiable product ions, relatively long sampling duration and thus high sample volumes are needed.

Identification of potential food spoilage indicators is not possible only by means of SIFT-MS or other quantitative methods. As discussed in Ch. 5.2.2, even though a VOC may be produced during refrigerated storage and correlate with microbial growth and/or sensory rejection, it may have a modest impact on the perceived quality. Examining the relations between microbial growth, VOC concentrations and sensory quality was thus considered highly beneficial for the identification of most potential spoilage indicators. However, it should be noted that in case the concentrations of given VOCs increase before the panel observes quality changes, the identification of these VOCs as early spoilage indicators could be interfered. However, such VOCs were not identified in the present dissertation (Ch. 5.2.2; P4), despite the fact that their identification would have been possible by PLS on the basis of following considerations:

- *Multiple response variables*. Selective PLS was carried out using TPC or sensory evaluation as the response variable; the results of both models were generally in good correspondence.
- *Collinearity*. As stated in Ch. 5.2.2, the VOCs could be classified into three main groups on the basis of similarity in patterns due to multicollinearity.

Furthermore, it should be noted that a negligible sensory impact does not necessarily prevent using a particular VOC in spoilage analysis and/or quality monitoring: for example, even though alcohols having high OT values cannot often be detected by the human nose, their production may correlate well with microbiological quality and other VOCs. However, a deeper understanding of spoilage processes and the impact of VOCs on sensory quality calls for enhanced methods for data analysis, preferably including the impact of detectability (OTs) and acceptability of VOCs in real food samples.

As suggested in Ch. 5.2.2, the decrease of concentration as a function of time indicates that a VOC cannot be used as a spoilage indicator. This kind of VOCs could be potential freshness indicators; however, identification of such compounds would require a negative correlation between the VOC and an applicable response variable, such as sensory quality. Given that in case of both Atlantic cod and brown shrimp, VOC concentrations were found to be very low and frequently even below the LOQ in the beginning of storage (Ch. 5.2.2, P3-P4), sufficiently accurate quantification of concentration decrease could be expected to be challenging. Furthermore, in case of VOCs becoming depleted from the package headspace, identification of potential freshness indicators on the basis of correlation might not be possible.

The compounds 2,3-butanediol, 3-methyl-1-butanol, dimethyl sulfide, ethanol, ethyl acetate, isobutyl alcohol and TMA were most frequently identified as potential spoilage indicators of both Atlantic cod and brown shrimp packaged under MAs (Ch. 5.2.2, P3-P4). It should be noted that their concentration levels depend on the applied storage conditions. A given single compound is thus likely to have limited capacity as a spoilage indicator. Simultaneous monitoring of multiple compounds should be promoted in further studies (Ch. 6.4).

6.4 Development of intelligent packaging technologies

Interest in food quality monitoring by means of intelligent packaging technologies is constantly increasing and has been considered as a potential solution for addressing challenges arising from shelf life labelling. Traditional shelf life labelling in form of expiry dates is based on generalized information about the progress of spoilage processes when the food product has been stored under defined conditions. [368.] This means that possible failures in the supply chain that can significantly reduce the shelf life – such as contamination, leakage or cold chain breakdown – cannot be taken into account. Intelligent packaging technologies could overcome these drawbacks and limitations by providing with a convenient, simple and real-time access to accurate and reliable information about the quality status of any individual food product. This approach could significantly reduce food waste (and consequently packaging material consumption) by allowing a shift from worst-case scenarios and assumptions to package-specific information.

Development of intelligent packaging technologies calls for extensive data collection regarding food quality status. Data collection should be aimed at the selection of VOCs that can be used as a target group in quality monitoring applications. Research should thus focus on determining following properties of the target VOCs:

1) Identity
2) Total number
3) Critical concentration levels

Advantages and challenges related to the identification of potential spoilage indicators have been discussed in Ch. 6.2.2.

The total number of VOCs that should be targeted when developing intelligent packaging technologies depends on the packaged product. As observed in the present study (Ch. 5.2.2, P3-P4), evolution of the VOC profile depends on the food product and its storage conditions. Even though several VOCs could be considered more universal spoilage indicators than others, extending the development of intelligent packaging solutions to other food products and/or storage conditions calls for more extensive research. Since the perceived odor of a food sample arises from multiple VOCs, a certain

concentration of a particular VOC does not exclusively indicate a certain quality status. For the development of food monitoring applications, the relation between sensory acceptability and a single VOC should thus be defined under all relevant packaging and storage conditions. Thus, monitoring of multiple compounds can be generally recommended not only for spoilage analysis (Ch. 6.3.2), but also for packaging technology development.

Determination of critical concentration levels depends on the desired sensitivity of the intelligent packaging solution. Data analysis should thus aim at establishing three concentration domains:

1) Acceptable concentrations
2) Intermediate concentrations
3) Unacceptable concentrations

In this dissertation (Ch. 5.2.2, P3), increasing VOC production and sensory rejection was typically observed only after reaching high microbial levels (TPC > 7.0 log CFU g^{-1}). Sensory rejection (50 % of panellists) occurred even later, typically at approximately 7.5 log CFU g^{-1}. This indicates that microbiological limits that have traditionally been assigned for different food products are not sufficient as sole quality indices. Instead, relation between microbial growth, VOC levels and sensory quality is essential for effective quality monitoring. In case of seafood, the three concentration domains could thus be suggested as follows:

1) Concentrations corresponding to TPC < 7.0 log CFU g^{-1} and rejection < 50 %
2) Concentrations corresponding to TPC > 7.0 log CFU g^{-1} and rejection < 50 %
3) Concentrations corresponding to rejection > 50 %

The minimum requirement for spoilage detection is that concentrations belonging to the 3rd domain will be successfully recognized. However, efficient quality monitoring requires that quality changes are detected before consumer rejection occurs. Intelligent packaging solutions should thus be sensitive enough to detect target VOCs in the 2nd domain. As observed in the present study (Ch. 5.2.2), concentration of any VOC rarely exceeded 10^4 µg/m^3 even in the end of storage time. It can thus be concluded that high sensitivity to low concentrations is of primary importance for the detection of early spoilage. Table 14 presents the concentration domains of most potential spoilage indicators (Ch. 6.3.2) for Atlantic cod stored under different gaseous atmospheres (based on P3).

Table 14. The concentration domains 1 (acceptable; green) and 3 (unacceptable; red) in Atlantic cod stored under conditions H4 (60 % CO_2 /40 & O_2/0 % N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and Air (air 4 °C) (based on P3). Possible intermediate days of storage were excluded from the table if sensory evaluation was not performed.

Condition	Day	TPC (log CFU/g)	Rejection (%)	2,3-butanediol	3-methyl-1-butanol	Dimethyl sulfide	Ethanol	Ethyl acetate	Isobutyl alcohol	TMA
H4	0	5.48	0	10	13	218	4914	23	44	19
	4	6.79	20	12	10	360	907	126	32	40
	8	7.58	60	220	232	288	6946	3686	59	142
	13	8.65	90	457	489	348	23584	7097	108	478
H8	0	5.75	0	20	23	315	899	32	40	43
	3	7.53	58	155	305	462	1885	4705	64	149
	5	8.41	75	424	658	458	6647	7729	104	921
	7	8.07	67	298	424	1219	8623	5482	107	1168
L4	0	6.75	10	11	11	561	3231	9	40	12
	3	8.08	50	153	238	1313	2350	3659	70	405
	5	8.25	70	559	381	3676	14456	4760	109	1556
	7	8.14	60	329	273	4974	24947	3184	138	1859
L8	0	5.75	13	11	4	110	2810	13	61	18
	4	7	50	14	24	680	786	293	30	56
	8	7.73	38	144	133	767	11913	2145	89	615
	13	7.15	75	207	74	3282	35782	638	172	2143
Air	0	5.65	10	14	17	737	1044	27	25	28
	1	6.16	10	14	22	1659	707	18	28	35
	2	6.99	30	16	22	1277	522	55	27	28
	3	7.71	60	42	72	1627	1139	865	36	813

The absence of domain 2 concentrations in Table 14 suggests that this domain is limited to a relatively narrow concentration range between domains 1 and 3; however, it should be noted that the evaluation of samples from the intermediate days of storage could have improved the domain analysis. It should also be noted that certain concentration levels of a given VOC may be acceptable under condition A and unacceptable under condition B. This is most likely due to the fact that the perceived quality arises from the mixture of all present VOCs. In future studies, determination of the three concentration domains could thus benefit from performing additional sensory evaluation tests in order to narrow the domain interfaces, as well as from enhanced preliminary identification of target VOCs (Ch. 6.3.2).

6.5 Final conclusions and summary

6.5.1 Answers to the research questions

The results of the dissertation answer to the research questions defined in Ch. 3:

S1 *What is the impact of silver-containing packaging films prepared by coextrusion or liquid flame spray (LFS) on the quality and spoilage of vacuum packaged pork? What are the critical factors determining their antimicrobial efficacy?*

No difference was found between the quality of pork packaged in 1) any of the tested silver-containing films or 2) silver-free control film. The results thus indicate that the antimicrobial efficiency of the silver-containing films was negligible in contact with meat, despite the high *in vitro* efficiency that LFS films showed against most of the tested microbes, including typical meat spoilage bacteria that were also found in the pork samples. Following factors were suggested to contribute to the observed antimicrobial efficiency:

- Packaging materials: preparation techniques and properties
    - Availability of active silver on the film surface
    - Concentration of active silver
    - Migration capacity

- Food packaging
    - Direct contact between silver and bacteria
    - Composition and properties of the food product
    - Food microbiota: possible resistance towards silver

For example, the observed inefficiency against *L. gelidum* subs. *gasicomitatum* could lead to enhancing its growth and thus associated spoilage phenomena.

S2 *How do different O_2 levels (high-residual-absent) affect the physicochemical properties (headspace gas composition, surface pH and color) of pork packaged under MAs?*

Differences in the studied physiochemical variables were in most cases limited between different days of storage and/or different MA conditions. On a given day of storage, following main results were obtained:

- Differences in O_2/CO_2 levels between the tested packaging conditions were usually associated with the selected MA composition
- No difference was observed in pH or lightness
- Color of meat was highly similar between the tested conditions, especially after blooming

Similarity of color under different MA conditions suggests that anoxic packaging could be beneficial for pork, where O_2 absorbers could have some additional benefit.

S3 *Which VOCs (identity and quantity) are potential spoilage indicators of Atlantic cod (Gadus morhua) and brown shrimp (Crangon crangon) stored under MAs? Which criteria can be used for their identification?*

The PIQES procedure was used for identifying potential spoilage indicators according to the following criteria:

- Preliminary identification
  - Literature survey and preliminary experiments
- Quantification
  - Determination of average $SD_{\%}$ and LOQ
- Exploration and selection
  - PLS and selection criteria: 1) positive correlation with TPC or sensory rejection, 2) VIP > 1, 3) positive regression coefficient

Both in case of Atlantic cod and brown shrimp, the VOCs 2,3-butanediol, 3-methyl-1-butanol, dimethyl sulfide, ethanol, ethyl acetate, isobutyl alcohol and TMA were frequently identified under different MA conditions. Critical concentration levels of these compounds have been given in Table 14.

M1 *What are the key aspects of quality characterization within the context of developing active and intelligent packaging technologies for muscle foods?*

Food quality characterization has a central role in packaging technology development and should thus be optimized in accordance to the features, requirements and limitations that affect it. Following main aspects related to food quality and packaging technology development should thus be considered:

- Requirements of packaging technology development
  - Applicability
  - Performance
  - Feasibility
- Characteristics of food quality information
  - Impact of food product-packaging combination
  - Impact of methodology
  - Impact of dataset characteristics

As shown throughout the present dissertation, the demands set by/for the specified and potential applications affect the whole food quality characterization process. The requirement of *applicability* means that the process should be designed in accordance with the intended application and the

meaning of the obtained results for packaging technology development should be clear. This can be achieved by collecting data under well-defined conditions and by subsequent systematic data analysis. On the other hand, optimizing the *performance* of the process requires extensive data collection and analysis and thus calls for the development of facilitating methods, selection criteria and thresholds. Furthermore, data analysis from the viewpoint of technical, legal, ecological and economic aspects enhances the *feasibility* of applying the data in practice.

Since each *food product-packaging combination* has its characteristic properties, the studied variables and analytical methods need to be selected in accordance with the spoilage mechanisms of the particular product; generally, preliminary screening of representative variables can be recommended in order to optimize the storage experiment *methodology*. In many cases, the development of non-invasive methods and/or repeated measurements could increase the applicability of the collected data in packaging technology development. Finally, typical food quality *dataset characteristics* can be described with the terms small, time-dependent, experimental and multivariate: in order to respond to the challenges arising from these features, optimized methods are needed for data analysis.

6.5.2 Originality and contribution

Development of active and intelligent packaging technologies calls for a multidisciplinary approach, combining expertise in microbiology, (bio)chemistry, sensory studies, materials science and multivariate statistics. This dissertation not only enhanced the communication between different research fields involved in packaging technology development, but also lead to the generation of extensive interdisciplinary datasets. The obtained results directly contribute to the development of active and intelligent packaging materials for muscle foods, not only within the framework of the Comeat and CheckPack projects, but also as a comprehensive background for further research.

The scientific contribution and relevance of the present dissertation can be summarized as follows:

1) *Contribution to the principles of food quality characterization*

In this dissertation, food quality characterization was examined as a systematic process within a specific context (development of active and intelligent packaging technologies) and its main aspects were defined: both internal (characteristics of food quality information) and external (requirements of packaging technology development) aspects could be identified. The general principles and workflow of quality characterization applied in the dissertation can be expected to advance the planning and realization of experimental setups in future studies.

2) *Contribution to methods, techniques and processes*

Traditional methods used in food quality characterization often have limited applicability when producing information for the development of active and intelligent packaging technologies. Alternative methods, techniques and processes were utilized and developed in the present dissertation:

- Application of coextrusion and LFS for the preparation of silver-containing packaging materials: even though prolonged antimicrobial activity was not achieved, new information about several techniques and parameters used in packaging material preparation was obtained
- Characterization of the antimicrobial activity of packaging materials with BLI: this method allowed fast, accurate and real-time screening of packaging materials for further use and thus considerably facilitated packaging material development
- PIQES procedure: overall, this method provided with a systematic tool combining both laboratory work and multivariate statistical analysis for the identification and real-time quantification of potential spoilage indicators

3) Contribution to scientific knowledge

The results of this dissertation provided new insights to several questions related to food quality. Following main advancements in the scientific knowledge were achieved:

- Impact of silver on typical meat spoilage organisms was demonstrated *in vitro* and *in situ* meat: high variation in *in vitro* susceptibility was found
- Inefficiency of silver of *L. gelidum* subs. *gasicomitatum* was demonstrated and associated with potential risks of growth promotion and/or silver resistance
- Impact of residual vs. absent O_2 levels on pork color was examined: the results complement the existing knowledge and suggest potential benefits of anoxic packaging
- Relevance of *P. phosphoreum* in the spoilage of Atlantic cod was demonstrated under all tested conditions (air/MAP)
- Several VOCs were identified as potential seafood spoilage indicators for quality monitoring purposes and their critical headspace concentration levels were determined under multiple packaging and storage conditions: in contrast to the majority of data available in the scientific literature, the results of this dissertation represented the exact reality that a quality monitoring system would meet

Overall, the dissertation highlights the importance of a multidisciplinary approach in food quality characterization. With the help of emphasizing multidisciplinary awareness and novel approaches in the characterization process, considerable advances in packaging technology development as well as a deeper understanding of food quality can eventually be expected, optimally advancing the whole supply chain.

References

[1] FAO Trade and Markets Division, Food Outlook: Biannual Report on Global Food Markets, FAO, 11/2017.

[2] FAO, Definitional Framework of Food Loss, FAO, 2015. Available online (accessed 13/07/2018): http://thinkeatsave.org/docs/FLW-Definition-and-Scope-version-2015.pdf.

[3] G. Chaboud, B. Daviron, Food losses and waste: Navigating the inconsistencies, Global Food Security, Vol. 12, 2017, pp. 1-7.

[4] J. Gustavson, C. Cederberg, R. van Otterdijk, A. Meybeck, Global Food Losses and Food Waste, FAO (Food and Agriculture Organization of the United Nations), 2011, 1-38 p.

[5] X. Wang, D. Li, A dynamic product quality evaluation based pricing model for perishable food supply chains, Omega, Vol. 40, No. 6, 2012, pp. 906-917.

[6] M.E. Buisman, R. Haijema, J.M. Bloemhof-Ruwaard, Discounting and dynamic shelf life to reduce fresh food waste at retailers, International Journal of Production Economics, available online 21/07/2017.

[7] J. Parfitt, M. Barthel, S. Macnaughton, Food waste within food supply chains: quantification and potential for change to 2050, Philos Trans R Soc Lond B Biol Sci, Vol. 365, No. 1554, 2010, pp. 3065.

[8] K. Schanes, K. Dobernig, B. Gözet, Food waste matters - A systematic review of household food waste practices and their policy implications, Journal of Cleaner Production, Vol. 182, 2018, pp. 978-991.

[9] H. Brown, J. Williams, 3 Packaged product quality and shelf life, in: R. Coles, D. McDowell, M.J. Kirwan (ed.), Food Packaging Technology, Blackwell Publishing Ltd, CRC Press LLC, Boca Raton, USA, 2003, pp. 65-94.

[10] P. Dalgaard, Modelling of microbial activity and prediction of shelf life for packed fresh fish, International Journal of Food Microbiology, Vol. 26, No. 3, 1995, pp. 305-317.

[11] L. Gram, P. Dalgaard, Fish spoilage bacteria – problems and solutions, Current opinion in biotechnology, Vol. 13, No. 3, 2002, pp. 262-266.

[12] J.H.J. Huis in't Veld, Microbial and biochemical spoilage of foods: an overview, International Journal of Food Microbiology, Vol. 33, No. 1, 1996, pp. 1-18.

[13] G.E. Nychas, P.N. Skandamis, C.C. Tassou, K.P. Koutsoumanis, Meat spoilage during distribution, Meat Science, Vol. 78, No. 1–2, 2008, pp. 77-89.

[14] FAO, The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome, 2016, 220 p.

[15] P. Appendini, J.H. Hotchkiss, Review of antimicrobial food packaging, Innovative Food Science & Emerging Technologies, Vol. 3, No. 2, 2002, pp. 113-126.

[16] K.L. Yam, P.T. Takhistov, J. Miltz, Intelligent Packaging: Concepts and Applications, Journal of Food Science, Vol. 70, No. 1, 2005, pp. R1-R10.

[17] S. Quintavalla, L. Vicini, Antimicrobial food packaging in meat industry, Meat Science, Vol. 62, No. 3, 2002, pp. 373-380.

[18] R. Ahvenainen, Active and intelligent packaging, An introduction, in: R. Ahvenainen (ed.), Novel Food Packaging Techniques, Woodhead Publishing Limited, Cambridge, England, 2003, pp. 6-18.

[19] M. Mullan, D. McDowell, 10 Modified atmosphere packaging, in: R. Coles, D. McDowell, M.J. Kirwan (ed.), Food Packaging Technology, Blackwell Publishing Ltd, CRC Press LLC, Boca Raton, USA, 2003, pp. 303-339.

[20] J. Cheng, D. Sun, X. Zeng, D. Liu, Recent Advances in Methods and Techniques for Freshness Quality Determination and Evaluation of Fish and Fish Fillets: A Review, Critical reviews in food science and nutrition, Vol. 55, No. 7, 2015, pp. 1012-1225.

[21] P.D. Warriss, Meat Science, An Introductory Text, 2nd Edition ed. CABI, Wallingford, Oxfordshire, UK, 2010, 234 p.

[22] M.R. Adams, M.O. Moss, P. McClure, Food Microbiology, 4th Edition ed. Royal Society of Chemistry, Cambridge, UK, 2015, 546 p.

[23] The European Comission, Commission Directive 2001/101/EC of 26 November 2001 amending Directive 2000/13/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs, Official Journal of the European Communities, Vol. 44, No. L 310, 2001, pp. 0019-0021.

[24] T. Ruethers, A.C. Taki, E.B. Johnston, R. Nugraha, T.T.K. Le, T. Kalic, T.R. McLean, S.D. Kamath, A.L. Lopata, Seafood allergy: A comprehensive review of fish and shellfish allergens, Molecular Immunology, Vol. 100, 2018, pp. 28-57.

[25] EFSA, Scientific Opinion on health benefits of seafood (fish and shellfish) consumption in relation to health risks associated with exposure to methylmercury, EFSA Journal, Vol. 12, No. 7, 2014, pp. 3761.

[26] J. Murray, J.R. Burt, The Composition of Fish, Torry Advisory Note No. 38, Torry Research Station, .

[27] A.H. Varnam, J.M. Sutherland, Meat and Meat Products: Technology, Chemistry and Microbiology, First ed. Chapman & Hall, London, 1995, .

[28] H.H. Huss, Fresh Fish -Quality and Quality Changes: A Training Manual Prepared for the FAO/DANIDA Training Programme on Fish Technology and Quality Control, FAO Fisheries Series 29, Food & Agriculture Organization, Rome, 1988, .

[29] C. Guérin, From Muscle to Meat and Meat Products, in: R. Jeantet, T. Croguennec, P. Schuck, G. Brule (ed.), Handbook of Food Science and Technology 3: Food Biochemistry and Technology, John Wiley & Sons, 2016, pp. 65-114.

[30] G. Olafsdóttir, E. Martinsdóttir, J. Oehlenschläger, P. Dalgaard, B. Jensen, I. Undeland, I.M. Mackie, G. Henehan, J. Nielsen, H. Nilsen, Methods to evaluate fish freshness in research and industry, Trends in Food Science & Technology, Vol. 8, No. 8, 1997, pp. 258-265.

[31] Y. Kobayashi, S.G. Mayer, J.W. Park, FT-IR and Raman spectroscopies determine structural changes of tilapia fish protein isolate and surimi under different comminution conditions, Food Chemistry, Vol. 226, 2017, pp. 156-164.

[32] A.M. Herrero, Raman Spectroscopy for Monitoring Protein Structure in Muscle Food Systems, Critical reviews in food science and nutrition, Vol. 48, No. 6, 2008, pp. 512-523.

[33] Verrez-Bagnis Véronique, L. Christine, M. Martine, Noël Joëlle, Fleurence Joël, Protein changes in post mortem sea bass (*Dicentrarchus labrax*) muscle monitored by one- and two-dimensional gel electrophoresis, Electrophoresis, Vol. 22, No. 8, 2001, pp. 1539-1544.

[34] F. Jessen, Two-Dimensional Gel Electrophoresis Detection of Protein Oxidation in Fresh and Tainted Rainbow Trout Muscle, Journal of Agricultural and Food Chemistry, Vol. 52, No. 23, 2004, pp. 7101-7107.

[35] L. Gram, H.H. Huss, Microbiological spoilage of fish and fish products, International Journal of Food Microbiology, Vol. 33, No. 1, 1996, pp. 121-137.

[36] R. Malcolm Love, The post-mortem ph of cod and haddock muscle and its seasonal variation, Journal of the Science of Food and Agriculture, Vol. 30, No. 4, 1979, pp. 433-438.

[37] H.H. Huss, Quality and quality changes in fresh fish, 348, Food and Agriculture Organization of the United Nations (FAO), Rome, 1995, .

[38] The European Commission, Commission Regulation (EC) No 1022/2008 of 17 October 2008 amending Regulation (EC) No 2074/2005 as regards the total volatile basic nitrogen (TVB-N) limits (Text with EEA relevance), Official Journal of the European Union, Vol. 51, No. L 277, 2008, pp. 18-20.

[39] The European Commission, Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004 (Text with EEA relevance), Official Journal of the European Union, Vol. 48, No. L 338, 2004, pp. 27-59.

[40] J. Debevere, G. Boskou, Effect of modified atmosphere packaging on the TVB/TMA-producing microflora of cod fillets, International Journal of Food Microbiology, Vol. 31, No. 1–3, 1996, pp. 221-229.

[41] L.C. Faustman, Postmortem Changes in Muscle Foods, in: D.M. Kinsman, A.W. Kotula, B.C. Breidenstein (ed.), Muscle Foods: Meat, Poultry and Seafood Technology, Springer Science + Business Media, Dordrecht, 1994, pp. 63-78.

[42] P.H. Yancey, J.F. Siebenaller, Trimethylamine oxide stabilizes teleost and mammalian lactate dehydrogenases against inactivation by hydrostatic pressure and trypsinolysis, Journal of Experimental Biology, Vol. 202, No. 24, 1999, pp. 3597-3603.

[43] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, G.N. Somero, Living with water stress: evolution of osmolyte systems, Science (New York, N.Y.), Vol. 217, No. 4566, 1982, pp. 1214-1222.

[44] Y. Qu, D.W. Bolen, Hydrogen Exchange Kinetics of RNase A and the Urea:TMAO Paradigm, Biochemistry, Vol. 42, No. 19, 2003, pp. 5837-5849.

[45] A.R.A. Villalobos, J.L. Renfro, Trimethylamine oxide suppresses stress-induced alteration of organic anion transport in choroid plexus, Journal of Experimental Biology, Vol. 210, No. 3, 2007, pp. 541-552.

[46] B.A. Seibel, P.J. Walsh, Trimethylamine oxide accumulation in marine animals: relationship to acylglycerol storage, Journal of Experimental Biology, Vol. 205, No. 3, 2002, pp. 297-306.

[47] A.B. Bockus, B.A. Seibel, Trimethylamine oxide accumulation as a function of depth in Hawaiian mid-water fishes, Deep Sea Research Part I: Oceanographic Research Papers, Vol. 112, 2016, pp. 37-44.

[48] L. Baliño-Zuazo, A. Barranco, A novel liquid chromatography–mass spectrometric method for the simultaneous determination of trimethylamine, dimethylamine and methylamine in fishery products, Food Chemistry, Vol. 196, 2016, pp. 1207-1214.

[49] A. Ruiter, Trimethylamine and the quality of fish, Voedingsmiddelentechnologie, Vol. 43, 1971, pp. 1-10.

[50] J. Debevere, F. Devlieghere, P. van Sprundel, B. De Meulenaer, Influence of acetate and CO2 on the TMAO-reduction reaction by *Shewanella baltica*, International Journal of Food Microbiology, Vol. 68, No. 1–2, 2001, pp. 115-123.

[51] U. Anthoni, T. Børresen, C. Christophersen, L. Gram, P.H. Nielsen, Is trimethylamine oxide a reliable indicator for the marine origin of fish? Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, Vol. 97, No. 3, 1990, pp. 569-571.

[52] N. Niizeki, T. Daikoku, T. Hirata, I. El-Shourbagy, X. Song, M. Sakaguchi, Mechanism of biosynthesis of trimethylamine oxide from choline in the teleost tilapia, *Oreochromis niloticus*, under freshwater conditions, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, Vol. 131, No. 3, 2002, pp. 371-386.

[53] W.J. Dyer, Amines in Fish Muscle: I. Colorimetric Determination of Trimethylamine as the Picrate Salt, Journal of the Fisheries Research Board of Canada, Vol. 6d, No. 5, 1945, pp. 351-358.

[54] H. Tozawa, K. Enokibara, K. Amano, Effect of Dimethylamine on the Value of Trimethylamine Determined by the D_{YER}'s Method, Nippon Suisan Gakkaishi, Vol. 36, No. 6, 1970, pp. 606-611.

[55] G. Duflos, V.M. Coin, M. Cornu, J. Antinelli, P. Malle, Determination of volatile compounds to characterize fish spoilage using headspace/mass spectrometry and solid-phase microextraction/gas chromatography/mass spectrometry, Journal of the Science of Food and Agriculture, Vol. 86, No. 4, 2006, pp. 600-611.

[56] R. Triqui, N. Bouchriti, Freshness Assessments of Moroccan Sardine (Sardina pilchardus): Comparison of Overall Sensory Changes to Instrumentally Determined Volatiles, Journal of Agricultural and Food Chemistry, Vol. 51, No. 26, 2003, pp. 7540-7546.

[57] Z. Zhang, G. Li, L. Luo, G. Chen, Study on seafood volatile profile characteristics during storage and its potential use for freshness evaluation by headspace solid phase microextraction coupled with gas chromatography–mass spectrometry, Analytica Chimica Acta, Vol. 659, No. 1–2, 2010, pp. 151-158.

[58] F. Özogul, K.D.A. Taylor, P. Quantick, Y. Özogul, Biogenic amines formation in Atlantic herring (*Clupea harengus*) stored under modified atmosphere packaging using a rapid HPLC method, International Journal of Food Science & Technology, Vol. 37, No. 5, 2002, pp. 515-522.

[59] E.M. Buys, Colour changes and consumer acceptability of bulk packaged pork retail cuts stored under O2, CO2 and N2, Meat Science, Vol. 68, No. 4, 2004, pp. 641-647.

[60] R.A. Mancini, M.C. Hunt, Current research in meat color, Meat Science, Vol. 71, No. 1, 2005, pp. 100-121.

[61] T.M. Ngapo, J.-. Martin, E. Dransfield, Consumer choices of pork chops: results from three panels in France, Food Quality and Preference, Vol. 15, No. 4, 2004, pp. 349-359.

[62] M.G. O'Sullivan, D.V. Byrne, H. Martens, L.H. Gidskehaug, H.J. Andersen, M. Martens, Evaluation of pork colour: prediction of visual sensory quality of meat from instrumental and computer vision methods of colour analysis, Meat Science, Vol. 65, No. 2, 2003, pp. 909-918.

[63] M. Zhang, S. Sundar, Effect of oxygen concentration on the shelf-life of fresh pork packed in a modified atmosphere, Packaging Technology and Science, Vol. 18, No. 4, 2005, pp. 217-222.

[64] K.W. McMillin, Where is MAP Going? A review and future potential of modified atmosphere packaging for meat, Meat Science, Vol. 80, No. 1, 2008, pp. 43-65.

[65] L. John, D. Cornforth, C.E. Carpenter, O. Sorheim, B.C. Pettee, D.R. Whittier, Color and thiobarbituric acid values of cooked top sirloin steaks packaged in modified atmospheres of 80% oxygen, or 0.4% carbon monoxide, or vacuum, Meat Science, Vol. 69, No. 3, 2005, pp. 441-449.

[66] R.A. Mancini, S.P. Suman, M.K.R. Konda, R. Ramanathan, Effect of carbon monoxide packaging and lactate enhancement on the color stability of beef steaks stored at 1 °C for 9 days, Meat Science, Vol. 81, No. 1, 2009, pp. 71-76.

[67] J.Y. Jeong, J.R. Claus, Color stability of ground beef packaged in a low carbon monoxide atmosphere or vacuum, Meat Science, Vol. 87, No. 1, 2011, pp. 1-6.

[68] P. Jayasingh, D.P. Cornforth, C.E. Carpenter, D. Whittier, Evaluation of carbon monoxide treatment in modified atmosphere packaging or vacuum packaging to increase color stability of fresh beef, Meat Science, Vol. 59, No. 3, 2001, pp. 317-324.

[69] A. Sakowska, D. Guzek, D. Głąbska, A. Wierzbicka, Carbon monoxide concentration and exposure time effects on the depth of CO penetration and surface color of raw and cooked beef longissimus lumborum steaks, Meat Science, Vol. 121, 2016, pp. 182-188.

[70] F. De Santos, M. Rojas, G. Lockhorn, M.S. Brewer, Effect of carbon monoxide in modified atmosphere packaging, storage time and endpoint cooking temperature on the internal color of enhanced pork, Meat Science, Vol. 77, No. 4, 2007, pp. 520-528.

[71] T.R. Krause, J.G. Sebranek, R.E. Rust, M.S. Honeyman, Use of Carbon Monoxide Packaging for Improving the Shelf Life of Pork, Journal of Food Science, Vol. 68, No. 8, 2003, pp. 2596-2603.

[72] O. Sørheim, H. Nissen, T. Nesbakken, The storage life of beef and pork packaged in an atmosphere with low carbon monoxide and high carbon dioxide, Meat Science, Vol. 52, No. 2, 1999, pp. 157-164.

[73] B.H.P. Wilkinson, J.A.M. Janz, P.C.H. Morel, R.W. Purchas, W.H. Hendriks, The effect of modified atmosphere packaging with carbon monoxide on the storage quality of master-packaged fresh pork, Meat Science, Vol. 73, No. 4, 2006, pp. 605-610.

[74] J.L. Norman, E.P. Berg, M.R. Ellersieck, C.L. Lorenzen, Prediction of color and pH measurement throughout boneless center-cut pork loins, Meat Science, Vol. 66, No. 2, 2004, pp. 273-278.

[75] M.S. Brewer, J. Novakofski, K. Freise, Instrumental evaluation of pH effects on ability of pork chops to bloom, Meat Science, Vol. 72, No. 4, 2006, pp. 596-602.

[76] M.S. Brewer, L.G. Zhu, B. Bidner, D.J. Meisinger, F.K. McKeith, Measuring pork color: effects of bloom time, muscle, pH and relationship to instrumental parameters, Meat Science, Vol. 57, No. 2, 2001, pp. 169-176.

[77] D.P. Sen, Advances in Fish Processing Technology, Allied Publishers Private Limited, New Delhi, 2010, 818 p.

[78] Y.H. Hui, N. Cross, H.G. Kristinsson, M.H. Lim, W.K. Nip, L.F. Siow, P.S. Stanfield, Biochemistry of Seafood Processing, in: B.K. Simpson (ed.), Food Biochemistry and Food Processing, Second Edition ed., Wiley-Blackwell, Oxford, UK, 2012, pp. 344-364.

[79] M. Rodriguez-Concepcion, J. Avalos, M.L. Bonet, A. Boronat, L. Gomez-Gomez, D. Hornero-Mendez, M.C. Limon, A.J. Meléndez-Martínez, B. Olmedilla-Alonso, A. Palou, J. Ribot, M.J. Rodrigo, L. Zacarias, C. Zhu, A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health, Progress in Lipid Research, Vol. 70, 2018, pp. 62-93.

[80] A.J. Meléndez-Martínez, P. Mapelli-Brahm, A. Benítez-González, C.M. Stinco, A comprehensive review on the colorless carotenoids phytoene and phytofluene, Archives of Biochemistry and Biophysics, Vol. 572, 2015, pp. 188-200.

[81] J.Á. Pérez-Alvarez, J. Fernández-López, Chemical and Biochemical Aspects of Color in Muscle Foods, in: L.M.L. Nollet (ed.), Handbook of Meat, Poultry and Seafood Quality, Second Edition ed., Wiley-Blackwell, Oxford, UK, 2012, pp. 1-24.

[82] W.N. Tapp III, J.W.S. Yancey, J.K. Apple, How is the instrumental color of meat measured? Meat Science, Vol. 89, No. 1, 2011, pp. 1-5.

[83] G.L. Robertson, Food Packaging: Principles and Practice, 3rd ed. CRC Press, Boca Raton, 2013, 733 p.

[84] D. Dave, A.E. Ghaly, Meat Spoilage Mechanisms and Preservation Techniques: A Critical Review, American Journal of Agricultural and Biological Sciences, Vol. 6, No. 4, 2011, pp. 486-510.

[85] A.I. Doulgeraki, D. Ercolini, F. Villani, G.E. Nychas, Spoilage microbiota associated to the storage of raw meat in different conditions, International Journal of Food Microbiology, Vol. 157, No. 2, 2012, pp. 130-141.

[86] P. Dalgaard, O. Mejlholm, T.J. Christiansen, H.H. Huss, Importance of *Photobacterium phosphoreum* in relation to spoilage of modified atmosphere-packed fish products, Letters in Applied Microbiology, Vol. 24, No. 5, 1997, pp. 373-378.

[87] F.F. Parlapani, A. Mallouchos, S.A. Haroutounian, I.S. Boziaris, Microbiological spoilage and investigation of volatile profile during storage of sea bream fillets under various conditions, International Journal of Food Microbiology, Vol. 189, 2014, pp. 153-163.

[88] F.F. Parlapani, G.I. Verdos, S.A. Haroutounian, I.S. Boziaris, The dynamics of Pseudomonas and volatilome during the spoilage of gutted sea bream stored at 2 °C, Food Control, Vol. 55, 2015, pp. 257-265.

[89] M. Mikš-Krajnik, Y. Yoon, D.O. Ukuku, H. Yuk, Volatile chemical spoilage indexes of raw Atlantic salmon (*Salmo salar*) stored under aerobic condition in relation to microbiological and sensory shelf lives, Food Microbiology, Vol. 53, Part B, 2016, pp. 182-191.

[90] M. Nuin, B. Alfaro, Z. Cruz, N. Argarate, S. George, Y. Le Marc, J. Olley, C. Pin, Modelling spoilage of fresh turbot and evaluation of a time–temperature integrator (TTI) label under fluctuating temperature, International Journal of Food Microbiology, Vol. 127, No. 3, 2008, pp. 193-199.

[91] L.V. Jørgensen, H.H. Huss, P. Dalgaard, The effect of biogenic amine production by single bacterial cultures and metabiosis on cold-smoked salmon, Journal of Applied Microbiology, Vol. 89, No. 6, 2000, pp. 920-934.

[92] V. Pothakos, F. Devlieghere, F. Villani, J. Björkroth, D. Ercolini, Lactic acid bacteria and their controversial role in fresh meat spoilage, Meat Science, Vol. 109, 2015, pp. 66-74.

[93] M.K. Morsy, K. Zór, N. Kostesha, T.S. Alstrøm, A. Heiskanen, H. El-Tanahi, A. Sharoba, D. Papkovsky, J. Larsen, H. Khalaf, M.H. Jakobsen, J. Emnéus, Development and validation of a colorimetric sensor array for fish spoilage monitoring, Food Control, Vol. 60, 2016, pp. 346-352.

[94] R.H. Dainty, Chemical/biochemical detection of spoilage, International Journal of Food Microbiology, Vol. 33, No. 1, 1996, pp. 19-33.

[95] A. Casaburi, P. Piombino, G. Nychas, F. Villani, D. Ercolini, Bacterial populations and the volatilome associated to meat spoilage, Food Microbiology, Vol. 45, Part A, 2015, pp. 83-102.

[96] Y. Wang, Y. Li, J. Yang, J. Ruan, C. Sun, Microbial volatile organic compounds and their application in microorganism identification in foodstuff, TrAC Trends in Analytical Chemistry, Vol. 78, 2016, pp. 1-16.

[97] L. Gram, Microbiological Spoilage of Fish and Seafood Products, in: W.H. Sperber, M.P. Doyle (ed.), Compendium of the Microbiological Spoilage of Foods and Beverages, Springer New York, New York, NY, 2009, pp. 87-119.

[98] S. Mayrhofer, T. Mikoviny, S. Waldhuber, A.O. Wagner, G. Innerebner, I.H. Franke-Whittle, T.D. Märk, A. Hansel, H. Insam, Microbial community related to volatile organic compound (VOC) emission in household biowaste, Environmental microbiology, Vol. 8, No. 11, 2006, pp. 1960-1974.

[99] H. Schleibinger, D. Laussmann, C.-. Bornehag, D. Eis, H. Rueden, Microbial volatile organic compounds in the air of moldy and mold-free indoor environments, Indoor air, Vol. 18, No. 2, 2008, pp. 113-124.

[100] P. Gao, F. Korley, J. Martin, B.T. Chen, Determination of Unique Microbial Volatile Organic Compounds Produced by Five Aspergillus Species Commonly Found in Problem Buildings, AIHA Journal, Vol. 63, No. 2, 2002, pp. 135-140.

[101] W. Carroll, W. Lenney, T. Wang, P. Španěl, A. Alcock, D. Smith, Detection of volatile compounds emitted by Pseudomonas aeruginosa using selected ion flow tube mass spectrometry, Pediatric pulmonology, Vol. 39, No. 5, 2005, pp. 452-456.

[102] W. Filipiak, A. Sponring, M.M. Baur, C. Ager, A. Filipiak, H. Wiesenhofer, M. Nagl, J. Troppmair, A. Amann, Characterization of volatile metabolites taken up by or released from *Streptococcus pneumoniae* and *Haemophilus influenzae* by using GC-MS, Microbiology, Vol. 158, No. 12, 2012, pp. 3044-3053.

[103] G. Nicolas, N.M. Ratcliffe, P.T. Spencer-Phillips, R.A. Howe, Identifying Bacteria in Human Urine: Current Practice and the Potential for Rapid, Near-Patient Diagnosis by Sensing Volatile Organic Compounds, Clinical Chemistry and Laboratory Medicine, Vol. 39, No. 10, 2005, pp. 893-906.

[104] C.E. Garner, S. Smith, B. de Lacy Costello, P. White, R. Spencer, C.S.J. Probert, N.M. Ratcliffe, Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease, The FASEB Journal, Vol. 21, No. 8, 2007, pp. 1675-1688.

[105] E. Jääskeläinen, J. Hultman, J. Parshintsev, M. Riekkola, J. Björkroth, Development of spoilage bacterial community and volatile compounds in chilled beef under vacuum or high oxygen atmospheres, International Journal of Food Microbiology, Vol. 223, 2016, pp. 25-32.

[106] M.M. Reis, M.G. Reis, J. Mills, C. Ross, G. Brightwell, Characterization of volatile metabolites associated with confinement odour during the shelf-life of vacuum packed lamb meat under different storage conditions, Meat Science, Vol. 113, 2016, pp. 80-91.

[107] W. Sun, Q. Zhao, H. Zhao, M. Zhao, B. Yang, Volatile compounds of Cantonese sausage released at different stages of processing and storage, Food Chemistry, Vol. 121, No. 2, 2010, pp. 319-325.

[108] A.I. Andrés, R. Cava, J. Ruiz, Monitoring volatile compounds during dry-cured ham ripening by solid-phase microextraction coupled to a new direct-extraction device, Journal of Chromatography A, Vol. 963, No. 1–2, 2002, pp. 83-88.

[109] A. Béné, A. Hayman, E. Reynard, J.L. Luisier, J.C. Villettaz, A new method for the rapid determination of volatile substances: the SPME-direct method: Part II. Determination of the freshness of fish, Sensors and Actuators B: Chemical, Vol. 72, No. 3, 2001, pp. 204-207.

[110] C.C. Grimm, S.W. Lloyd, R. Batista, P.V. Zimba, Using Microwave Distillation-Solid-Phase Microextraction—Gas Chromatography—Mass Spectrometry for Analyzing Fish Tissue, Journal of chromatographic science, Vol. 38, No. 7, 2000, pp. 289-296.

[111] R.K.B. Edirisinghe, A.J. Graffham, S.J. Taylor, Characterisation of the volatiles of yellowfin tuna (*Thunnus albacares*) during storage by solid phase microextraction and GC-MS and their relationship to fish quality parameters, International Journal of Food Science & Technology, Vol. 42, No. 10, 2007, pp. 1139-1147.

[112] E. Jaffrès, V. Lalanne, S. Macé, J. Cornet, M. Cardinal, T. Sérot, X. Dousset, J. Joffraud, Sensory characteristics of spoilage and volatile compounds associated with bacteria isolated from cooked and peeled tropical shrimps using SPME–GC–MS analysis, International Journal of Food Microbiology, Vol. 147, No. 3, 2011, pp. 195-202.

[113] F. Leduc, F. Krzewinski, B. Le Fur, A. N'Guessan, P. Malle, O. Kol, G. Duflos, Differentiation of fresh and frozen/thawed fish, European sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), cod (*Gadus morhua*) and salmon (*Salmo salar*), using volatile compounds by SPME/GC/MS, Journal of the Science of Food and Agriculture, Vol. 92, No. 12, 2012, pp. 2560-2568.

[114] G. Duflos, F. Leduc, A. N'Guessan, F. Krzewinski, O. Kol, P. Malle, Freshness characterisation of whiting (*Merlangius merlangus*) using an SPME/GC/MS method and a statistical multivariate approach, Journal of the Science of Food and Agriculture, Vol. 90, No. 15, 2010, pp. 2568-2575.

[115] I. Fernández-Segovia, I. Escriche, M. Gómez-Sintes, A. Fuentes, J.A. Serra, Influence of different preservation treatments on the volatile fraction of desalted cod, Food Chemistry, Vol. 98, No. 3, 2006, pp. 473-482.

[116] D. Smith, P. Španěl, Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis, Mass Spectrometry Reviews, Vol. 24, No. 5, 2005, pp. 661-700.

[117] P. Španěl, D. Smith, Quantitative selected ion flow tube mass spectrometry: the influence of ionic diffusion and mass discrimination, Journal of the American Society for Mass Spectrometry, Vol. 12, No. 7, 2001, pp. 863-872.

[118] M.K. Storer, K. Hibbard-Melles, B. Davis, J. Scotter, Detection of volatile compounds produced by microbial growth in urine by selected ion flow tube mass spectrometry (SIFT-MS), Journal of Microbiological Methods, Vol. 87, No. 1, 2011, pp. 111-113.

[119] A. Hryniuk, B.M. Ross, Detection of acetone and isoprene in human breath using a combination of thermal desorption and selected ion flow tube mass spectrometry, International Journal of Mass Spectrometry, Vol. 285, No. 1–2, 2009, pp. 26-30.

[120] J.M. Scotter, R.A. Allardyce, V.S. Langford, A. Hill, D.R. Murdoch, The rapid evaluation of bacterial growth in blood cultures by selected ion flow tube–mass spectrometry (SIFT-MS) and comparison with the BacT/ALERT automated blood culture system, Journal of Microbiological Methods, Vol. 65, No. 3, 2006, pp. 628-631.

[121] J. Julák, E. Stránská, V. Rosová, H. Geppert, P. Španěl, D. Smith, Bronchoalveolar lavage examined by solid phase microextraction, gas chromatography–mass spectrometry and selected ion flow tube mass spectrometry, Journal of Microbiological Methods, Vol. 65, No. 1, 2006, pp. 76-86.

[122] J.M. Scotter, V.S. Langford, P.F. Wilson, M.J. McEwan, S.T. Chambers, Real-time detection of common microbial volatile organic compounds from medically important fungi by Selected

Ion Flow Tube-Mass Spectrometry (SIFT-MS), Journal of Microbiological Methods, Vol. 63, No. 2, 2005, pp. 127-134.

[123] A. Gibson, L. Malek, R.F.H. Dekker, B. Ross, Detecting volatile compounds from Kraft lignin degradation in the headspace of microbial cultures by selected ion flow tube mass spectrometry (SIFT-MS), Journal of Microbiological Methods, Vol. 112, 2015, pp. 40-45.

[124] D. Smith, P. Španěl, J.B. Jones, Analysis of volatile emissions from porcine faeces and urine using selected ion flow tube mass spectrometry, Bioresource technology, Vol. 75, No. 1, 2000, pp. 27-33.

[125] C. Walgraeve, J. Bruneel, K. Van Huffel, K. Demeestere, L. Vincze, B. De Meulenaer, H. Van Langenhove, Sorption behaviour of targeted volatile organic compounds on airborne particulate matter using selected ion flow tube mass spectrometry, Biosystems Engineering, Vol. 131, 2015, pp. 84-94.

[126] C. Walgraeve, K. Van Huffel, J. Bruneel, H. Van Langenhove, Evaluation of the performance of field olfactometers by selected ion flow tube mass spectrometry, Biosystems Engineering, Vol. 137, 2015, pp. 84-94.

[127] A.I. Carrapiso, B. Noseda, C. García, R. Reina, J. Sánchez del Pulgar, F. Devlieghere, SIFT-MS analysis of Iberian hams from pigs reared under different conditions, Meat Science, Vol. 104, 2015, pp. 8-13.

[128] A. Olivares, K. Dryahina, P. Španěl, M. Flores, Rapid detection of lipid oxidation in beef muscle packed under modified atmosphere by measuring volatile organic compounds using SIFT-MS, Food Chemistry, Vol. 135, No. 3, 2012, pp. 1801-1808.

[129] B. Noseda, M.T. Islam, M. Eriksson, M. Heyndrickx, K. De Reu, H. Van Langenhove, F. Devlieghere, Microbiological spoilage of vacuum and modified atmosphere packaged Vietnamese *Pangasius hypophthalmus* fillets, Food Microbiology, Vol. 30, No. 2, 2012, pp. 408-419.

[130] B. Noseda, P. Ragaert, D. Pauwels, T. Anthierens, H. Van Langenhove, J. Dewulf, F. Devlieghere, Validation of Selective Ion Flow Tube Mass Spectrometry for Fast Quantification of Volatile Bases Produced on Atlantic Cod (*Gadus morhua*), Journal of Agricultural and Food Chemistry, Vol. 58, No. 9, 2010, pp. 5213-5219.

[131] B. Noseda, J. Goethals, L. De Smedt, J. Dewulf, S. Samapundo, H. Van Langenhove, F. Devlieghere, Effect of O2CO2 enriched atmospheres on microbiological growth and volatile metabolite production in packaged cooked peeled gray shrimp (*Crangon crangon*), International Journal of Food Microbiology, Vol. 160, No. 1, 2012, pp. 65-75.

[132] B. Zhang, S. Samapundo, M. Rademaker, B. Noseda, Q. Denon, I. de Baenst, G. Sürengil, B. De Baets, F. Devlieghere, Effect of initial headspace oxygen level on growth and volatile metabolite production by the specific spoilage microorganisms of fresh-cut pineapple, LWT - Food Science and Technology, Vol. 55, No. 1, 2014, pp. 224-231.

[133] B. Zhang, S. Samapundo, V. Pothakos, I. de Baenst, G. Sürengil, B. Noseda, F. Devlieghere, Effect of atmospheres combining high oxygen and carbon dioxide levels on microbial spoilage and sensory quality of fresh-cut pineapple, Postharvest Biology and Technology, Vol. 86, 2013, pp. 73-84.

[134] B. Zhang, S. Samapundo, V. Pothakos, G. Sürengil, F. Devlieghere, Effect of high oxygen and high carbon dioxide atmosphere packaging on the microbial spoilage and shelf-life of fresh-cut honeydew melon, International Journal of Food Microbiology, Vol. 166, No. 3, 2013, pp. 378-390.

[135] V. Pothakos, C. Nyambi, B. Zhang, A. Papastergiadis, B. De Meulenaer, F. Devlieghere, Spoilage potential of psychrotrophic lactic acid bacteria (LAB) species: *Leuconostoc gelidum* subsp. *gasicomitatum* and *Lactococcus piscium*, on sweet bell pepper (SBP) simulation medium under different gas compositions, International Journal of Food Microbiology, Vol. 178, 2014, pp. 120-129.

[136] P. Španěl, D. Smith, Selected ion flow tube – mass spectrometry: detection and real-time monitoring of flavours released by food products, Rapid Communications in Mass Spectrometry, Vol. 13, No. 7, 1999, pp. 585-596.

[137] H.Z. Castada, C. Wick, K. Taylor, W.J. Harper, Analysis of selected volatile organic compounds in split and nonsplit swiss cheese samples using selected-ion flow tube mass spectrometry (SIFT-MS), Journal of Food Science, Vol. 79, No. 4, 2014, pp. C489-98.

[138] H.Z. Castada, C. Wick, W.J. Harper, S. Barringer, Headspace quantification of pure and aqueous solutions of binary mixtures of key volatile organic compounds in Swiss cheeses using selected ion flow tube mass spectrometry, Rapid Communications in Mass Spectrometry : RCM, Vol. 29, No. 1, 2015, pp. 81-90.

[139] V.S. Langford, C.J. Reed, D.B. Milligan, M.J. McEwan, S.A. Barringer, W.J. Harper, Headspace analysis of Italian and New Zealand parmesan cheeses, Journal of Food Science, Vol. 77, No. 6, 2012, pp. C719-26.

[140] F. Devlieghere, J. Debevere, J. Van Impe, Concentration of carbon dioxide in the water-phase as a parameter to model the effect of a modified atmosphere on microorganisms, International Journal of Food Microbiology, Vol. 43, No. 1–2, 1998, pp. 105-113.

[141] F. Devlieghere, J. Debevere, Influence of Dissolved Carbon Dioxide on the Growth of Spoilage Bacteria, LWT - Food Science and Technology, Vol. 33, No. 8, 2000, pp. 531-537.

[142] B.A. Blakistone, 10 Meats and poultry, in: B.A. Blakistone (ed.), Principles and Applications of Modified Atmosphere Packaging of Foods, Springer US, 1998, pp. 240-290.

[143] A.E. Kapetanakou, E.I. Agathaggelou, P.N. Skandamis, Storage of pork meat under modified atmospheres containing vapors from commercial alcoholic beverages, International Journal of Food Microbiology, Vol. 178, No. 0, 2014, pp. 65-75.

[144] M.N. Lund, M. Heinonen, C.P. Baron, M. Estévez, Protein oxidation in muscle foods: A review, Molecular Nutrition & Food Research, Vol. 55, No. 1, 2011, pp. 83-95.

[145] D.P. Cornforth, M.C. Hunt, Low-Oxygen Packaging of Fresh Meat with Carbon Dioxide, 2, American Meat Science Association White Paper Series, 2008, .

[146] A.M. Rulis, Agency Response Letter GRAS Notice No. GRN 000083, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2002, February 21.

[147] L.M. Tarantino, Agency Response Letter GRAS Notice No. GRN 000143, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2004, July 29.

[148] K. Koutsoumanis, A. Stamatiou, P. Skandamis, G.-.E. Nychas, Development of a Microbial Model for the Combined Effect of Temperature and pH on Spoilage of Ground Meat, and Validation of the Model under Dynamic Temperature Conditions, Applied and Environmental Microbiology, Vol. 72, No. 1, 2006, pp. 124-134.

[149] D. Ercolini, F. Russo, G. Blaiotta, O. Pepe, G. Mauriello, F. Villani, Simultaneous Detection of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* from Meat by Use of a Multiplex PCR Assay Targeting the carA Gene, Applied and Environmental Microbiology, Vol. 73, No. 7, 2007, pp. 2354-2359.

[150] D. Ercolini, F. Russo, E. Torrieri, P. Masi, F. Villani, Changes in the Spoilage-Related Microbiota of Beef during Refrigerated Storage under Different Packaging Conditions, Applied and Environmental Microbiology, Vol. 72, No. 7, 2006, pp. 4663-4671.

[151] D. Ercolini, A. Casaburi, A. Nasi, I. Ferrocino, R. Di Monaco, P. Ferranti, G. Mauriello, F. Villani, Different molecular types of *Pseudomonas fragi* have the same overall behaviour as meat spoilers, International Journal of Food Microbiology, Vol. 142, No. 1–2, 2010, pp. 120-131.

[152] J.M. Jay, J.P. Vilai, M.E. Hughes, Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5–7 °C, International Journal of Food Microbiology, Vol. 81, No. 2, 2003, pp. 105-111.

[153] D. Ercolini, I. Ferrocino, A. Nasi, M. Ndagijimana, P. Vernocchi, A. La Storia, L. Laghi, G. Mauriello, M.E. Guerzoni, F. Villani, Monitoring of Microbial Metabolites and Bacterial Diversity in Beef Stored under Different Packaging Conditions, Applied and Environmental Microbiology, Vol. 77, No. 20, 2011, pp. 7372-7381.

[154] C. Pennacchia, D. Ercolini, F. Villani, Spoilage-related microbiota associated with chilled beef stored in air or vacuum pack, Food Microbiology, Vol. 28, No. 1, 2011, pp. 84-93.

[155] M.Y. Li, G.H. Zhou, X.L. Xu, C.B. Li, W.Y. Zhu, Changes of bacterial diversity and main flora in chilled pork during storage using PCR-DGGE, Food Microbiology, Vol. 23, No. 7, 2006, pp. 607-611.

[156] G. Molin, A. Ternström, Numerical Taxonomy of Psychrotrophic Pseudomonads, Microbiology, Vol. 128, No. 6, 1982, pp. 1249-1264.

[157] T. Susiluoto, H. Korkeala, K.J. Björkroth, Leuconostoc gasicomitatum is the dominating lactic acid bacterium in retail modified-atmosphere-packaged marinated broiler meat strips on sell-by-day, International Journal of Food Microbiology, Vol. 80, No. 1, 2003, pp. 89-97.

[158] E. Borch, M. Kant-Muermans, Y. Blixt, Specific Spoilage Organisms Bacterial spoilage of meat and cured meat products, International Journal of Food Microbiology, Vol. 33, No. 1, 1996, pp. 103-120.

[159] C.O. Gill, K.H. Tan, Effect of Carbon Dioxide on Growth of Meat Spoilage Bacteria, Applied and Environmental Microbiology, Vol. 39, No. 2, 1980, pp. 317-319.

[160] E. Borch, M. Kant-Muermans, Y. Blixt, Bacterial spoilage of meat and cured meat products, International Journal of Food Microbiology, Vol. 33, No. 1, 1996, pp. 103-120.

[161] J. Labadie, Consequences of packaging on bacterial growth. Meat is an ecological niche, Meat Science, Vol. 52, No. 3, 1999, pp. 299-305.

[162] F. Russo, D. Ercolini, G. Mauriello, F. Villani, Behaviour of *Brochothrix thermosphacta* in presence of other meat spoilage microbial groups, Food Microbiology, Vol. 23, No. 8, 2006, pp. 797-802.

[163] Y. Jiang, F. Gao, X.L. Xu, Y. Su, K.P. Ye, G.H. Zhou, Changes in the bacterial communities of vacuum-packaged pork during chilled storage analyzed by PCR–DGGE, Meat Science, Vol. 86, No. 4, 2010, pp. 889-895.

[164] R.M. Sakala, H. Hayashidani, Y. Kato, T. Hirata, Y. Makino, A. Fukushima, T. Yamada, C. Kaneuchi, M. Ogawa, Change in the composition of the microflora on vacuum-packaged beef during chiller storage, International Journal of Food Microbiology, Vol. 74, No. 1–2, 2002, pp. 87-99.

[165] F. Leroi, Occurrence and role of lactic acid bacteria in seafood products, Food Microbiology, Vol. 27, No. 6, 2010, pp. 698-709.

[166] C.K. Yost, F.M. Nattress, Molecular typing techniques to characterize the development of a lactic acid bacteria community on vacuum-packaged beef, International Journal of Food Microbiology, Vol. 72, No. 1–2, 2002, pp. 97-105.

[167] A.I. Doulgeraki, S. Paramithiotis, D.M. Kagkli, G.E. Nychas, Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions, Food Microbiology, Vol. 27, No. 8, 2010, pp. 1028-1034.

[168] T.T. Nieminen, E. Vihavainen, A. Paloranta, J. Lehto, L. Paulin, P. Auvinen, M. Solismaa, K.J. Björkroth, Characterization of psychrotrophic bacterial communities in modified atmosphere-packed meat with terminal restriction fragment length polymorphism, International Journal of Food Microbiology, Vol. 144, No. 3, 2011, pp. 360-366.

[169] E.J. Vihavainen, K.J. Björkroth, Spoilage of value-added, high-oxygen modified-atmosphere packaged raw beef steaks by *Leuconostoc gasicomitatum* and *Leuconostoc gelidum*, International Journal of Food Microbiology, Vol. 119, No. 3, 2007, pp. 340-345.

[170] E.J. Vihavainen, M.A. FAU, K.J. Bjorkroth, Leuconostoc spoilage of vacuum-packaged vegetable sausages, Journal of food protection, Vol. 71, No. 11, 2008, pp. 2312-2315..

[171] U. Lyhs, J.M.K. Koort, H. Lundström, K.J. Björkroth, *Leuconostoc gelidum* and *Leuconostoc gasicomitatum* strains dominated the lactic acid bacterium population associated with strong slime formation in an acetic-acid herring preserve, International Journal of Food Microbiology, Vol. 90, No. 2, 2004, pp. 207-218.

[172] P. Johansson, L. Paulin, E. Sade, N. Salovuori, E.R. Alatalo, K.J. Bjorkroth, P. Auvinen, Genome sequence of a food spoilage lactic acid bacterium, *Leuconostoc gasicomitatum* LMG 18811T, in association with specific spoilage reactions, Applied and Environmental Microbiology, Vol. 77, No. 13, 2011, pp. 4344-4351.

[173] D. Ercolini, I. Ferrocino, A. La Storia, G. Mauriello, S. Gigli, P. Masi, F. Villani, Development of spoilage microbiota in beef stored in nisin activated packaging, Food Microbiology, Vol. 27, No. 1, 2010, pp. 137-143.

[174] B. Groth Laursen, L. Bay, I. Cleenwerck, M. Vancanneyt, J. Swings, P. Dalgaard, J.J. Leisner, *Carnobacterium divergens* and *Carnobacterium maltaromaticum* as spoilers or protective cultures in meat and seafood: phenotypic and genotypic characterization, Systematic and Applied Microbiology, Vol. 28, No. 2, 2005, pp. 151-164.

[175] A.R. Silva, É.N. Paulo, A.S. Sant'Ana, R.D. Chaves, P.R. Massaguer, Involvement of *Clostridium gasigenes* and *C. algidicarnis* in 'blown pack' spoilage of Brazilian vacuum-packed beef, International Journal of Food Microbiology, Vol. 148, No. 3, 2011, pp. 156-163.

[176] K.H. Adam, S.H. Flint, G. Brightwell, Psychrophilic and psychrotrophic clostridia: sporulation and germination processes and their role in the spoilage of chilled, vacuum-packaged beef, lamb and venison, International Journal of Food Science & Technology, Vol. 45, No. 8, 2010, pp. 1539-1544.

[177] A.I. Doulgeraki, S. Paramithiotis, G.E. Nychas, Characterization of the *Enterobacteriaceae* community that developed during storage of minced beef under aerobic or modified atmosphere packaging conditions, International Journal of Food Microbiology, Vol. 145, No. 1, 2011, pp. 77-83.

[178] O. Sørheim, D.H. Kropf, M.C. Hunt, M.T. Karwoski, K.E. Warren, Effects of modified gas atmosphere packaging on pork loin colour, display life and drip loss, Meat Science, Vol. 43, No. 2, 1996, pp. 203-212.

[179] J. Martin, J. Lyte, J. Legako, L. Thompson, K. Surowiec, J.C. Brooks, Volatile compounds related to ground beef spoilage, Meat Science, Vol. 101, 2015, pp. 141-142.

[180] T.T. Nieminen, P. Dalgaard, J. Björkroth, Volatile organic compounds and *Photobacterium phosphoreum* associated with spoilage of modified-atmosphere-packaged raw pork, International Journal of Food Microbiology, Vol. 218, 2016, pp. 86-95.

[181] M. Jakobsen, G. Bertelsen, Colour stability and lipid oxidation of fresh beef. Development of a response surface model for predicting the effects of temperature, storage time, and modified atmosphere composition, Meat Science, Vol. 54, No. 1, 2000, pp. 49-57.

[182] L. Martínez, D. Djenane, I. Cilla, J.A. Beltrán, P. Roncalés, Effect of varying oxygen concentrations on the shelf-life of fresh pork sausages packaged in modified atmosphere, Food Chemistry, Vol. 94, No. 2, 2006, pp. 219-225.

[183] O.K. Esmer, R. Irkin, N. Degirmencioglu, A. Degirmencioglu, The effects of modified atmosphere gas composition on microbiological criteria, color and oxidation values of minced beef meat, Meat Science, Vol. 88, No. 2, 2011, pp. 221-226.

[184] O. Sørheim, T. Erlandsen, H. Nissen, P. Lea, T. Høyem, Effects of modified atmosphere storage on colour and microbiological shelf life of normal and pale, soft and exudative pork, Meat Science, Vol. 47, No. 1–2, 1997, pp. 147-155.

[185] E.S. Viana, L.A.M. Gomide, M.C.D. Vanetti, Effect of modified atmospheres on microbiological, color and sensory properties of refrigerated pork, Meat Science, Vol. 71, No. 4, 2005, pp. 696-705.

[186] A.M. Doherty, P. Allen, The effect of oxygen scavengers on the color stability and shelf-life of CO, master packaged pork, Journal of Muscle Foods, Vol. 9, No. 4, 1998, pp. 351-363.

[187] D. Spanos, M. Christensen, M.A. Tørngren, C.P. Baron, Visible spectroscopy as a tool for the assessment of storage conditions of fresh pork packaged in modified atmosphere, Meat Science, Vol. 113, 2016, pp. 154-161.

[188] M. Beggan, P. Allen, F. Butler, Shelf-life of retail beef muscles following storage un a low oxygen environment, Journal of Muscle Foods, Vol. 15, No. 4, 2004, pp. 269-285.

[189] A.C. Venturini, C.J.C. Contreras, C.I.G.L. Sarantópoulos, N.D.M. Villanueva, The Effects of Residual Oxygen on the Storage Life of Retail-Ready Fresh Beef Steaks Masterpackaged Under a CO2 Atmosphere, Journal of Food Science, Vol. 71, No. 7, 2006, pp. S560-S566.

[190] L.G. Zhu, B. Bidner, M.S. Brewer, Postmortem pH, Muscle, and Refrigerated Storage Effects on Ability of Vacuum-Packaged Pork to Bloom, Journal of Food Science, Vol. 66, No. 8, 2001, pp. 1230-1235.

[191] M.D. Aaslyng, M.A. Tørngren, N.T. Madsen, Scandinavian consumer preference for beef steaks packed with or without oxygen, Meat Science, Vol. 85, No. 3, 2010, pp. 519-524.

[192] C. Stannard, Development and use of microbiological criteria for foods, Food Science and Technology Today, Vol. 11, No. 3, 1997, pp. 137-176.

[193] P. Dalgaard, Qualitative and quantitative characterization of spoilage bacteria from packed fish, International Journal of Food Microbiology, Vol. 26, No. 3, 1995, pp. 319-333.

[194] G. Boskou, J. Debevere, Reduction of trimethylamine oxide by *Shewanella* spp. under modified atmospheresin vitro, Food Microbiology, Vol. 14, No. 6, 1997, pp. 543-553.

[195] L. Gram, G. Trolle, H.H. Huss, Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures, International Journal of Food Microbiology, Vol. 4, No. 1, 1987, pp. 65-72.

[196] M.E. López-Caballero, J.A. Sánchez-Fernández, A. Moral, Growth and metabolic activity of *Shewanella putrefaciens* maintained under different CO2 and O2 concentrations, International Journal of Food Microbiology, Vol. 64, No. 3, 2001, pp. 277-287.

[197] M.B. Hovda, B.T. Lunestad, M. Sivertsvik, J.T. Rosnes, Characterisation of the bacterial flora of modified atmosphere packaged farmed Atlantic cod (*Gadus morhua*) by PCR-DGGE of conserved 16S rRNA gene regions, International Journal of Food Microbiology, Vol. 117, No. 1, 2007, pp. 68-75.

[198] G. Olafsdottir, R. Jonsdottir, H.L. Lauzon, J. Luten, K. Kristbergsson, Characterization of Volatile Compounds in Chilled Cod (*Gadus morhua*) Fillets by Gas Chromatography and Detection of Quality Indicators by an Electronic Nose, Journal of Agricultural and Food Chemistry, Vol. 53, No. 26, 2005, pp. 10140-10147.

[199] Q.Z. Zeng, K.A. Thorarinsdottir, G. Olafsdottir, Quality Changes of Shrimp (*Pandalus borealis*) Stored under Different Cooling Conditions, Journal of Food Science, Vol. 70, No. 7, 2005, pp. s459-s466.

[200] D.S. Dabadé, H.M.W. den Besten, P. Azokpota, M.J.R. Nout, D.J. Hounhouigan, M.H. Zwietering, Spoilage evaluation, shelf-life prediction, and potential spoilage organisms of tropical brackish water shrimp (*Penaeus notialis*) at different storage temperatures, Food Microbiology, Vol. 48, 2015, pp. 8-16.

[201] E. Jaffrès, D. Sohier, F. Leroi, M.F. Pilet, H. Prévost, J.J. Joffraud, X. Dousset, Study of the bacterial ecosystem in tropical cooked and peeled shrimps using a polyphasic approach, International Journal of Food Microbiology, Vol. 131, No. 1, 2009, pp. 20-29.

[202] G. Jeyasekaran, P. Ganesan, R. Anandaraj, R. Jeya Shakila, D. Sukumar, Quantitative and qualitative studies on the bacteriological quality of Indian white shrimp (*Penaeus indicus*) stored in dry ice, Food Microbiology, Vol. 23, No. 6, 2006, pp. 526-533.

[203] F. Calliauw, T. De Mulder, K. Broekaert, G. Vlaemynck, C. Michiels, M. Heyndrickx, Assessment throughout a whole fishing year of the dominant microbiota of peeled brown shrimp (*Crangon crangon*) stored for 7 days under modified atmosphere packaging at 4 °C without preservatives, Food Microbiology, Vol. 54, 2016, pp. 60-71.

[204] P. Dalgaard, M. Vancanneyt, N. Euras Vilalta, J. Swings, P. Fruekilde, J.J. Leisner, Identification of lactic acid bacteria from spoilage associations of cooked and brined shrimps stored under modified atmosphere between 0°C and 25°C, Journal of Applied Microbiology, Vol. 94, No. 1, 2003, pp. 80-89.

[205] K. Broekaert, M. Heyndrickx, L. Herman, F. Devlieghere, G. Vlaemynck, Molecular identification of the microbiota of peeled and unpeeled brown shrimp (*Crangon crangon*) during storage on ice and at 7.5 °C, Food Microbiology, Vol. 36, No. 2, 2013, pp. 123-134.

[206] O. Mejlholm, N. Bøknæs, P. Dalgaard, Shelf life and safety aspects of chilled cooked and peeled shrimps (*Pandalus borealis*) in modified atmosphere packaging, Journal of Applied Microbiology, Vol. 99, No. 1, 2005, pp. 66-76.

[207] M. Sivertsvik, The optimized modified atmosphere for packaging of pre-rigor filleted farmed cod (*Gadus morhua*) is 63 ml/100 ml oxygen and 37 ml/100 ml carbon dioxide, LWT - Food Science and Technology, Vol. 40, No. 3, 2007, pp. 430-438.

[208] T. Aro, R. Tahvonen, L. Koskinen, H. Kallio, Volatile compounds of Baltic herring analysed by dynamic headspace sampling-gas chromatography-mass spectrometry, European Food Research and Technology, Vol. 216, No. 6, 2003, pp. 483-488.

[209] A. Miller, R.A. Scanlan, J.S. Lee, L.M. Libbey, Identification of the Volatile Compounds Produced in Sterile Fish Muscle (*Sebastes melanops*) by *Pseudomonas* fragi, Applied Microbiology, Vol. 25, No. 6, 1973, pp. 952-955.

[210] J.J. Joffraud, F. Leroi, C. Roy, J.L. Berdagué, Characterisation of volatile compounds produced by bacteria isolated from the spoilage flora of cold-smoked salmon, International Journal of Food Microbiology, Vol. 66, No. 3, 2001, pp. 175-184.

[211] The Good Scents Company, The Good Scents Company Information System, web page. Available online (accessed 13/07/2018): http://www.thegoodscentscompany.com/.

[212] S. Ui, T. Masuda, H. Masuda, H. Muraki, Mechanism for the formation of 2,3-butanediol stereoisomers in *Bacillus polymyxa*, Journal of Fermentation Technology, Vol. 64, No. 6, 1986, pp. 481-486.

[213] L. Gram, L. Ravn, M. Rasch, J.B. Bruhn, A.B. Christensen, M. Givskov, Food spoilage—interactions between food spoilage bacteria, International Journal of Food Microbiology, Vol. 78, No. 1–2, 2002, pp. 79-97.

[214] S. Macé, M. Cardinal, E. Jaffrès, J. Cornet, V. Lalanne, F. Chevalier, T. Sérot, M. Pilet, X. Dousset, J. Joffraud, Evaluation of the spoilage potential of bacteria isolated from spoiled cooked whole tropical shrimp (*Penaeus vannamei*) stored under modified atmosphere packaging, Food Microbiology, Vol. 40, 2014, pp. 9-17.

[215] P. Dalgaard, L. Gram, H.H. Huss, Spoilage and shelf-life of cod fillets packed in vacuum or modified atmospheres, International Journal of Food Microbiology, Vol. 19, No. 4, 1993, pp. 283-294.

[216] M.C. Easter, D.M. Gibson, F.B. Ward, A conductance method for the assay and study of bacterial trimethylamine oxide reduction, Journal of Applied Bacteriology, Vol. 52, No. 3, 1982, pp. 357-365.

[217] G. Boskou, J. Debevere, In vitro study of TMAO reduction by *Shewanella putrefaciens* isolated from cod fillets packed in modified atmosphere, Food additives and contaminants, Vol. 15, No. 2, 1998, pp. 229-236.

[218] B.G. Laursen, J. Leisner, P. Dalgaard, *Carnobacterium* Species: Effect of Metabolic Activity and Interaction with *Brochothrix thermosphacta* on Sensory Characteristics of Modified Atmosphere Packed Shrimp, Journal of Agricultural and Food Chemistry, Vol. 54, No. 10, 2006, pp. 3604-3611.

[219] K. Broekaert, B. Noseda, M. Heyndrickx, G. Vlaemynck, F. Devlieghere, Volatile compounds associated with *Psychrobacter* spp. and *Pseudoalteromonas* spp., the dominant microbiota of brown shrimp (*Crangon crangon*) during aerobic storage, International Journal of Food Microbiology, Vol. 166, No. 3, 2013, pp. 487-493.

[220] P.A. Fall, M.F. Pilet, F. Leduc, M. Cardinal, G. Duflos, C. Guérin, J. Joffraud, F. Leroi, Sensory and physicochemical evolution of tropical cooked peeled shrimp inoculated by *Brochothrix thermosphacta* and *Lactococcus piscium* CNCM I-4031 during storage at 8 °C, International Journal of Food Microbiology, Vol. 152, No. 3, 2012, pp. 82-90.

[221] C. Pin, G. Garcí-a de Fernando D., J. Ordóñez A., Effect of Modified Atmosphere Composition on the Metabolism of Glucose by *Brochothrix thermosphacta*, Applied and Environmental Microbiology, Vol. 68, No. 9, 2002, pp. 4441-4447.

[222] T.P. Labuza, W.M. Breene, Applications of "active packaging" for improvement of shelf-life and nutritional quality of fresh and extended shelf-life foods, Journal of Food Processing and Preservation, Vol. 13, No. 1, 1989, pp. 1-69.

[223] J.H. Han, Antimicrobial Food Packaging, Food Technology, Vol. 54, No. 3, 2000, pp. 56-65.

[224] W. Michaeli, Plastics Processing - An Introduction, Carl Hanser Verlag, Munich, Vienna, New York, 1995, 211 p.

[225] S. Levy, J.F. Carley, Plastics Extrusion Technology Handbook, 2nd Edition ed. Industrial Press Inc., New York, 1989, 388 p.

[226] S.M. Weiss, Extrusion Coating Lines, in: W.M. Karszes (ed.), Extrusion Coating of Paper and Paperboard - Equipment and Materials, 3rd Edition ed., TAPPI, Atlanta, 1990, pp. 5-9.

[227] J. Kuusipalo, A. Savolainen, E. Laiho, T. Penttinen, Extrusion coating and products, in: J. Kuusipalo (ed.), Paper and Paperboard Converting, Finnish Paper Engineers' Association/Paperi ja Puu Oy, Helsinki, 2008, pp. 108-166.

[228] The European Parliament and the Council, Regulation (EC) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come

into contact with food and repealing Directives 80/590/EEC and 89/109/EEC, Official Journal of the European Union, Vol. 47, No. L 338, 2004.

[229] The Commission of the European Communities, Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food Text with EEA relevance, Official Journal of the European Union, Vol. 54, No. L 12, 2009, pp. 1-89.

[230] EFSA, Provisional list of additives used in Plastics, EFSA, 28/10/2011. Available online (accessed 13/07/2018): https://ec.europa.eu/food/sites/food/files/safety/docs/cs_fcm_legis_additives-prov-list.pdf.

[231] The Commission of the European Communities, Commission Regulation (EC) No 450/2009 of 29 May 2009 on active and intelligent materials and articles intended to come into contact with food (Text with EEA relevance), Official Journal of the European Union, Vol. 52, No. L 135, 2009, pp. 3-11.

[232] N. Silvestry-Rodriguez, E.E. Sicairos-Ruelas, C.P. Gerba, K.R. Bright, Silver as a Disinfectant, Reviews of Environmental Contamination and Toxicology, Vol. 191, 2007, pp. 23-45.

[233] A. Fernández, E. Soriano, P. Hernández-Muñoz, R. Gavara, Migration of Antimicrobial Silver from Composites of Polylactide with Silver Zeolites, Journal of Food Science, Vol. 75, No. 3, 2010, pp. E186-E193.

[234] P. Lalueza, M. Monzón, M. Arruebo, J. Santamaría, Bactericidal effects of different silver-containing materials, Materials Research Bulletin, Vol. 46, No. 11, 2011, pp. 2070-2076.

[235] Q.L. Feng, J. Wu, G.Q. Chen, F.Z. Cui, T.N. Kim, J.O. Kim, A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*, Journal of Biomedical Materials Research, Vol. 52, No. 4, 2000, pp. 662-668.

[236] R.L. Davies, S.F. Etris, Copper, Silver and Gold in Catalysis The development and functions of silver in water purification and disease control, Catalysis Today, Vol. 36, No. 1, 1997, pp. 107-114.

[237] S.Y. Liau, D.C. Read, W.J. Pugh, J.R. Furr, A.D. Russell, Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions, Letters in Applied Microbiology, Vol. 25, No. 4, 1997, pp. 279-283.

[238] K.B. Holt, A.J. Bard, Interaction of Silver(I) Ions with the Respiratory Chain of *Escherichia coli*: An Electrochemical and Scanning Electrochemical Microscopy Study of the Antimicrobial Mechanism of Micromolar Ag+, Biochemistry, Vol. 44, No. 39, 2005, pp. 13214-13223.

[239] A. Top, S. Ülkü, Silver, zinc, and copper exchange in a Na-clinoptilolite and resulting effect on antibacterial activity, Applied Clay Science, Vol. 27, No. 1–2, 2004, pp. 13-19.

[240] D. Guldiren, S. Aydin, Characterization and antimicrobial properties of soda lime glass prepared by silver/sodium ion exchange, Materials Science and Engineering: C, Vol. 67, 2016, pp. 144-150.

[241] H. Jia, W. Hou, L. Wei, B. Xu, X. Liu, The structures and antibacterial properties of nano-SiO2 supported silver/zinc–silver materials, Dental Materials, Vol. 24, No. 2, 2008, pp. 244-249.

[242] R. Tekin, N. Bac, Antimicrobial behavior of ion-exchanged zeolite X containing fragrance, Microporous and Mesoporous Materials, Vol. 234, 2016, pp. 55-60.

[243] L. Ferreira, J.F. Guedes, C. Almeida-Aguiar, A.M. Fonseca, I.C. Neves, Microbial growth inhibition caused by Zn/Ag-Y zeolite materials with different amounts of silver, Colloids and Surfaces B: Biointerfaces, Vol. 142, 2016, pp. 141-147.

[244] K. Kawahara, K. Tsuruda, M. Morishita, M. Uchida, Antibacterial effect of silver-zeolite on oral bacteria under anaerobic conditions, Dental Materials, Vol. 16, No. 6, 2000, pp. 452-455.

[245] H. Pehlivan, D. Balköse, S. Ülkü, F. Tihminliog˘lu, Characterization of pure and silver exchanged natural zeolite filled polypropylene composite films, Composites Science and Technology, Vol. 65, No. 13, 2005, pp. 2049-2058.

[246] H. Dogan, M. Koral, T.Y. Inan, Ag/Zn Zeolite Containing Antibacterial Coating for Food-Packaging Substrates, Journal of Plastic Film and Sheeting, Vol. 25, No. 3-4, 2009, pp. 207-220.

[247] D.L. Boschetto, L. Lerin, R. Cansian, S.B.C. Pergher, M. Di Luccio, Preparation and antimicrobial activity of polyethylene composite films with silver exchanged zeolite-Y, Chemical Engineering Journal, Vol. 204–206, No. 0, 2012, pp. 210-216.

[248] C. Lok, C. Ho, R. Chen, Q. He, W. Yu, H. Sun, P.K. Tam, J. Chiu, C. Che, Proteomic Analysis of the Mode of Antibacterial Action of Silver Nanoparticles, Journal of Proteome Research, Vol. 5, No. 4, 2006, pp. 916-924.

[249] J. Lee, Y. Lee, K. Jones, E. Sharek, M.A. Pascall, Antimicrobial packaging of raw beef, pork and turkey using silver-zeolite incorporated into the material, International Journal of Food Science & Technology, Vol. 46, No. 11, 2011, pp. 2382-2386.

[250] S. Kittler, C. Greulich, J. Diendorf, M. KÃ¶ller, M. Epple, Toxicity of Silver Nanoparticles Increases during Storage Because of Slow Dissolution under Release of Silver Ions, Chemistry of Materials, Vol. 22, No. 16, 2010, pp. 4548-4554.

[251] J.S. Möhler, W. Sim, M.A.T. Blaskovich, M.A. Cooper, Z.M. Ziora, Silver bullets: A new lustre on an old antimicrobial agent, Biotechnology Advances, Vol. 36, No. 5, 2018, pp. 1391-1411.

[252] J.S. Kim, J.H. Sung, J.H. Ji, K.S. Song, J.H. Lee, C.S. Kang, I.J. Yu, In vivo Genotoxicity of Silver Nanoparticles after 90-day Silver Nanoparticle Inhalation Exposure, Safety and Health at Work, Vol. 2, No. 1, 2011, pp. 34-38.

[253] S. Sweeney, B.F. Leo, S. Chen, N. Abraham-Thomas, A.J. Thorley, A. Gow, S. Schwander, J.J. Zhang, M.S.P. Shaffer, K.F. Chung, M.P. Ryan, A.E. Porter, T.D. Tetley, Pulmonary surfactant mitigates silver nanoparticle toxicity in human alveolar type-I-like epithelial cells, Colloids and Surfaces B: Biointerfaces, Vol. 145, 2016, pp. 167-175.

[254] M. Ahamed, M.S. AlSalhi, M.K.J. Siddiqui, Silver nanoparticle applications and human health, Clinica Chimica Acta, Vol. 411, No. 23, 2010, pp. 1841-1848.

[255] N. Hadrup, H.R. Lam, Oral toxicity of silver ions, silver nanoparticles and colloidal silver – A review, Regulatory Toxicology and Pharmacology, Vol. 68, No. 1, 2014, pp. 1-7.

[256] N.G. Marchiore, I.J. Manso, K.C. Kaufmann, G.F. Lemes, A.P.d.O. Pizolli, A.A. Droval, L. Bracht, O.H. Gonçalves, F.V. Leimann, Migration evaluation of silver nanoparticles from antimicrobial edible coating to sausages, LWT - Food Science and Technology, Vol. 76, 2017, pp. 203-208.

[257] J.C. Hannon, J.P. Kerry, M. Cruz-Romero, S. Azlin-Hasim, M. Morris, E. Cummins, Migration assessment of silver from nanosilver spray coated low density polyethylene or polyester films into milk, Food Packaging and Shelf Life, Vol. 15, 2018, pp. 144-150.

[258] L. Wu, Q. Su, Q. Lin, W. Peng, C. Chen, Z. Wang, C. Hu, Impact of migration test method on the release of silver from nanosilver-polyethylene composite films into an acidic food simulant, Food Packaging and Shelf Life, Vol. 14, 2017, pp. 83-87.

[259] EFSA, Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain, EFSA Journal, Vol. 9, No. 5, 2011, pp. 2140.

[260] EFSA, 1-21 Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request related to a 12th list of substances for food contact materials, The EFSA Journal, 2006, pp. 395-401.

[261] The European Parliament and the Council, Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives (Text with EEA relevance), Official Journal of the European Union, Vol. 51, No. L 354, 2008, pp. 16-33.

[262] EFSA, Scientific opinion on the re-evaluation of silver (E 174) as food additive, EFSA Journal, Vol. 14, No. 1, 2016, pp. 4364.

[263] V. Coma, Bioactive packaging technologies for extended shelf life of meat-based products, Meat Science, Vol. 78, No. 1–2, 2008, pp. 90-103.

[264] J.P. Kerry, M.N. O'Grady, S.A. Hogan, Past, current and potential utilisation of active and intelligent packaging systems for meat and muscle-based products: A review, Meat Science, Vol. 74, No. 1, 2006, pp. 113-130.

[265] L. Vermeiren, F. Devlieghere, M. van Beest, N. de Kruijf, J. Debevere, Developments in the active packaging of foods, Trends in Food Science & Technology, Vol. 10, No. 3, 1999, pp. 77-86.

[266] G. Saravacos, P. Taoukis, M. Krokida, V. Karathanos, H. Lazarides, N. Stoforos, C. Tzia, S. Yanniotis, D. Gibis, K. Rieblinger, 11th International Congress on Engineering and Food (ICEF11) Oxygen scavenging films for food application, Procedia Food Science, Vol. 1, 2011, pp. 229-234.

[267] T.P. Labuza, Oxygen scavenger sachets, Food Research, Vol. 32, 1987, pp. 276-277.

[268] M.L. Rooney, Oxygen Scavenging from Air in Package Headspaces by Singlet Oxygen Reactions in Polymer Media, Journal of Food Science, Vol. 47, No. 1, 1982, pp. 291-294.

[269] R.S. Cruz, G.P. Camilloto, dos Santos Pires, Ana Clarissa, Oxygen Scavengers: An Approach on Food Preservation, Structure and Function of Food Engineering, in: A.E. Ayman (ed.), Structure and Function of Food Engineering, InTech, 2012. Available online (accessed 13/07/2018: https://www.intechopen.com/books/structure-and-function-of-food-engineering/oxygen-scavengers-an-approach-on-food-preservation.

[270] P. Suppakul, J. Miltz, K. Sonneveld, S.W. Bigger, Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications, Journal of Food Science, Vol. 68, No. 2, 2003, pp. 408-420.

[271] M.S. Aday, C. Caner, The shelf life extension of fresh strawberries using an oxygen absorber in the biobased package, LWT - Food Science and Technology, Vol. 52, No. 2, 2013, pp. 102-109.

[272] F. Charles, C. Guillaume, N. Gontard, Effect of passive and active modified atmosphere packaging on quality changes of fresh endives, Postharvest Biology and Technology, Vol. 48, No. 1, 2008, pp. 22-29.

[273] M.S. Aday, C. Caner, F. Rahvalı, Effect of oxygen and carbon dioxide absorbers on strawberry quality, Postharvest Biology and Technology, Vol. 62, No. 2, 2011, pp. 179-187.

[274] S.F. Mexis, E. Chouliara, M.G. Kontominas, Combined effect of an O2 absorber and oregano essential oil on shelf-life extension of Greek cod roe paste (tarama salad) stored at 4 °C, Innovative Food Science & Emerging Technologies, Vol. 10, No. 4, 2009, pp. 572-579.

[275] E. Latou, S.F. Mexis, A.V. Badeka, M.G. Kontominas, Shelf life extension of sliced wheat bread using either an ethanol emitter or an ethanol emitter combined with an oxygen absorber as alternatives to chemical preservatives, Journal of cereal science, Vol. 52, No. 3, 2010, pp. 457-465.

[276] S.F. Mexis, E. Chouliara, M.G. Kontominas, Shelf life extension of ground chicken meat using an oxygen absorber and a citrus extract, LWT - Food Science and Technology, Vol. 49, No. 1, 2012, pp. 21-27.

[277] T. Bolumar, D. LaPeña, L.H. Skibsted, V. Orlien, Rosemary and oxygen scavenger in active packaging for prevention of high-pressure induced lipid oxidation in pork patties, Food Packaging and Shelf Life, Vol. 7, 2016, pp. 26-33.

[278] S.F. Mexis, E. Chouliara, M.G. Kontominas, Combined effect of an oxygen absorber and oregano essential oil on shelf life extension of rainbow trout fillets stored at 4 °C, Food Microbiology, Vol. 26, No. 6, 2009, pp. 598-605.

[279] C.O. Mohan, C.N. Ravishankar, T.K. Srinivasa Gopal, K.V. Lalitha, K. Asok Kumar, Effect of reduced oxygen atmosphere and sodium acetate treatment on the microbial quality changes of seer fish (*Scomberomorus commerson*) steaks stored in ice, Food Microbiology, Vol. 27, No. 4, 2010, pp. 526-534.

[280] C.E. Realini, B. Marcos, Active and intelligent packaging systems for a modern society, Meat Science, Vol. 98, No. 3, 2014, pp. 404-419.

[281] M. Vanderroost, P. Ragaert, F. Devlieghere, B. De Meulenaer, Intelligent food packaging: The next generation, Trends in Food Science & Technology, Vol. 39, No. 1, 2014, pp. 47-62.

[282] I.S. Arvanitoyannis, A.C. Stratakos, Application of Modified Atmosphere Packaging and Active/Smart Technologies to Red Meat and Poultry: A Review, Food and Bioprocess Technology, Vol. 5, No. 5, 2012, pp. 1423-1446.

[283] P.S. Taoukis, T.P. Labuza, Time-temperature indicators (TTIs), in: R. Ahvenainen (ed.), Novel Food Packaging Techniques, Woodhead Publishing Limited, Cambridge, England, 2003, pp. 103-126.

[284] A.P.D.R. Brizio, C. Prentice, Use of smart photochromic indicator for dynamic monitoring of the shelf life of chilled chicken based products, Meat Science, Vol. 96, No. 3, 2014, pp. 1219-1226.

[285] M.C. Giannakourou, K. Koutsoumanis, G.J.E. Nychas, P.S. Taoukis, Field evaluation of the application of time temperature integrators for monitoring fish quality in the chill chain, International Journal of Food Microbiology, Vol. 102, No. 3, 2005, pp. 323-336.

[286] P.S. Taoukis, K. Koutsoumanis, G.J.E. Nychas, Use of time–temperature integrators and predictive modelling for shelf life control of chilled fish under dynamic storage conditions, International Journal of Food Microbiology, Vol. 53, No. 1, 1999, pp. 21-31.

[287] M. Ellouze, J. Augustin, Applicability of biological time temperature integrators as quality and safety indicators for meat products, International Journal of Food Microbiology, Vol. 138, No. 1–2, 2010, pp. 119-129.

[288] H. Vaikousi, C.G. Biliaderis, K.P. Koutsoumanis, Development of a Microbial Time/Temperature Indicator Prototype for Monitoring the Microbiological Quality of Chilled Foods, Applied and Environmental Microbiology, Vol. 74, No. 10, 2008, pp. 3242-3250.

[289] H.R. Park, Y.A. Kim, S.W. Jung, H.C. Kim, S.J. Lee, Response of microbial time temperature indicator to quality indices of chicken breast meat during storage, Food Science and Biotechnology, Vol. 22, No. 4, 2013, pp. 1145-1152.

[290] E. Kim, D.Y. Choi, H.C. Kim, K. Kim, S.J. Lee, Calibrations between the variables of microbial TTI response and ground pork qualities, Meat Science, Vol. 95, No. 2, 2013, pp. 362-367.

[291] T.F. Mendoza, B.A. Welt, S. Otwell, A.A. Teixeira, H. Kristonsson, M.O. Balaban, Kinetic Parameter Estimation of Time-temperature Integrators Intended for Use with Packaged Fresh Seafood, Journal of Food Science, Vol. 69, No. 3, 2004, pp. FMS90-FMS96.

[292] T. Tsironi, E. Gogou, E. Velliou, P.S. Taoukis, Application and validation of the TTI based chill chain management system SMAS (Safety Monitoring and Assurance System) on shelf life optimization of vacuum packed chilled tuna, International Journal of Food Microbiology, Vol. 128, No. 1, 2008, pp. 108-115.

[293] M. Smolander, H. Alakomi, T. Ritvanen, J. Vainionpää, R. Ahvenainen, Monitoring of the quality of modified atmosphere packaged broiler chicken cuts stored in different temperature conditions. A. Time–temperature indicators as quality-indicating tools, Food Control, Vol. 15, No. 3, 2004, pp. 217-229.

[294] J.K. Heising, M. Dekker, P.V. Bartels, M.A.J.S. van Boekel, A non-destructive ammonium detection method as indicator for freshness for packed fish: Application on cod, Journal of Food Engineering, Vol. 110, No. 2, 2012, pp. 254-261.

[295] J.K. Heising, M.A.J.S. van Boekel, M. Dekker, Simulations on the prediction of cod (*Gadus morhua*) freshness from an intelligent packaging sensor concept, Food Packaging and Shelf Life, Vol. 3, 2015, pp. 47-55.

[296] M. Peris, L. Escuder-Gilabert, Electronic noses and tongues to assess food authenticity and adulteration, Trends in Food Science & Technology, Vol. 58, 2016, pp. 40-54.

[297] L. Huang, J. Zhao, Q. Chen, Y. Zhang, Nondestructive measurement of total volatile basic nitrogen (TVB-N) in pork meat by integrating near infrared spectroscopy, computer vision and electronic nose techniques, Food Chemistry, Vol. 145, 2014, pp. 228-236.

[298] O.S. Papadopoulou, E.Z. Panagou, F.R. Mohareb, G.E. Nychas, Sensory and microbiological quality assessment of beef fillets using a portable electronic nose in tandem with support vector machine analysis, Food Research International, Vol. 50, No. 1, 2013, pp. 241-249.

[299] H. Guohua, W. Lvye, M. Yanhong, Z. Lingxia, Study of grass carp (Ctenopharyngodon idellus) quality predictive model based on electronic nose, Sensors and Actuators B: Chemical, Vol. 166–167, 2012, pp. 301-308.

[300] S. Panigrahi, S. Balasubramanian, H. Gu, C.M. Logue, M. Marchello, Design and development of a metal oxide based electronic nose for spoilage classification of beef, Sensors and Actuators B: Chemical, Vol. 119, No. 1, 2006, pp. 2-14.

[301] P. Zaragozá, I. Fernández-Segovia, A. Fuentes, J. Vivancos, J.V. Ros-Lis, J.M. Barat, R. Martínez-Máñez, Monitorization of Atlantic salmon (*Salmo salar*) spoilage using an optoelectronic nose, Sensors and Actuators B: Chemical, Vol. 195, 2014, pp. 478-485.

[302] G. Ólafsdóttir, E. Martinsdóttir, E.H. Jónsson, Rapid Gas Sensor Measurements To Determine Spoilage of Capelin (*Mallotus villosus*), Journal of Agricultural and Food Chemistry, Vol. 45, No. 7, 1997, pp. 2654-2659.

[303] C.D. Natale, G. Olafsdottir, S. Einarsson, E. Martinelli, R. Paolesse, A. D'Amico, Comparison and integration of different electronic noses for freshness evaluation of cod-fish fillets, Sensors and Actuators B: Chemical, Vol. 77, No. 1–2, 2001, pp. 572-578.

[304] A. Pacquit, K.T. Lau, H. McLaughlin, J. Frisby, B. Quilty, D. Diamond, Development of a volatile amine sensor for the monitoring of fish spoilage, Talanta, Vol. 69, No. 2, 2006, pp. 515-520.

[305] A. Pacquit, J. Frisby, D. Diamond, K.T. Lau, A. Farrell, B. Quilty, D. Diamond, Development of a smart packaging for the monitoring of fish spoilage, Food Chemistry, Vol. 102, No. 2, 2007, pp. 466-470.

[306] B. Kuswandi, Jayus, R. Oktaviana, A. Abdullah, L.Y. Heng, A Novel On-Package Sticker Sensor Based on Methyl Red for Real-Time Monitoring of Broiler Chicken Cut Freshness, Packaging Technology and Science, Vol. 27, No. 1, 2014, pp. 69-81.

[307] B. Kuswandi, Jayus, A. Restyana, A. Abdullah, L.Y. Heng, M. Ahmad, A novel colorimetric food package label for fish spoilage based on polyaniline film, Food Control, Vol. 25, No. 1, 2012, pp. 184-189.

[308] N.D. Boscher, T. Bohn, P. Heier, F. Moisy, B. Untereiner, K. Heinze, P. Choquet, Optical sensing responses of CrIIICl(TPP)(H2O)-based coatings obtained by an atmospheric pressure plasma method – Application to the detection of volatile amines, Sensors and Actuators B: Chemical, Vol. 191, 2014, pp. 553-560.

[309] K. Pospiskova, I. Safarik, M. Sebela, G. Kuncova, Magnetic particles-based biosensor for biogenic amines using an optical oxygen sensor as a transducer, Microchimica Acta, Vol. 180, No. 3, 2013, pp. 311-318.

[310] L.C. Umuhumuza, X. Sun, Rapid detection of pork meat freshness by using L-cysteine-modified gold electrode, European Food Research and Technology, Vol. 232, No. 3, 2011, pp. 425-431.

[311] R. Devi, B. Batra, S. Lata, S. Yadav, C.S. Pundir, A method for determination of xanthine in meat by amperometric biosensor based on silver nanoparticles/cysteine modified Au electrode, Process Biochemistry, Vol. 48, No. 2, 2013, pp. 242-249.

[312] R. Devi, S. Yadav, R. Nehra, S. Yadav, C.S. Pundir, Electrochemical biosensor based on gold coated iron nanoparticles/chitosan composite bound xanthine oxidase for detection of xanthine in fish meat, Journal of Food Engineering, Vol. 115, No. 2, 2013, pp. 207-214.

[313] M. Aromaa, A. Arffman, H. Suhonen, J. Haapanen, J. Keskinen, M. Honkanen, J. Nikkanen, E. Levänen, M.E. Messing, K. Deppert, H. Teisala, M. Tuominen, J. Kuusipalo, M. Stepien, J.J. Saarinen, M. Toivakka, J.M. Mäkelä, Atmospheric synthesis of superhydrophobic TiO2 nanoparticle deposits in a single step using Liquid Flame Spray, Journal of Aerosol Science, Vol. 52, No. 0, 2012, pp. 57-68.

[314] S. Frackman, M. Anhalt, K.H. Nealson, Cloning, organization, and expression of the bioluminescence genes of *Xenorhabdus luminescens*, Journal of Bacteriology, Vol. 172, No. 10, 1990, pp. 5767-5773.

[315] N.M. Männistö, N. Ahola, M.T. Karp, M. Veiranto, M. Kellomäki, *In vitro* Bioluminescence Used as a Method for Real-Time Inhibition Zone Testing for Antibiotic-Releasing Composites, British Microbiology Research Journal, Vol. 4, No. 2, 2014, pp. 235-254.

[316] G. Kampf, B. Dietze, C. Grosse-Siestrup, C. Wendt, H. Martiny, Microbicidal activity of a new silver-containing polymer, SPI-ARGENT II, Antimicrobial Agents and Chemotherapy, Vol. 42, No. 9, 1998, pp. 2440-2442.

[317] J. Mocak, A.M. Bond, S. Mitchell, G. Scollary, A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of detection and quantification: Application to voltammetric and stripping techniques (Technical Report), Pure and Applied Chemistry, Vol. 69, No. 2, 1997, pp. 297-328.

[318] U. Edwards, T. Rogall, H. Blocker, M. Emde, E.C. Bottger, Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA, Nucleic acids research, Vol. 17, No. 19, 1989, pp. 7843-7853.

[319] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Pena, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, R. Knight, QIIME allows analysis of high-throughput community sequencing data, Nature methods, Vol. 7, No. 5, 2010, pp. 335-336.

[320] R.C. Edgar, Search and clustering orders of magnitude faster than BLAST, Bioinformatics (Oxford, England), Vol. 26, No. 19, 2010, pp. 2460-2461.

[321] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, Journal of Molecular Biology, Vol. 215, No. 3, 1990, pp. 403-410.

[322] T.Z. DeSantis, P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, G.L. Andersen, Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB, Applied and Environmental Microbiology, Vol. 72, No. 7, 2006, pp. 5069-5072.

[323] J. De Vrieze, M. Coma, M. Debeuckelaere, P. Van der Meeren, K. Rabaey, High salinity in molasses wastewaters shifts anaerobic digestion to carboxylate production, Water research, Vol. 98, 2016, pp. 293-301.

[324] J.J. Kozich, S.L. Westcott, N.T. Baxter, S.K. Highlander, P.D. Schloss, Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform, Applied and Environmental Microbiology, Vol. 79, 2013, pp. 5112-5120.

[325] MiSeq SOP, web page. Available online (accessed 13/07/2018): http://www.mothur.org/wiki/MiSeq_SOP.

[326] P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R.A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G.G. Thallinger, D.J. Van Horn, C.F. Weber, Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities, Applied and Environmental Microbiology, Vol. 75, No. 23, 2009, pp. 7537-7541.

[327] E. Pruesse, C. Quast, K. Knittel, B.M. Fuchs, W. Ludwig, J. Peplies, F.O. Glöckner, SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB, Nucleic acids research, Vol. 35, No. 21, 2007, pp. 7188-7196.

[328] R.C. Edgar, B.J. Haas, J.C. Clemente, C. Quince, R. Knight, UCHIME improves sensitivity and speed of chimera detection, Bioinformatics, Vol. 27, No. 16, 2011, pp. 2194-2200.

[329] J.R. Cole, Q. Wang, E. Cardenas, J. Fish, B. Chai, R.J. Farris, A.S. Kulam-Syed-Mohideen, D.M. McGarrell, T. Marsh, G.M. Garrity, J.M. Tiedje, The Ribosomal Database Project: improved alignments and new tools for rRNA analysis, Nucleic acids research, Vol. 37, No. suppl 1, 2009, pp. D141-D145.

[330] R Core Team, The Comprehensive R Archive Network, web page. Available (accessed 13/07/2018): https://cran.r-project.org/index.html.

[331] H.F. Kaiser, A second generation little jiffy, Psychometrika, Vol. 35, No. 4, 1970, pp. 401-415.

[332] A.S. Ethiraj, S. Jayanthi, C. Ramalingam, C. Banerjee, Control of size and antimicrobial activity of green synthesized silver nanoparticles, Materials Letters, Vol. 185, 2016, pp. 526-529.

[333] M.A. Raza, Z. Kanwal, A. Rauf, A.N. Sabri, S. Riaz, S. Naseem, Size- and Shape-Dependent Antibacterial Studies of Silver Nanoparticles Synthesized by Wet Chemical Routes, Nanomaterials, Vol. 6, No. 4, 2016, pp. 74.

[334] M. Ip, S.L. Lui, V.K.M. Poon, I. Lung, A. Burd, Antimicrobial activities of silver dressings: an in vitro comparison, Journal of medical microbiology, Vol. 55, No. 1, 2006, pp. 59-63.

[335] A. Martinez-Abad, J.M. Lagaron, M.J. Ocio, Development and characterization of silver-based antimicrobial ethylene-vinyl alcohol copolymer (EVOH) films for food-packaging applications, Journal of Agricultural and Food Chemistry, Vol. 60, No. 21, 2012, pp. 5350-5359.

[336] Y. Ilg, J. Kreyenschmidt, Effects of food components on the antimicrobial activity of polypropylene surfaces containing silver ions (Ag+), International Journal of Food Science & Technology, Vol. 46, No. 7, 2011, pp. 1469-1476.

[337] A. Martínez-Abad, J.M. Lagarón, M.J. Ocio, Characterization of transparent silver loaded poly(l-lactide) films produced by melt-compounding for the sustained release of antimicrobial silver ions in food applications, Food Control, Vol. 43, No. 0, 2014, pp. 238-244.

[338] J.N. Bhakta, K. Ohnishi, Y. Munekage, K. Iwasaki, M.Q. Wei, Characterization of lactic acid bacteria-based probiotics as potential heavy metal sorbents, Journal of Applied Microbiology, Vol. 112, No. 6, 2012, pp. 1193-1206.

[339] E.J. Vihavainen, K.J. Björkroth, Diversity of Leuconostoc gasicomitatum associated with meat spoilage, International Journal of Food Microbiology, Vol. 136, No. 1, 2009, pp. 32-36.

[340] C. Rukchon, A. Nopwinyuwong, S. Trevanich, T. Jinkarn, P. Suppakul, Development of a food spoilage indicator for monitoring freshness of skinless chicken breast, Talanta, Vol. 130, 2014, pp. 547-554.

[341] P. Hu, G. Zhou, X. Xu, C. Li, Y. Han, Characterization of the predominant spoilage bacteria in sliced vacuum-packed cooked ham based on 16S rDNA-DGGE, Food Control, Vol. 20, No. 2, 2009, pp. 99-104.

[342] J. Kreyenschmidt, A. Hubner, E. Beierle, L. Chonsch, A. Scherer, B. Petersen, Determination of the shelf life of sliced cooked ham based on the growth of lactic acid bacteria in different steps of the chain, Journal of Applied Microbiology, Vol. 108, No. 2, 2010, pp. 510-520.

[343] K.R. Adams, S.E. Niebuhr, J.S. Dickson, Dissolved carbon dioxide and oxygen concentrations in purge of vacuum-packaged pork chops and the relationship to shelf life and models for estimating microbial populations, Meat Science, Vol. 110, No. 0, 2015, pp. 1-8.

[344] M. Sivertsvik, 18 - Active packaging in practice: fish, in: R. Ahvenainen (ed.), Novel Food Packaging Techniques, Woodhead Publishing, 2003, pp. 384-400.

[345] K. Broekaert, M. Heyndrickx, L. Herman, F. Devlieghere, G. Vlaemynck, Seafood quality analysis: Molecular identification of dominant microbiota after ice storage on several general growth media, Food Microbiology, Vol. 28, No. 6, 2011, pp. 1162-1169.

[346] P. Dalgaard, O. Mejlholm, H.H. Huss, Application of an iterative approach for development of a microbial model predicting the shelf-life of packed fish, International Journal of Food Microbiology, Vol. 38, No. 2–3, 1997, pp. 169-179.

[347] J.M. Jay, Microbial spoilage indicators and metabolites, in: M.D. Pierson, N.J. Sterm (ed.), Food-borne microorganisms and their toxins: Developing methodology, Marcel Dekker, Inc., New York, 1986, pp. 219-240.

[348] A. Küntzel, S. Fischer, A. Bergmann, P. Oertel, M. Steffens, P. Trefz, W. Miekisch, J.K. Schubert, P. Reinhold, H. Köhler, Effects of biological and methodological factors on volatile organic compound patterns during cultural growth of *Mycobacterium avium* ssp . *paratuberculosis*, Journal of Breath Research, Vol. 10, No. 3, 2016, pp. 037103.

[349] N.L. Afanador, T.N. Tran, L.M.C. Buydens, An assessment of the jackknife and bootstrap procedures on uncertainty estimation in the variable importance in the projection metric, Chemometrics and Intelligent Laboratory Systems, Vol. 137, 2014, pp. 162-172.

[350] W. Grosch, Determination of Potent Odourants in Foods by Aroma Extract Dilution Analysis (AEDA) and Calculation of Odour Activity Values (OAVs), Flavour and Fragrance Journal, Vol. 9, No. 4, 1994, pp. 147-158.

[351] M. Devos, F. Patte, J. Rouault, P. Laffort, L.J. Van Gemert, Standardized human olfactory thresholds, Oxford University Press, New York, US, 1990, .

[352] Y. Nagata, Measurement of odor threshold by triangle odor bag method, Odor Measurement Review, 2003, pp. 118-127.

[353] M. Czerny, M. Christlbauer, M. Christlbauer, A. Fischer, M. Granvogl, M. Hammer, C. Hartl, N.M. Hernandez, P. Schieberle, Re-investigation on odour thresholds of key food aroma compounds and development of an aroma language based on odour qualities of defined aqueous odorant solutions, European Food Research and Technology, Vol. 228, No. 2, 2008, pp. 265-273.

[354] J. Albrecht, T. Schreder, Kleemann A.M., V. Schöpf, R. Kopietz, A. Anzinger, M. Demmel, J. Linn, B. Kettenmann, M. Wiesmann, Olfactory detection thresholds and pleasantness of a food-related and a non-food odour in hunger and satiety. Rhinology, Vol. 47, No. 2, 2009, pp. 160-165.

[355] W.M. Yoder, S.W. Currlin, A. Larue, K.M. Fernandez, D. King, D.W. Smith, Interaction of guaiacol and methyl salicylate in binary mixture significantly lowers perceptual threshold in human observers, Journal of Sensory Studies, Vol. 27, No. 3, 2012, pp. 161-167.

[356] A. Plotto, C.A. Margaria, K.L. Goodner, R. Goodrich, E.A. Baldwin, Odour and flavour thresholds for key aroma components in an orange juice matrix: terpenes and aldehydes, 2004, .

[357] B.T. Rotabakk, O. Lekang, M. Sivertsvik, Solubility, absorption and desorption of carbon dioxide in chicken breast fillets, LWT - Food Science and Technology, Vol. 43, No. 3, 2010, pp. 442-446.

[358] B.T. Rotabakk, O.I. Lekang, M. Sivertsvik, Volumetric method to determine carbon dioxide solubility and absorption rate in foods packaged in flexible or semi rigid package, Journal of Food Engineering, Vol. 82, No. 1, 2007, pp. 43-50.

[359] Yildirim Selçuk, Röcker Bettina, M.K. Pettersen, Nilsen-Nygaard Julie, A. Zehra, R. Ramune, R. Tanja, S. Patrycja, M. Begonya, Coma Véronique, Active Packaging Applications for Food, Comprehensive Reviews in Food Science and Food Safety, Vol. 17, No. 1, 2018, pp. 165-199.

[360] M.A. Andrade, R. Ribeiro-Santos, M.C. Costa Bonito, M. Saraiva, A. Sanches-Silva, Characterization of rosemary and thyme extracts for incorporation into a whey protein based film, LWT, Vol. 92, 2018, pp. 497-508.

[361] R. Ribeiro-Santos, M. Andrade, N.R.d. Melo, A. Sanches-Silva, Use of essential oils in active food packaging: Recent advances and future trends, Trends in Food Science & Technology, Vol. 61, 2017, pp. 132-140.

[362] Z. Dong, F. Xu, I. Ahmed, Z. Li, H. Lin, Characterization and preservation performance of active polyethylene films containing rosemary and cinnamon essential oils for Pacific white shrimp packaging, Food Control, Vol. 92, 2018, pp. 37-46.

[363] J.C.P. Santos, R.C.S. Sousa, C.G. Otoni, A.R.F. Moraes, V.G.L. Souza, E.A.A. Medeiros, P.J.P. Espitia, A.C.S. Pires, J.S.R. Coimbra, N.F.F. Soares, Nisin and other antimicrobial peptides: Production, mechanisms of action, and application in active food packaging, Innovative Food Science & Emerging Technologies, Vol. 48, 2018, pp. 179-194.

[364] J.P. Correa, V. Molina, M. Sanchez, C. Kainz, P. Eisenberg, M.B. Massani, Improving ham shelf life with a polyhydroxybutyrate/polycaprolactone biodegradable film activated with nisin, Food Packaging and Shelf Life, Vol. 11, 2017, pp. 31-39.

[365] E. Roni, N. Michal, P. Avishay, H. Anina, B. Ehud, D. Ana, O. Amos, The combined effect of additives and processing on the thermal stability and controlled release of essential oils in antimicrobial films, Journal of Applied Polymer Science, Vol. 131, No. 15, 2014, .

[366] T. Phakawat, B. Soottawat, Essential Oils: Extraction, Bioactivities, and Their Uses for Food Preservation, Journal of Food Science, Vol. 79, No. 7, 2014, pp. R1231-R1249.

[367] C.G. Otoni, P.J.P. Espitia, R.J. Avena-Bustillos, T.H. McHugh, Trends in antimicrobial food packaging systems: Emitting sachets and absorbent pads, Food Research International, Vol. 83, 2016, pp. 60-73.

[368] N. Rosetta, C.G. Balestrini, M.D. Baum, C. Joseph, F. William, G. Kaarin, T.P. Labuza, P. Gale, H.S. Thesmar, Y. Frank, Applications and Perceptions of Date Labeling of Food, Comprehensive Reviews in Food Science and Food Safety, Vol. 13, No. 4, 2014, pp. 745-769.

[369] R.L. Wierda, G. Fletcher, L. Xu, J. Dufour, Analysis of Volatile Compounds as Spoilage Indicators in Fresh King Salmon (*Oncorhynchus tshawytscha*) During Storage Using SPME-GC-MS, Journal of Agricultural and Food Chemistry, Vol. 54, No. 22, 2006, pp. 8480-8490.

[370] C. Alasalvar, K.D.A. Taylor, F. Shahidi, Comparison of Volatiles of Cultured and Wild Sea Bream (*Sparus aurata*) during Storage in Ice by Dynamic Headspace Analysis/Gas Chromatography-Mass Spectrometry, Journal of Agricultural and Food Chemistry, Vol. 53, No. 7, 2005, pp. 2616-2622.

[371] F.F. Parlapani, S.A. Haroutounian, G.E. Nychas, I.S. Boziaris, Microbiological spoilage and volatiles production of gutted European sea bass stored under air and commercial modified atmosphere package at 2 °C, Food Microbiology, Vol. 50, 2015, pp. 44-53.

[372] Y. Xu, Y. Liu, C. Jiang, C. Zhang, X. Li, D. Zhu, J. Li, Determination of volatile compounds in turbot (*Psetta maxima*) during refrigerated storage by headspace solid-phase microextraction and gas chromatography?mass spectrometry, Journal of the Science of Food and Agriculture, Vol. 94, No. 12, 2014, pp. 2464-2471.

[373] N.P.L. Tuckey, J.R. Day, M.R. Miller, Determination of volatile compounds in New Zealand Greenshell™ mussels (*Perna canaliculus*) during chilled storage using solid phase microextraction gas chromatography–mass spectrometry, Food Chemistry, Vol. 136, No. 1, 2013, pp. 218-223.

Appendix 1.

Table A1. Volatile organic compounds (VOCs) associated with fish spoilage under different atmospheres. Raw products were examined if not otherwise indicated.

VOC	Product	Atmosphere (% $CO_2/O_2/N_2$)	Temperature (°C)	Reference
Acids				
3-methylbutanoic acid	King salmon	air	ice	[369]
Acetic acid	Atlantic cod	air	0.5	[198]
	Atlantic salmon	air	4/10/21	[89]
	Brown shrimp, cooked and peeled	anaerobic or aerobic MAP	4	[131]
	King salmon	air	ice	[369]
	Pangasius hypophthalmus	air, vacuum, 50/0/50 or 50/50/0	4	[129]
	Sea bream	air or 60/10/30	0/5	[87]
	Sea bream	air	ice	[370]
Aldehydes				
(E)-2-pentenal	Whiting	air	ice	[114]
2-methylbutanal	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Sea bass	60/10/30	2	[371]
	Whiting	air	4	[55]
2-methylpropanal	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Whiting	air	4	[55]
3-methylbutanal	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Sea bass	air or 60/10/30	2	[371]
	Sea bream	air or 60/10/30	5/15	[87]
	Whiting	air	4	[55]
Benzene acetaldehyde	Turbot	air	4	[372]
Butanal	Whiting	air	ice	[114]
Heptanal	Whiting	air	ice	[114]
Hexanal	Whiting	air	ice	[114]
Octanal	Whiting	air	ice	[114]
Pentanal	Whiting	air	ice	[114]
Propanal	Whiting	air	ice	[114]
Alcohols				
1-hexanol	Atlantic cod	air	4	[55]

	Mackerel	air	4	[55]
	Whiting	air	ice	[114]
	Whiting	air	4	[55]
1-hexen-3-ol	GreenshellTM mussels	air	6.5	[373]
1-octen-3-ol	GreenshellTM mussels	air	6.5	[373]
1-pentanol	Whiting	air	ice	[114]
1-penten-3-ol	GreenshellTM mussels	air	6.5	[373]
	Sea bream	air	ice	[370]
	Whiting	air	ice	[114]
2,3-butanediol	Atlantic cod	air	4	[55]
	Atlantic cod	air	0.5	[198]
	Mackerel	air	4	[55]
	Pangasius hypophthalmus	vacuum	4	[129]
	Whiting	air	4	[55]
2-ethyl-1-hexanol	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Sea bass	air	2	[371]
	Whiting	air	4	[55]
2-methyl-1-butanol	Sea bass	air	2	[371]
2-methyl-1-propanol	Atlantic cod	air	4	[55]
	Atlantic cod	air	0.5	[198]
	Mackerel	air	4	[55]
	Whiting	air	4	[55]
2-penten-1-ol	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Whiting	air	ice	[114]
	Whiting	air	4	[55]
3-methyl-1-butanol	Atlantic cod	air	4	[55]
	Atlantic cod	air	0.5	[198]
	Atlantic salmon	air	4/10/21	[89]
	Mackerel	air	4	[55]
	Sea bass	air or 60/10/30	2	[371]
	Sea bream	air	ice	[370]
	Turbot	air	4	[372]
	Whiting	air	4	[55]
	Yellowfin tuna	air	ice/30	[111]
4,4-dimethyl-1,3-dioxane	Whiting	air	ice	[114]

Ethanol	Atlantic cod	air	4	[55]
	Atlantic salmon	air	4/10/21	[89]
	Brown shrimp, cooked and peeled	anaerobic MAP	4	[131]
	Mackerel	air	4	[55]
	Pangasius hypophthalmus	air, vacuum, 50/0/50 or 50/50/0	4	[129]
	Sea bass	air or 60/10/30	2	[371]
	Sea bream	air or 60/10/30	0/5/15	[87]
	Whiting	air	4	[55]
Amine compounds				
Ammonia	Brown shrimp, cooked and peeled	anaerobic or aerobic MAP	4	[131]
Dimethyl amine	Brown shrimp, cooked and peeled	anaerobic or aerobic MAP	4	[131]
Piperidine	Sea bream	air	ice	[370]
Trimethylamine	Atlantic cod	air	4	[55]
	Atlantic cod	air	0.5	[198]
	Atlantic salmon	air	4/10/21	[89]
	Brown shrimp, cooked and peeled	anaerobic or aerobic MAP	4	[131]
	Mackerel	air	4	[55]
	Sea bream	air	ice	[370]
	Turbot	air	4	[372]
	Whiting	air	4	[55]
Ketones				
1-penten-3-one	Whiting	air	ice	[114]
2,3-butanedione	*Pangasius hypophthalmus*	vacuum or 50/50/0	4	[129]
2,3-octanedione	Whiting	air	ice	[114]
2,3-pentanedione	Whiting	air	ice	[114]
2-heptanone	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Whiting	air	4	[55]
2-nonanone	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Turbot	air	4	[372]
	Whiting	air	4	[55]
2-pentanone	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Whiting	air	4	[55]
2-undecanone	Atlantic cod	air	4	[55]

	Mackerel	air	4	[55]
	Whiting	air	4	[55]
3-pentanone	Atlantic cod	air	4	[55]
	Mackerel	air	4	[55]
	Whiting	air	4	[55]
6-methyl-5-hepten-2-one	Turbot	air	4	[372]
Acetoin	Atlantic cod	air	4	[55]
	Atlantic cod	air	0.5	[198]
	Atlantic salmon	air	4/10/21	[89]
	King salmon	air	ice	[369]
	Mackerel	air	4	[55]
	Pangasius hypophthalmus	vacuum	4	[129]
	Sea bream	air	ice	[370]
	Whiting	air	4	[55]
Acetone	Brown shrimp, cooked and peeled	aerobic MAP	4	[131]
Esters				
Acetic acid butyl ester	Turbot	air	4	[372]
Ethyl acetate	Atlantic cod	air	4	[55]
	Atlantic cod	air	0.5	[198]
	Brown shrimp, cooked and peeled	anaerobic or aerobic MAP	4	[131]
	Mackerel	air	4	[55]
	Pangasius hypophthalmus	air, vacuum, 50/0/50 or 50/50/0	4	[129]
	Sea bass	air	2	[371]
	Whiting	air	4	[55]
Ethyl isobutyrate	Sea bass	air	2	[371]
Ethyl propionate	Sea bass	air	2	[371]
Ethyl-3-methylbutanonate	Whiting	air	4	[55]
Other				
3,5,5-trimethyl-2-hexene	Whiting	air	ice	[114]
Ethyl benzene	King salmon	air	ice	[369]
Octane	*Pangasius hypophthalmus*	50/0/50	4	[129]
Pentadecane	Yellowfin tuna	air	ice/30	[111]
Propyl benzene	King salmon	air	ice	[369]
Styrene	King salmon	air	ice	[369]
Sulfuric compounds				
Carbon disulfide	Brown shrimp, cooked and peeled	CO_2-free MAP	4	[131]

	Pangasius hypophthalmus	air, vacuum, 50/0/50 or 50/50/0	4	[129]
Dimethyl disulfide	*Pangasius hypophthalmus*	vacuum or 50/0/50	4	[129]
	Sea bream	air	ice	[370]
Dimethyl sulfide	Brown shrimp, cooked and peeled	anaerobic MAP	4	[131]
	GreenshellTM mussels	air	6.5	[373]
Dimethyl trisulfide	Sea bream	air	ice	[370]
Hydrogen sulfide	Brown shrimp, cooked and peeled	CO_2-free MAP	4	[131]
	Pangasius hypophthalmus	air, vacuum, 50/0/50 or 50/50/0	4	[129]
Methyl mercaptan	Sea bream	air	ice	[370]
	Brown shrimp, cooked and peeled	CO_2-free MAP	4	[131]
	Pangasius hypophthalmus	air, vacuum, 50/0/50 or 50/50/0	4	[129]

ORIGINAL PAPERS

P1

PREPARATION AND ANTIMICROBIAL CHARACTERIZATION OF SILVER-CONTAINING PACKAGING MATERIALS FOR MEAT

by

Kuuliala, L., Pippuri, T., Hultman, J., Auvinen, S.-M., Kolppo, K., Nieminen, T., Karp, M., Björkroth, J., Kuusipalo, J. & Jääskeläinen, E., 2015

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Preparation and antimicrobial characterization of silver-containing packaging materials for meat

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ABSTRACT

In food technology, antimicrobial packaging materials could inhibit or limit the growth of spoilage bacteria and thus improve the shelf life of packaged products. The present study provides new insights into the preparation and antimicrobial characterization of silver-containing packaging materials and their efficacy against typical meat spoilage bacteria. Antimicrobial efficacy of packaging films produced by coextrusion or liquid flame spray process was determined by bioluminescence imaging and conventional antimicrobial assay. Fresh pork sirloin was packaged in selected films and composition of meat microbiota was analyzed by 16S rRNA amplicon sequencing. Shelf life of meat was not affected by any of the silver-containing packaging films, even though meat microbiota mostly consisted of bacteria that were inhibited or retarded *in vitro* by nanoscale silver coating. This may be due to different release dynamics of silver ions on meat surfaces compared to the circumstances in the antimicrobial assay or interactions between silver and amino acids.

1. Introduction

Raw red meat has a high water activity and plenty of nutrients that enable bacterial growth. Thus, meat rapidly loses its quality even at chill temperatures because of microbial activity and subsequent sensory changes (Sun & Holley, 2012). Carbon dioxide tolerant psychrotropic lactic acid bacteria (LAB) are typically associated with spoilage of vacuum or modified atmosphere (MA) packaged meat. *Carnobacterium*,*Lactobacillus* and *Leuconostoc* spp. are most commonly detected in spoiled, cold stored meat products packaged under MA (Borch, Kant-Muermans, & Blixt, 1996; Susiluoto, Korkeala, & Björkroth, 2003). Since these bacteria cannot be completely eradicated from raw meat even if high processing hygiene is maintained, new packaging technologies are needed for improving shelf life and quality of the packaged product.

Meat spoilage occurs primarily on the surface of meat. To limit the growth of spoilage microbes, antimicrobial substances have been tested in direct contact with food products by dipping or spraying, even though this kind of approach has relatively short-time effects on microbial growth due to neutralization, diffusion or inactivation of active ingredients (Appendini & Hotchkiss, 2002; Quintavalla & Vicini, 2002). A more durable solution could be the incorporation of antimicrobial agents as a part of the packaging material. Controlled and extended release of antimicrobial agents could inhibit or retard bacterial growth throughout storage, thus leading into benefits in the whole supply chain (Appendini & Hotchkiss, 2002; Han, 2000; Quintavalla & Vicini, 2002).

Silver has been known since historical times for its antimicrobial activity and several mechanisms of activity against bacteria have been proposed (Lalueza, Monzón, Arruebo, & Santamaría, 2011; Silvestry-Rodriguez, Sicairos-Ruelas, Gerba, & Bright, 2007). These include extracellular binding or precipitation of silver to cell walls, active transport of silver into cells *via* transport systems of essential metals, and binding of silver to DNA or electron donor groups. Silver can also bind to sulfhydryl groups (—SH) of proteins, which causes protein inactivation and inhibition of metabolic processes (Silvestry-Rodriguez et al., 2007). Interaction of silver with ribosomes inhibits enzyme expression (Llorens, Lloret, Picouet, Trbojevich, & Fernandez, 2012). Silver can be introduced in different forms such as ions, complexes, salts and in metallic form. Activity of silver ions is dependent on the anions and

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biological molecules of the environment and is also affected by redox processes that depend on light, temperature and oxygen level. In case of nanoparticles, factors such as size, surface area, surface charge and geometry of the particles affect their activity (Lalueza et al., 2011). Silver-exchanged zeolites consist of aluminosilicates where complexed alkaline or earth alkaline metals have been partially replaced with silver ions (Fernández, Soriano, Hernández-Muñoz, & Gavara, 2010).

Although various techniques have been used in preparing silver-containing polymeric materials (Muñoz-Bonilla & Fernández-García, 2012) and antimicrobial effects have been shown *in vitro* in several studies (Boschetto, Lerin, Cansian, Pergher, & Di Luccio, 2012; Dogan, Koral, & Inan, 2009; Fernández et al., 2010; Lalueza et al., 2011; Pehlivan, Balköse, Ülkü, & Tihminlioğlu, 2005), fewer studies have been published about the effects of silver in storage of real food, such as meat (Lee, Lee, Jones, Sharek, & Pascall, 2011), fruit or vegetables (Costa, Conte, Buonocore, & Del Nobile, 2011; Martínez-Abad, Lagarón, & Ocio, 2014) or cheese (Gammariello, Conte, Buonocore, & Del Nobile, 2011; Incoronato, Conte, Buonocore, & Del Nobile, 2011). The aim of this study was to prepare silver-containing packaging materials and evaluate their antimicrobial effect against several bacterial strains associated with meat spoilage. Potential packaging films were selected by initial *in vitro* tests against genetically modified luminescent bacteria and conventional antimicrobial assay against various spoilage bacteria. Selected films were used in packaging of pork sirloin to evaluate their effect on pork shelf life. Characterization of meat spoilage under different packaging conditions was performed by 16S rRNA amplicon sequencing.

2. Materials and methods

2.1. Preparation of packaging materials

2.1.1. Coextrusion

Two silver-containing low density polyethylene (LDPE) masterbatches were used in the trials. A commercial masterbatch contained silver-zinc zeolite in LDPE matrix (Irgaguard® B 5120, BASF, Ludwigshafen, Germany). The other masterbatch was prepared by melt mixing of 4 kg LDPE (CA7230, Borealis, Wien, Austria) and 200 g silver substituted titanium dioxide (SG TP8, Silvergreen Oy Ltd., Helsinki, Finland) in Brabender DSE25 twin screw extruder (Duisburg, Germany) at 200 °C, screw speed 89 rpm, torque 93 Nm and mass pressure 6.0 bar.

Coextruded packaging films were produced in a continuous roll process at Tampere University of Technology (TUT, Finland) on the Paper Converting and Packaging Technology pilot line. Food-grade paperboard (Stora Enso Oyj, Imatra, Finland) was used as a substrate for coating. Silver masterbatches were dry blended with CA7230 to obtain concentrations presented in Table 1. The resulting films consisted of a thin top layer of silver-containing polymer blend and bottom layer of pure CA7230. Total coating weight of approximately 20 g/m^2 was achieved by adjusting the screw speed or line speed. Pure LDPE film (control) was analogously produced. Adhesion between the substrate and the film was adjusted to be very low to enable substrate removal without damaging the produced film.

In this paper, coextruded packaging films are referred to according to their active ingredient (Irgaguard or SG TP8) and its concentration, *e.g.*, Irgaguard 2%. Silver-free coextruded film is referred to as control.

2.1.2. Liquid flame spray

Liquid flame spray (LFS) technology was used for coating LDPE films with nano-scale metallic silver particles. Principles of the process have been described earlier (Aromaa, Keskinen, & Mäkelä, 2007; Aromaa et al., 2012; Teisala et al., 2010; Tikkanen et al., 1997). The laboratory scale conveyor line described by Aromaa et al. (2012) was used in the experiments. Silver nitrate (Sigma–Aldrich, Germany) in ion exchanged H_2O was used as a precursor and coextruded control film sheets (27.5 × 19.5 cm) as substrate for nanoparticle deposition. Size of nanoparticles was adjusted by using either 500 mg/mL (big particle size) or 125 mg/mL (small particle size) precursor concentration. Each substrate sheet was coated 1, 2 or 4 times. Other parameters remained constant: precursor feed rate 2 mL/min, burner distance 20 cm, line speed 50 m/min and gas flow rate 40/20 lpm (H_2/O_2).

In this paper, packaging films prepared by LFS are referred to as B1–B4 (big particle size, coated 1–4 times) or S1–S4 (small particle size, coated 1–4 times). Control film is the same as for coextruded films.

Silver nanoparticles produced by LFS were studied with transmission electron microscopy (TEM). During LFS, nanoparticle deposit was collected on a TEM grid (S160-3, Agar Scientific) by a sampling device (alumina stick and sample holder). The grid was centered on the device and wiped perpendicularly through the flame. Separate grids were produced for big and small particle size. The JEOL JEM-2010 instrument operating at 200 kV acceleration voltage was used for studying the size and distribution of the produced nanoparticles.

2.2. Bioluminesence imaging for initial testing of antimicrobial activity

Xenogen IVIS 200 Optical Imaging System (Caliperl Life Sciences, USA) was used for bioluminescence imaging of bacterial growth. The method uses whole bacterial cells as biosensors and is based on the expression of *Photorhabdus luminescens* genes coding for luciferase enzyme in *Escherichia coli*. In the presence of oxygen, luciferase catalyzes light producing reactions that are directly linked to energy balance in cell. The photons are captured in real time by a charge coupled device (CCD) camera in the bioluminescence imaging (BLI) system. This information is converted into figures that express emitted photons or counts on a color scale. Since the amount of emitted light correlates to the well-being of the bacterial strain (Tenhami, Hakkila, & Karp, 2001; Virta et al., 1998), the figures indicate the presence and growth of bacteria.

E. coli K12 carrying a plasmid pCGSL-1 (Frackman, Anhalt, & Nealson, 1990) received from Department of Chemistry and Bioengineering (TUT) was cultured overnight at 30 °C on antibiotic L-agar (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar and 200 μg/mL ampicillin). Luminescence was examined with Xenogen and light-emitting colonies were transferred to antibiotic Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and 200 μg/mL ampicillin) and incubated overnight at 30 °C

Table 1
Masterbatches and their active ingredient concentrations in coextruded films.

Masterbatch	Active ingredient	Concentration (%) of active ingredient in packaging films
Irgaguard® B5120	Irgaguard B 5000 (silver–zinc zeolite)	2; 3
SG TP8 masterbatch	SG TP8 (silver substituted titanium dioxide)	1

and 300 rpm. Luminescence levels were examined with Plate Chameleon™ multilabel counter 1.001 (Hidex Ltd., Turku, Finland) and cultures with highest luminescence levels (over 10^5 counts) were selected.

BLI plates were prepared by placing round packaging film samples (ø 14 mm) onto 2 mL L-agar in a six-well plate, one sample per well and active film surface facing up. The samples were covered by a mixture of 1 mL of soft L-agar (7.5 g/L agar), 500 μL of selected liquid culture and 200 μg/mL ampicillin. Formation of air bubbles that could interfere with the imaging results was avoided. The plate was placed under the CCD camera and images were taken at one hour interval for 16 h, exposure time being five seconds. Images were analyzed with the Living Image® 3.1 program (Caliperl Life Sciences, USA).

Images were interpreted on the basis of color scale ranging from dark purple (lowest counts) to dark red (highest counts) according to Männistö, Ahola, Karp, Veiranto, & Kellomäki (2014). Dark purple and blue indicate sick or dead bacteria, whereas red and yellow are interpreted as stress zones where high light emission occurs due to sub-inhibitory levels of antimicrobial agents. Movement of zones outside from the packaging film samples by time was interpreted to result from liberation of silver by diffusion. Green color represents the growth of unaffected bacteria.

2.3. Antimicrobial assay

Antimicrobial effects of selected packaging films were tested against bacteria closely associated with meat spoilage. The tested microbes were *Leuconostoc gelidum* subsp. *gasicomitatum* (LMG 18811^T), *Lactobacillus sakei* (23K), *Lactococcus piscium* (MKFS47), *Carnobacterium divergens* (DSMZ 20623^T), *Brochothrix thermosphacta* (DSM20171^T) and *Hafnia alvei* (DSM30163). The tested films were B4, S4, Irgaguard 2%, SG TP8 1% and control. Testing was based on ASTM E2180-07 (2012) that can be used for evaluating the efficacy of incorporated antimicrobials in polymeric materials. Bacteria were cultured for 24 h at 25 °C using Oxoid (Basingstoke, UK) growth media GM17 (*L. piscium* and *C. divergens*), de Man-Rogosa-Sharpe (MRS; *L. gelidum* subsp. *gasicomitatum* and *L. sakei*) or Tryptic Soy Broth (TSB; *B. thermosphacta* and *H. alvei*) before testing. 1 mL of bacterial culture was mixed with 99 mL NaCl-water agar (0.85% NaCl + 0.3% agar, 0.5% glucose for *L. gelidum* subsp. *gasicomitatum* and *L. piscium*). The mixture (0.5 mL) was pipetted on 2 × 2 cm packaging film samples and incubated at 25 °C for 24 ± 2 h. Lactic acid bacteria were incubated in anaerobic jars in a CO_2 enriched atmosphere (AnaeroGen, Oxoid). After incubation, agars were moved to stomacher bags, mixed with 1:10 peptone saline and 5% horse serum for neutralization (Kampf, Dietze, Grosse-Siestrup, Wendt, & Martiny, 1998) and homogenized for 1 min, but were not sonicated. Dilution series was prepared for TSA (*L. piscium*, *C. divergens*, *B. thermosphacta* and *H. alvei*) or MRS (*L. gelidum* subsp. *gasicomitatum* and *L. sakei*) agar plates and incubated at 25 °C for 5 days. LAB were incubated in anaerobic jars.

2.4. Meat packaging experiments

Vacuum packaged fresh pork sirloin (slaughtered <24 h) was bought from a commercial retailer. Meat was cut into ca. 20 g pieces, wrapped in one of the three silver-containing packaging films (B4, S4 or Irgaguard 3%) or pure LDPE (control) and packaged under vacuum (Multivac A 300/168, Sepp. 160 Haggenmuller KG, Wolfertschwenden, Germany) using a high barrier film of 90 μm thickness with oxygen transmission rate of 1 $cm^3\,m^{-2}\,24\,h^{-1}\,atm^{-1}$ at 23 °C and 75% relative humidity (Finnvacum, Östersundom, Finland). Thirty pieces of meat were packaged in each packaging material. Samples were stored at +6 °C in the dark.

Three parallel packages per each film were analyzed after 0, 3, 5, 7, 10, 13, 17, 19, 21, 24 and 28 days of storage. Ten grams of meat was aseptically weighed and homogenized with 0.1% peptone saline in a stomacher blender (Seward, Worthing, UK). Serial 10-fold dilutions were placed in triplicate on the growth media (Table 2). Meat pH was measured by pH meter (Inolab 720, WTW, Weilheim, Germany) after 1 min homogenization (Stomacher, Seward, Worthing, UK) with 0.1% peptone saline solution at a ratio of 1:10. Sensory analysis was performed by a trained panel of 5 persons for meat samples equilibrated to room temperature. Fresh pork from the same meat batch was stored in the freezer and used as a reference. Sample appearance and odor was evaluated on a five-point scale (1 = severe defect, spoiled, 2 = clear defect, spoiled, 3 = mild defect, satisfactory, 4 = good, 5 = excellent). Meat was considered spoiled when median of results was 2 or below.

2.5. 16S rRNA amplicon sequencing and sequence-based identification

16S amplicon sequencing was performed on days 10, 17 and 28 of the meat storage trial for triplicate samples. DNA was extracted directly from the meat samples using 15 mL of the 1:10 homogenate used for dilution series. The eukaryotic cells were removed by centrifugation (3 min, 200 g, eppendorf 5810 R, Eppendorf AG, Hamburg, Germany). Bacterial cells were collected from supernatant after the second centrifugation (3 min, 10,000 × *g*). DNA was extracted from the cell pellets with bed-beating and GES–phenol–chloroform extraction. First, 500 μL of denaturing buffer (4 M Guanidium thiocyanate, 25 mM Sodium citrate, 0.5% Sarcosyl, 0.1 M 2-mercaptoethanol) and 500 μL of phenol–chloroform–isoamylalcohol (ph 8.8, Sigma–Aldrich) were added to the tubes with cell pellets, vortexed and transferred to FastPrep Lysing matrix E tubes (MP Biomedicals, Santa Ana, CA, USA). The lysing matrix tubes were bead beaten for 40 s (5.5 m/s) in a FastPrep-24 instrument (MP Biomedicals), followed by incubation on ice for 5 min and centrifugation for 10 min at 13,000 rcf

Table 2
Growth media for bacterial cultivation.

Medium	Bacteria	Incubation
Man–Rogosa–Sharpe agar (MRS, pH 6.2, Oxoid, Basingstoke, UK)	LAB	25 °C 5 days, anaerobic (O_2 <1%)
Plate count agar (PC, pH 7.0, Oxoid, Basingstoke, UK)	Aerobic bacteria	25 °C 5 days
Pseudomonas CFC selective agar (pH 7.1, Merck, Darmstadt, Germany)	*Pseudomonas* spp.	37 °C 2 days
Streptomycin sulfate thallium acetate actidione agar (STAA, pH 7.0, Oxoid, Basingstoke, UK)	*B. thermosphacta*	25 °C 2 days
Violet red bile glucose agar (VRBG, pH 7.4, Lab M, Bury, UK)	Enterobacteria	25 °C 2 days

(Eppendorf, Hamburg, Germany). Chloroform (500 μL) was added to the upper layer and tubes were centrifuged for 10 min after vortexing. The nucleic acids in the upper layer were precipitated with 1:10 volume of 3 M sodium acetate, 1 μL of glycoblue (Invitrogen, Carlsbad, CA, USA) and 3x ethanol. Pellet was washed with 70% ethanol and eluted to 50 μL of sterile nuclease free water.

To characterize the microbiome, the V1–V3 area of the 16S rRNA gene was PCR-amplified with primers 8f and 518r (Edwards, Rogall, Blocker, Emde, & Bottger, 1989). The PCR reaction consisted of 1x Phusion GC buffer, 200 uM of dNTP, 0.2 uM of each primer, 2.5% of DMSO and 50–250 ng of community DNA. After heating the PCR mix to 98 °C, 1U of Phusion polymerase (Life Technologies, Carlsbad, CA, USA) was added to the reaction. The following PCR program was used: denaturation at 98 °C for 30 s, 20 cycles at 98 °C for 10 s, 65 °C for 30 s and 72 °C for 10 s followed by 72 °C for 5 min and cooling down to 4 °C. The PCR products were purified with 0.9X Ampure beads (Beckman Coulter, Pasadena, CA, USA) and eluted to 40 μL of 0.1x TE buffer. The sample specific barcodes (8 bp) and sequencing adapters were added to the amplified PCR fragments in a second PCR with 1 × Phusion GC buffer, 200 μM of dNTP, 0.05 μM of each primer, 2.5% of DMSO and approximately 50 ng of purified PCR product. Again, after heating the reaction to 98 °C, the Phusion polymerase (1U) was added. Three replicate PCR reactions were done per sample and the products were pooled prior to purification. PCR product purification and sequencing was conducted at Institute of Biotechnology, University of Helsinki, with Roche 454 Titanium FLX protocol.

Sequences were analyzed with QIIME (version 1.7., Caporaso et al., 2010). First, the sequence reads were filtered for quality and reads with length less than 200 bp, containing ambiqous bases, with quality score below q30 or mismatches in the primer sequence were discarded and remaining reads assigned to samples based on the sample specific barcode. Operational taxonomic units (OTUs) were picked using the uclust (Edgar, 2010) algorithm with 97% similarity and representative sequence read from each OTU were assigned to taxonomy with BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) against the Greengenes database (Version 13.8.2014) (DeSantis et al., 2006). Significant differences between materials at each time point were analyzed by one-way analysis of variance (ANOVA) and Tukey's post hoc tests (SPSS Statistics v. 22 for Windows).

3. Results

3.1. TEM

TEM images (Fig. 1) of silver nanoparticle deposits (LFS) indicate variation in particle size. Deposit of big particles (Fig. 1(A)) has particle diameters between 3 and 20 nm and small particles (Fig. 1(B)) between 3 and 15 nm. However, particles with over 10 nm diameter occur frequently in Fig. 1(A) and rarely in Fig. 1(B). Even though particle size could not be fully predetermined by liquid precursor concentration, size distribution could be adjusted.

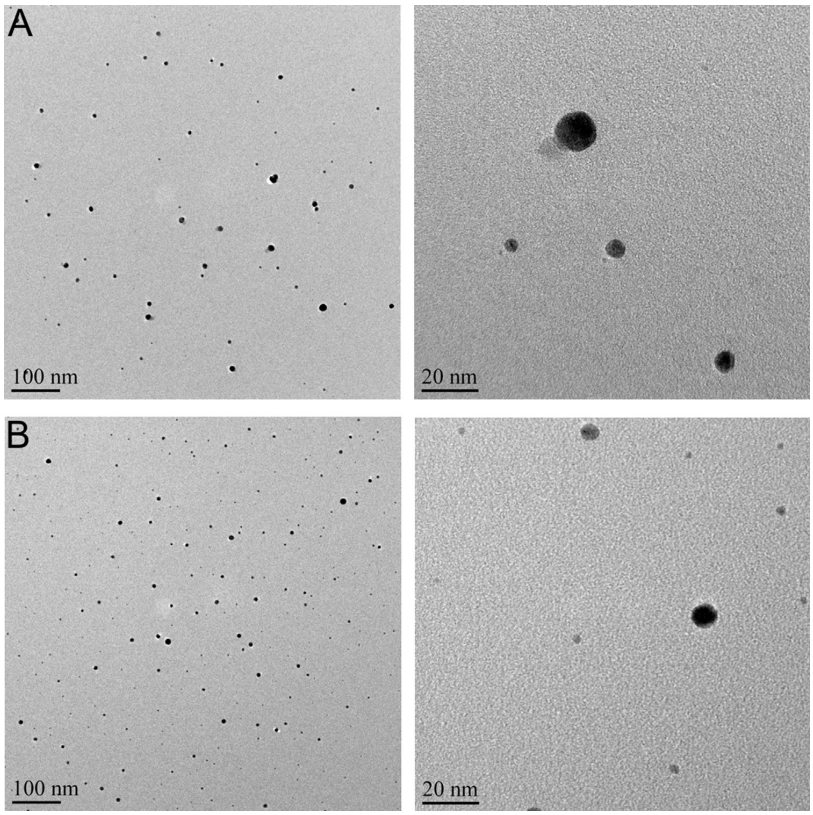

Fig. 1. TEM images of silver nanoparticles for (a) big particle size and (b) small particle size. The scale bars: 100 nm on the left, 20 nm on the right.

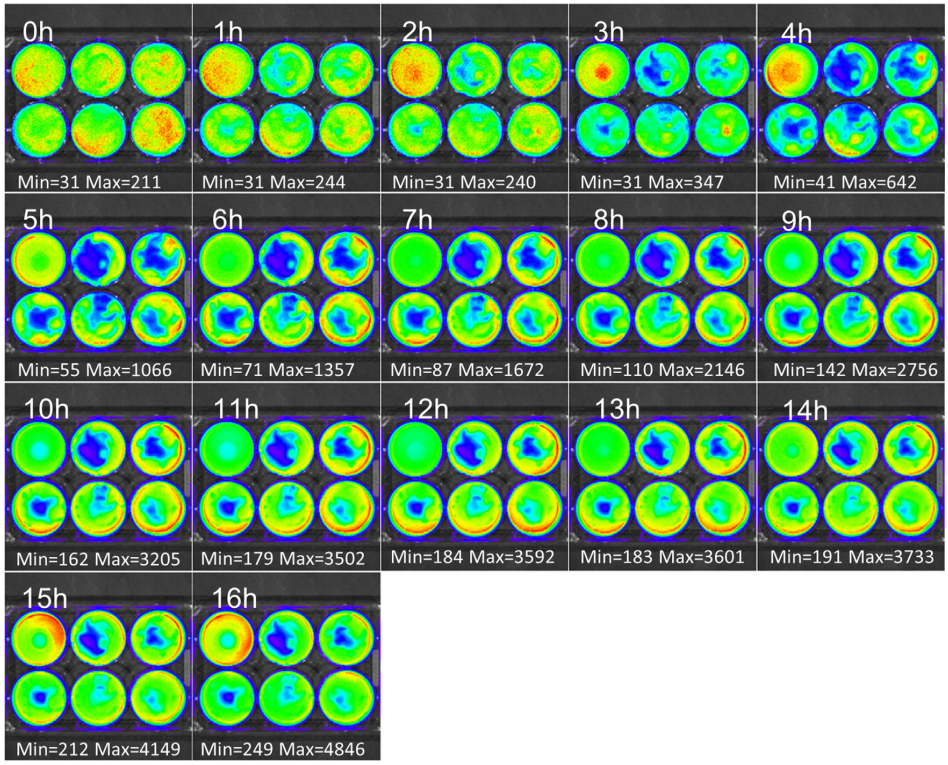

Fig. 2. Effects of LFS films on the bioluminescent *E. coli* strain during 16 h at one hour interval. Top row from left to right: control, B4, S4. Bottom row from left to right: B2, S2, B1. Each image (0–16 h) has individual color scale expressed as minimum and maximum counts.

3.2. Bioluminescence imaging

High concentrations of nano-scale silver affected the bioluminescence of *E. coli* (Fig. 2). Decreasing bioluminescence expressed as dark blue zones suggest the death of bacteria even within the early hours of testing. All samples exhibit inhibitory capacity when compared to the control well. After 4–5 h, all LFS films produced inhibition areas surrounded by yellow-red stress zones. These zones suggest for sub-lethal concentrations of antimicrobial agents shutting down metabolic pathways consuming energy that can be utilized for light-emitting reactions. Highest effect was associated with increasing coating times and thus increasing silver concentrations, whereas no clear difference between different particle sizes can be seen. Inhibition areas were not restricted to the sample area, indicating that during plate preparation, silver was at least partially diffused from the packaging film surface to the agar containing the *E. coli* sensor bacterial cells. On the other hand, coextruded films did not affect bacterial bioluminescence when compared to control (Supplementary Fig. 1). Although yellow-to-red stress zones appear on and around sample area, increasing blue zones appear similarly in silver-containing films and controls. However, this occurred at the late hours, when bacterial bioluminescence is no longer only affected by the presence of antimicrobial substances.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.fpsl.2015.09.004.

3.3. Antimicrobial assay

Results from the antimicrobial assay against LAB and other meat spoilage bacteria are presented in Table 3. Films containing nano-scale silver were highly effective against *L. piscium*, *B.*

Table 3
Antimicrobial efficacy of LFS films (B4, S4) and coextruded films (Irgaguard 2%, SG TP8 1%) against selected bacteria expressed as colony forming units ($\log_{10}$ CFU) in the beginning (0) and after incubation for sample (S) and control (C). Values are averages from three parallel plates, standard deviation <20%.

	B4			S4			Irgaguard 2%			SG TP8 1%		
	0	C	S	0	C	S	0	C	S	0	C	S
L. gelidum subsp. *gasicomitatum*	7.4	8.4	8.0	8.3	7.5	7.4	7.7	7.6	7.6	7.4	8.3	7.7
L. sakei	7.2	7.6	6.0	7.4	7.5	5.7	7.2	7.3	8.1	7.2	7.6	7.5
L. piscium	6.7	7.5	<1	7.8	5.9	<1	6.1	6.6	6.2	6.7	7.8	7.5
C. divergens	7.9	9.4	7.3	8.0	9.2	7.0	8.0	9.2	9.3	7.9	9.1	8.8
B. thermosphacta	7.8	8.9	<1	7.5	5.3	<1	7.2	8.8	8.4	7.8	9.5	9.2
H. alvei	7.6	10	<1	7.6	9.9	<1	7.6	>9.0	9.8	7.6	>9.0	>9.0

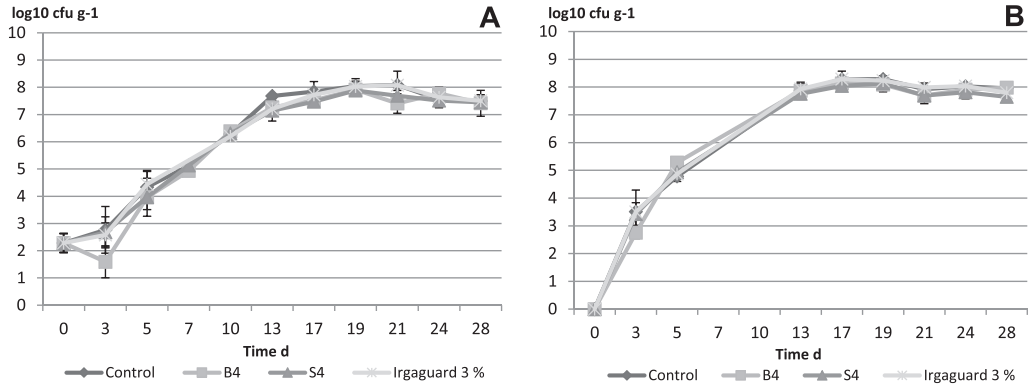

Fig. 3. (a) Total bacterial growth and (b) LAB growth in pork samples during 28 days of storage in contact with packaging films B4, S4, Irgaguard 3% or control.

thermosphacta and *H. alvei*, resulting in low logarithmic CFU levels (<1) after incubation. These films also affected the CFU levels of *L. sakei* and *C. divergens* when compared to the control. Initial $\log_{10}$ CFU level of *L. sakei* was reduced from 7 to 5, whereas increase in CFU of *C. divergens* was prevented when compared to control. Since the used growth medium was not optimal for some species (*L. piscium* and *B. thermosphacta*), some decrease of viability was observed during storage. However, clear difference between controls and nanosilver samples was observed. On the other hand, no significant growth reduction of any of the tested bacteria was obtained with coextruded films. Furthermore, growth of *L. gelidum* subsp. *gasicomitatum* was not affected by any of the tested films.

3.4. Meat packaging experiments

Quality of meat used in the experiments was high. Only low levels of *B. thermosphacta*, pseudomonads or enterobacteria were detected during storage (data not shown) and the initial total bacterial concentration was below 3 log cfu/g. Fig. 3(a) presents the growth of total bacteria and Fig. 3(b) presents the growth of LAB throughout storage.

Based on our experiments, silver-containing packaging films did not affect bacterial community development in pork throughout storage when compared to control (Fig. 3). Although total bacterial concentrations in B4 was slightly lower on day 3, the effect was lost on day 5. Concentration of LAB was uniform in all samples.

All pork samples remained acceptable according to the sensory evaluation throughout storage: the lowest median of grades was 2.5. Initial pH 5.67 declined rapidly to approximately 5.3 after 17–19 days. This was followed by an increase in pH, resulting in max. 0.2 units lower values than initial pH. In the end, control has the highest and B4 the lowest pH.

3.5. Identification of meat spoilage bacteria based on 16S sequencing

Fig. 4 presents the bacterial genera or orders deduced from the OTU similarities and their relative distributions among the total microbiota on days 10, 17 and 28 of storage. The average of number of reads was 6953 per sample (Supplementary Table 1). In most cases, the genera were successfully identified.

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Throughout the experiments, composition of the microbiota was relatively uniform in all meat samples, consisting mainly of lactobacilli, lactococci and leuconostocs. At a given time point, the same bacterial genera were mostly present in all the samples and no statistically significant differences ($p < 0.05$) were found in the

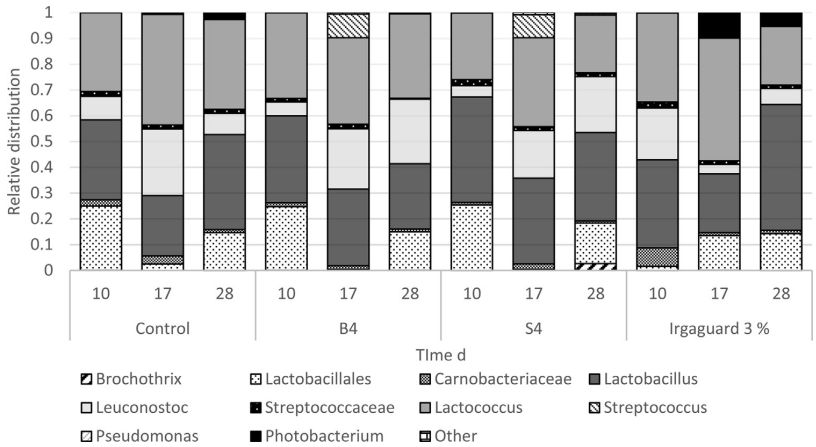

Fig. 4. Composition of pork microbiota after 10, 17 and 28 days of storage in contact with packaging films B4, S4, Irgaguard 3% or control, determined by 16S rRNA amplicon sequencing and sequence-based identification. Main OTU (>90%) of the *Lactobacillales* column was identified as *L. sakei*.

relative distributions of each identified genera between different packaging films. None of the studied silver-containing films thus affected the composition of meat microbiota when compared to control.

4. Discussion

Results obtained with BLI and conventional antimicrobial assay are in line, indicating high antimicrobial efficiency of LFS films when compared to coextruded films. BLI allowed fast and detailed characterization of antimicrobial effects in real time, enhancing material development considerably. The method could be used for effective screening and selection of potential packaging materials and further development of material properties.

Efficiency of LFS films *in vitro* is likely due to high concentration and availability of nano-scale silver on the polymer surface. The silver coating was in direct contact with the bacteria and loose enough to spread to the surroundings as indicated by BLI results (Fig. 2). The difference between the two particle sizes (big and small) was not clear, probably because of a wide particle size distribution as detected by TEM. Silver nanoparticles have typically higher antimicrobial efficiency compared to larger particles: high and broad efficiency scale of materials containing silver nanoparticles has been demonstrated in several studies (Kumar & Münstedt, 2005). Sotiriou & Pratsinis (2010) found the antimicrobial effect of nano-scale silver to be dependent on particle size: activity of particles below ca. 10 nm was found to be based on silver ion release, whereas larger particles released less Ag^+ and antimicrobial activity was more affected by the actual nanoparticles. In this research, particle size distribution of each LFS film covers both types.

Antimicrobial effect of silver-containing materials is often considered being dependent on silver ion (Ag^+) release (Kumar & Münstedt, 2005). Effect of both silver zeolite and silver substituted titanium dioxide used in this study is based on diffusion of Ag^+ from the carrier matrix in the presence of moisture. In this study, inefficiency of coextruded films is probably due to their structure. Cross-sectional microscopic images of these films show that silver-containing particles are often covered by a thin polyethylene layer (data not shown). Thus, migration and availability of silver ions might not have been sufficient to retard bacterial growth when silver was mixed in the matrix instead of being applied as a coating. Fernández et al. (2010) found that polylactic acid (PLA)/silver zeolite composites prepared by melt mixing did not exhibit antimicrobial activity when compared to composites prepared by solvent casting: this was related to the formation of PLA structures that prevent silver migration. In this study, the amount of silver was also notably lower in coextruded films than in LFS films. For example, Irgaguard B5000 contains 0.44% elemental silver, resulting in up to 0.0132% silver in the films used in this study. Antimicrobial effects have been achieved earlier using higher concentrations (Dogan et al., 2009; Fernández et al., 2010).

Results of the antimicrobial assay show that most of the tested spoilage bacteria are susceptible to the LFS silver coatings, suggesting that nano-scale silver could be used for limiting the growth of these meat spoilage bacteria. Gram-negative species are typically more sensitive to silver than Gram-positive ones (Ip, Lui, Poon, Lung, & Burd, 2006; Kawahara, Tsuruda, Morishita, & Uchida, 2000). In our study, *E. coli* and *H. alvei* were among the most susceptible bacteria. Correspondingly, Sondi & Salopek-Sondi (2004) found that silver nanoparticles were highly effective against *E. coli* on LB plates, whereas interactions between nanoparticles and intracellular substances of destroyed bacterial cells resulted in growth reduction only. However, differences in susceptibility were also observed in our study between different Gram-positive LAB. LFS films affected all the tested bacteria *in vitro* at least to some extent except *L. gelidum* subsp. *gasicomitatum*. Resistance of certain LAB toward heavy metals has been reported earlier (Bhakta, Ohnishi, Munekage, Iwasaki, & Wei, 2012). Since *L. gelidum* subsp. *gasicomitatum* is a dominating spoilage microbe in various meat products (Susiluoto et al., 2003; Vihavainen & Björkroth, 2007; Vihavainen & Björkroth, 2009), its resistance to silver could even lead into more optimized growth conditions inside silver-containing packages while other bacteria are suppressed. Thus, spoilage phenomena characteristic to the *Leuconostoc* family (Johansson et al., 2011) could be consequently enhanced.

Real food packaging requires sufficient and prolonged release of silver since fresh meat and meat products can be stored for days or even weeks. In this study, the tested films did not affect meat shelf life. However, 16S rRNA amplicon sequencing results show that meat microbiota mostly consisted of bacteria that were inhibited or retarded by the same nano-scale silver coating (B4 and S4) *in vitro* (Table 3). Since vacuum packaging allows direct contact between the coating and meat surface, inefficiency of LFS films in meat packaging could be due to properties of meat. Reduced activity of silver in food applications when compared to growth media has also been reported by others (Appendini & Hotchkiss, 2002 ; Ilg & Kreyenschmidt, 2011; Lee et al., 2011; Martinez-Abad, Lagaron, & Ocio, 2012; Martínez-Abad et al., 2014). Lee et al. (2011) examined the effect of silver zeolite incorporated in wrapping paper on the shelf life of meat inoculated with *Pseudomonas putida* and found that the growth rate was slower on 4% silver zeolite paper at 10 °C when compared to control. Incoronato et al. (2011) and Gammariello et al. (2011) observed the shelf life of Fior di Latte cheese to be improved when in contact with hydrogel containing silver montmorillonite nanoparticles. On the other hand, Martínez-Abad et al. (2014) found that films prepared by incorporating silver in PLA by melt compounding had reduced antimicrobial effect in contact with artificially inoculated lettuce when compared to antimicrobial assay based on JIS Z2801. This reduction was suggested to be caused by food sample roughness that prevented optimal surface contact, or silver being inactivated by food compounds. Analogously, Martinez-Abad et al. (2012) reported that antimicrobial efficacy of silver is greatly decreased especially in the presence of food samples that have high protein content. This is suggested to be related to inactivation of silver ions, their reactions with thiol compounds in food or reduction to elemental silver. Interactions between silver and amino acids in meat proteins or meat surface properties seem thus capable to limit the efficiency of LFS films when compared to the results obtained in antimicrobial assay against psychrotrophic spoilage bacteria. The nanoparticle coating provides high concentrations of silver in the beginning of storage but may lead into initial burst effect instead of prolonged release. Overall, the high antimicrobial efficacy obtained by standard surface assays may lead into overestimation of antimicrobial efficacy that is expected in prolonged food contact (Martínez-Abad et al., 2014).

In this study, efficiency of nano-scale silver was demonstrated against several meat spoilage bacteria *in vitro*, whereas no significant effect was seen against the same bacterial genera in meat packaging. Further development of food packaging systems is thus necessary in order to achieve the observed effects also in contact with meat. Setting the concentration of active agents higher than in the present study or allowing higher and more controlled release of silver could enhance these aims. According to Fernández et al. (2010), higher concentration of active silver is needed in food packaging applications than in growth media. A general migration limit of 0.05 mg Ag/kg food has been given by the European Food Safety Authority (EFSA) concerning the use of several silver carrier systems (EFSA, 2006). To achieve adequate silver ion release that simultaneously corresponds with the current

legislation, further development of packaging systems combined with migration tests is thus needed so that optimal antimicrobial efficacy could be obtained.

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References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410.

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science & Emerging Technologies*, *3*(2), 113–126.

Aromaa, M., Keskinen, H., & Mäkelä, J. M. (2007). The effect of process parameters on the liquid flame spray generated titania nanoparticles. *Biomolecular Engineering*, *24*(5), 543–548.

Aromaa, M., Arffman, A., Suhonen, H., Haapanen, J., Keskinen, J., Honkanen, M., & Mäkelä, J. M. (2012). Atmospheric synthesis of superhydrophobic TiO_2 nanoparticle deposits in a single step using liquid flame spray. *Journal of Aerosol Science*, *52*(0), 57–68.

ASTM Standard E2180-07 (2012). *Standard test method for determining the activity of incorporated antimicrobial agent(s) in polymeric or hydrophobic materials*. West Conshohocken, PA: ASTM International. http://dx.doi.org/10.1520/E2180-07R12. www.astm.org.

Bhakta, J. N., Ohnishi, K., Munekage, Y., Iwasaki, K., & Wei, M. Q. (2012). Characterization of lactic acid bacteria-based probiotics as potential heavy metal sorbents. *Journal of Applied Microbiology*, *112*(6), 1193–1206.

Borch, E., Kant-Muermans, M., & Blixt, Y. (1996). Bacterial spoilage of meat and cured meat products. *International Journal of Food Microbiology*, *33*(1), 103–120.

Boschetto, D. L., Lerin, L., Cansian, R., Pergher, S. B. C., & Di Luccio, M. (2012). Preparation and antimicrobial activity of polyethylene composite films with silver exchanged zeolite-Y. *Chemical Engineering Journal*, *204–206*, 210–216.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., & Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336.

Costa, C., Conte, A., Buonocore, G. G., & Del Nobile, M. A. (2011). Antimicrobial silver-montmorillonite nanoparticles to prolong the shelf life of fresh fruit salad. *International Journal of Food Microbiology*, *148*(3), 164–167.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., & Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072.

Dogan, H., Koral, M., & Inan, T. Y. (2009). Ag/Zn zeolite containing antibacterial coating for food-packaging substrates. *Journal of Plastic Film and Sheeting*, *25*(3–4), 207–220.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England)*, *26*(19), 2460–2461. http://dx.doi.org/10.1093/bioinformatics/btq461.

Edwards, U., Rogall, T., Blocker, H., Emde, M., & Bottger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research*, *17*(19), 7843–7853.

EFSA (2006). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request related to a 12th list of substances for food contact materials. *The EFSA Journal* 395–401 1–21.

Fernández, A., Soriano, E., Hernández-Muñoz, P., & Gavara, R. (2010). Migration of antimicrobial silver from composites of polylactide with silver zeolites. *Journal of Food Science*, *75*(3), E186–E193.

Frackman, S., Anhalt, M., & Nealson, K. H. (1990). Cloning, organization, and expression of the bioluminescence genes of xenorhabdus luminescens. *Journal of Bacteriology*, *172*(10), 5767–5773.

Gammariello, D., Conte, A., Buonocore, G. G., & Del Nobile, M. A. (2011). Bio-based nanocomposite coating to preserve quality of fior di latte cheese. *Journal of Dairy Science*, *94*(11), 5298–5304.

Han, J. H. (2000). Antimicrobial food packaging. *Food Technology*, *54*(3), 56–65.

Ilg, Y., & Kreyenschmidt, J. (2011). Effects of food components on the antimicrobial activity of polypropylene surfaces containing silver ions (Ag^+). *International Journal of Food Science & Technology*, *46*(7), 1469–1476.

Incoronato, A. L., Conte, A., Buonocore, G. G., & Del Nobile, M. A. (2011). Agar hydrogel with silver nanoparticles to prolong the shelf life of fior di latte cheese. *Journal of Dairy Science*, *94*(4), 1697–1704.

Ip, M., Lui, S. L., Poon, V. K. M., Lung, I., & Burd, A. (2006). Antimicrobial activities of silver dressings: an in vitro comparison. *Journal of Medical Microbiology*, *55*(1), 59–63.

Johansson, P., Paulin, L., Sade, E., Salovuori, N., Alatalo, E. R., Bjorkroth, K. J., et al. (2011). Genome sequence of a food spoilage lactic acid bacterium, leuconostoc gasicomitatum LMG 18811T, in association with specific spoilage reactions. *Applied and Environmental Microbiology*, *77*(13), 4344–4351.

Kampf, G., Dietze, B., Grosse-Siestrup, C., Wendt, C., & Martiny, H. (1998). Microbicidal activity of a new silver-containing polymer, SPI-ARGENT II. *Antimicrobial Agents and Chemotherapy*, *42*(9), 2440–2442.

Kawahara, K., Tsuruda, K., Morishita, M., & Uchida, M. (2000). Antibacterial effect of silver-zeolite on oral bacteria under anaerobic conditions. *Dental Materials*, *16*(6), 452–455.

Kumar, R., & Münstedt, H. (2005). Silver ion release from antimicrobial polyamide/silver composites. *Biomaterials*, *26*(14), 2081–2088.

Lalueza, P., Monzón, M., Arruebo, M., & Santamaría, J. (2011). Bactericidal effects of different silver-containing materials. *Materials Research Bulletin*, *46*(11), 2070–2076.

Lee, J., Lee, Y., Jones, K., Sharek, E., & Pascall, M. A. (2011). Antimicrobial packaging of raw beef, pork and turkey using silver-zeolite incorporated into the material. *International Journal of Food Science & Technology*, *46*(11), 2382–2386.

Llorens, A., Lloret, E., Picouet, P. A., Trbojevich, R., & Fernandez, A. (2012). Metallic-based micro and nanocomposites in food contact materials and active food packaging. *Trends in Food Science & Technology*, *24*(1), 19–29.

Männistö, N. M., Ahola, N., Karp, M. T., Veiranto, M., & Kellomäki, M. (2014). In vitro Bioluminescence used as a method for real-time inhibition zone testing for antibiotic-releasing composites. *British Microbiology Research Journal*, *4*(2), 235–254.

Martínez-Abad, A., Lagarón, J. M., & Ocio, M. J. (2014). Characterization of transparent silver loaded poly(L-lactide) films produced by melt-compounding for the sustained release of antimicrobial silver ions in food applications. *Food Control*, *43*(0), 238–244.

Martinez-Abad, A., Lagaron, J. M., & Ocio, M. J. (2012). Development and characterization of silver-based antimicrobial ethylene-vinyl alcohol copolymer (EVOH) films for food-packaging applications. *Journal of Agricultural and Food Chemistry*, *60*(21), 5350–5359.

Muñoz-Bonilla, A., & Fernández-García, M. (2012). Polymeric materials with antimicrobial activity. *Progress in Polymer Science*, *37*(2), 281–339.

Pehlivan, H., Balköse, D., Ülkü, S., & Tihminlioğlu, F. (2005). Characterization of pure and silver exchanged natural zeolite filled polypropylene composite films. *Composites Science and Technology*, *65*(13), 2049–2058.

Quintavalla, S., & Vicini, L. (2002). Antimicrobial food packaging in meat industry. *Meat Science*, *62*(3), 373–380.

Silvestry-Rodriguez, N., Sicairos-Ruelas, E. E., Gerba, C. P., & Bright, K. R. (2007). Silver as a disinfectant. *Reviews of Environmental Contamination and Toxicology*, *191*, 23–45.

Sondi, I., & Salopek-Sondi, B. (2004). Silver nanoparticles as antimicrobial agent: A case study on *E. coli* as a model for gram-negative bacteria. *Journal of Colloid and Interface Science*, *275*(1), 177–182.

Sotiriou, G. A., & Pratsinis, S. E. (2010). Antibacterial activity of nanosilver ions and particles. *Environmental Science & Technology*, *44*(14), 5649–5654.

Sun, X. D., & Holley, R. A. (2012). Antimicrobial and antioxidative strategies to reduce pathogens and extend the shelf life of fresh red meats. *Comprehensive Reviews in Food Science and Food Safety*, *11*(4), 340–354.

Susiluoto, T., Korkeala, H., & Björkroth, K. J. (2003). *Leuconostoc gasicomitatum* is the dominating lactic acid bacterium in retail modified-atmosphere-packaged marinated broiler meat strips on sell-by-day. *International Journal of Food Microbiology*, *80*(1), 89–97.

Teisala, H., Tuominen, M., Aromaa, M., Mäkelä, J. M., Stepien, M., Saarinen, J. J., & Kuusipalo, J. (2010). Development of superhydrophobic coating on paperboard surface using the liquid flame spray. *Surface and Coatings Technology*, *205*(2), 436–445.

Tenhami, M., Hakkila, K., & Karp, M. (2001). Measurement of effects of antibiotics in bioluminescent staphylococcus aureus RN4220. *Antimicrobial Agents and Chemotherapy*, *45*(12), 3456–3461.

Tikkanen, J., Gross, K. A., Berndt, C. C., Pitkänen, V., Keskinen, J., Raghu, S., & Karthikeyan, J. (1997). Characteristics of the liquid flame spray process. *Surface and Coatings Technology*, *90*(3), 210–216.

Vihavainen, E. J., & Björkroth, K. J. (2007). Spoilage of value-added, high-oxygen modified-atmosphere packaged raw beef steaks by leuconostoc gasicomitatum and leuconostoc gelidum. *International Journal of Food Microbiology*, *119*(3), 340–345.

Vihavainen, E. J., & Björkroth, K. J. (2009). Diversity of leuconostoc gasicomitatum associated with meat spoilage. *International Journal of Food Microbiology*, *136*(1), 32–36.

Virta, M., Lineri, S., Kankaanpaa, P., Karp, M., Peltonen, K., Nuutila, J., et al. (1998). Determination of complement-mediated killing of bacteria by viability staining and bioluminescence. *Applied and Environmental Microbiology*, *64*(2), 515–519.

P2

EFFECT OF HIGH, RESIDUAL AND ABSENT OXYGEN ON THE DEVELOPMENT OF HEADSPACE GAS CONCENTRATIONS, SURFACE PH AND COLOR IN PORK SIRLOIN PACKAGED UNDER MODIFIED ATMOSPHERES

by

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Effect of high, residual and absent oxygen on the development of headspace gas concentrations, surface pH and color in pork sirloin packaged under modified atmospheres

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Abstract

Modified atmosphere packaging (MAP) is typically applied for the storage and transport of fresh retail meat. Even though high oxygen concentration is commonly used for maintaining the red color associated with freshness, under certain conditions it can cause discoloration, lack of blooming and other sensory defects. In the present study, quality of boneless pork sirloin cutlets packaged under modified ($O_2/CO_2/N_2$ %) high-O_2 (80/20/0), common anoxic (0/20/80) and enhanced anoxic (0/20/80 + O_2 absorber) atmospheres and stored at 4 °C for up to 17 days was examined with Mixed Analysis of Variance (ANOVA). Headspace gas composition, surface pH and color were monitored throughout storage time. Several variables were found to remain constant over time under a certain atmosphere, which limits their applicability in pork quality characterization. However, the similarity of color between high-O_2 and common anoxic conditions could support the use of anoxic packaging for pork. Furthermore, O_2 absorbers could have an additional favorable impact on meat color under anoxic conditions.

Keywords

Anoxic, blooming; meat; oxygen absorber; residual oxygen

1. Introduction

Modified atmospheres (MAs) have been used for several decades to extend the shelf life of raw meat and meat products [1], typically consisting of oxygen (O_2), carbon dioxide (CO_2) and/or nitrogen (N_2). CO_2 dissolves in the packaged product until saturation point, leading to the production of carbonic acid that limits the growth of spoilage bacteria [1, 2]. Subsequent package collapse can be avoided by using N_2 as a filler gas. O_2 is typically applied to preserve the red color although it also promotes lipid and pigment oxidation, rancidity, off-odors, brown coloration and aerobic microbial growth [1, 3, 4]. Oxidation of meat proteins can also cause several unfavorable phenomena, such as protein breakdown, deterioration of nutritional value and reduced juiciness and tenderness [5].

Meat color has been identified as a major factor affecting consumer acceptance [6-9] and is dependent on several factors, including the form and content of myoglobin [10]. Under anaerobic conditions, deoxymyoglobin produces purple color that is associated with newly cut or vacuum packaged meat, whereas oxygenation of myoglobin leads to redness associated with fresh meat [11, 12]. Color change resulting from exposure to O_2 is referred to as blooming [10]. Very low O_2 concentrations or prolonged exposure to light, heat or microbial activity promote the formation of metmyoglobin and subsequently brown discoloration [11, 12]. Even though O_2-sensitive food products are often packaged under MAs, residual O_2 may remain in the package and promote microbiological and chemical deterioration [13]. Low O_2 partial pressure has also been found to inhibit blooming under MAP [14, 15]. According to Mancini and Hunt [11], less than 1 % residual O_2 is needed to preserve the blooming ability.

Since the complete removal of O_2 can be challenging in traditional MAP, this could be achieved by active packaging technologies [13, 16, 17]. The use of O_2 absorbers provides an indirect antimicrobial effect against aerobic microorganisms by creating and maintaining a low O_2 atmosphere; typically, a sachet is placed in a sealed package to remove residual O_2 and O_2 that permeates through the packaging materials [13, 18]. In order to ensure sufficient absorption throughout storage time, absorbers can be selected to surpass the needed capacity, estimated on the basis of the initial O_2 concentration and packaging material permeability [17]. O_2 absorbers have been used with several O_2-sensitive food products, including meat [6, 14, 19-21], fish [18, 22, 23] and vegetables [24].

Currently, a high O_2 content (ca. 80 %) is commonly used for red meat to achieve a desirable color, combined with at least 20 % CO_2 to inhibit the growth of aerobic bacteria [4]. However, since pork has a light red color, reduction of O_2 levels could be beneficial in inhibiting several deteriorative reactions despite possible discoloration, especially in the absence of residual O_2. Although the emphasis of red meat quality studies has often been on beef [20, 25-28] and pork [3, 6, 29-31], a limited amount of studies have focused on the comparison of residual and absent O_2 levels in pork packaging. In the present study, the effects of different O_2 concentrations on physicochemical properties of pork sirloin were examined. Headspace gas composition, sirloin surface pH and color (CIELAB) of pork packaged under high-O_2 (80 % O_2, 20 % CO_2), common anoxic conditions (0 % O_2, 20 % CO_2) and enhanced anoxic conditions (0 % O_2, 20 % CO_2 + O_2 absorber) and stored at 4 °C for up to 17 days were analyzed. Mixed Analysis of Variance (ANOVA) was used for examining the impact of storage time, atmosphere and blooming time on the studied variables.

2. Materials and methods

2.1. Sampling and storage

Pork sirloin packages were obtained from a meat packing plant. Meat originated from mixed breed pigs slaughtered at < 7 months age and 80-90 kg carcass weight. Each package consisted of a tray (O_2 transmission rate 90 cc/m^2/24h, 23 °C, 50 % R.H., measured by MOCON Ox-Tran 2/21 MH, Mocon, Inc., Minneapolis, MN, USA) and top film (O_2 transmission rate 30 cc/m^2/24h, respectively) and contained 310 ± 10 g boneless pork sirloin as four stacked cutlets (thickness 1.5-2 cm) at 1:2 product-headspace ratio. Packages were prepared with Sealpack SP 800 (Oberburg, Germany) using vacuum time 0.5 s, vacuum pressure 230 mbar and packaging time 7.91 s. Gas atmosphere was adjusted with Dansensor MAP Mix 9000-2 (Dansensor, Ringsted, Denmark). The packages were transported to Tampere University of Technology (TUT, Tampere, Finland) the day following packaging (day 1). Maximum temperature during transport was 4 °C.

Data collection was carried out as two independent storage experiments with different MAs (O_2/CO_2/N_2 %). In the first experiment (A/B), high-O_2 (80/20/0; A) and common anoxic (0/20/80; B) atmospheres were examined. In the second experiment (C/D), common anoxic (0/20/80; C) and enhanced anoxic (0/20/80 + O_2 absorber; D) atmospheres were examined. ATCO™ FT-210 O_2 absorbers (Standa Industries, Caen, France) were used in the study. All packages (32 per atmosphere) were stored at (4 ± 0.5) °C in dark from day 1 to the day of analysis. Four randomly selected packages per atmosphere were examined on days 1, 3, 6, 8, 10, 13, 15 and 17.

2.2. Determination of headspace gas concentrations (O_2/CO_2)

Headspace gas composition (O_2/CO_2 %) was determined with CheckPoint O_2/CO_2 gas analyzer (Dansensor, Ringsted, Denmark) through a septum (white, 15 mm diameter, Dansensor) inserted on the package lid. Three consecutive gas samples (15 ml) were analyzed from each package.

2.3. Determination of color

Meat color was determined with CR-210 portable chromameter (Konica Minolta, Osaka, Japan) immediately and 20 min after opening under room temperature and a mixture of natural and fluorescent illumination. The CIELAB system (L*, a*, b*), illuminant C and 2° observation angle were used. Three consecutive measurements were carried out from randomly chosen spots on the surface of the topmost cutlet that had been exposed to the headspace gases throughout storage time.

2.4. Determination of surface pH

HI1413B surface pH electrode and HI7662 temperature electrode connected with HI2210 pH meter (Hanna Instruments, Woonsocket, RI, USA) were used for determining surface pH immediately after opening. Surface pH was determined from six randomly chosen spots of the topmost cutlet. Simultaneous pH and temperature measurement enabled automatic temperature compensation of the measured pH values.

2.5. Statistical analysis

All statistical analyses were carried out with R 3.3.1 [32]. Individual pork sirloin packages were treated as samples and the two independent experiments (A/B and C/D) were separately analyzed. One sample was omitted from all analyses because of malfunctioning O_2 absorber and three samples from color analysis because of insufficient sampling area and/or missing values. Averages and standard deviations were calculated from the measured data and the comparison of

means was carried out with Mixed Analysis of Variance (ANOVA) and Tukey HSD post hoc tests. Prior ANOVA, homogeneity of variance was examined with Levene's test using function leveneTest() from package **car** [33] and normality of residuals using functions aov() from package **stats** [34] and hist() from package **graphics** [34]. Throughout the present study, level of statistical significance was $p < 0.05$.

Two-way mixed ANOVA and Tukey HSD test were used for analyzing significant differences in headspace gas concentrations and pH using functions lme() from package **nlme** [35], anova() from package **stats** and lsmeans() from package **lsmeans** [36]. Atmosphere (A-D) and storage time (d) were treated as between samples factors and sample ID as a within samples factor:

O_2, CO_2 or pH ~ Atmosphere*Storage time, random=~1|SampleID (1)

where the notation Atmosphere*Storage time indicates that the main effects of atmosphere and storage time as well as their interaction were analyzed. In case of a non-significant two-way interaction, post hoc tests were carried out for significant main effects. Respectively, three-way mixed ANOVA and Tukey HSD test were used for analyzing significant differences in L*, a* and b*. Atmosphere and storage time were treated as between samples factors, blooming time (0 or 20 min) and sample ID as within samples factors:

L*, a* or b* ~ Atmosphere*Storage time*Blooming time, random=~1|SampleID (2)

where the notation Atmosphere*Storage time*Blooming time indicates that all the main effects and their interactions were analyzed. In case of a non-significant three-way interaction, post hoc tests were carried out for significant two-way interactions or main effects.

3. Results and discussion

For pork packaged under MAs and stored under refrigerated conditions, shelf life of approximately nine days can be expected [37]. In the present study, the samples were stored longer in order to observe the changes during the expected shelf life as well as during extended storage time. The effects of atmosphere, storage time, blooming time and their interactions on the studied variables are presented in Table 1 and the evolution of these variables as a function of storage time in Tables 2-5. Significant ($p < 0.05$) differences between means are indicated with appropriate superscripts in Tables 2-4. Since the interaction of atmosphere x storage time x blooming time was not significant in experiment C/D, Tukey HSD test was not carried out for Table 5 means. Levene's test and histogram of residuals preceding each ANOVA indicated that in most cases, homogeneity of variance was sufficient ($p < 0.05$) and the residuals were approximately normally distributed (results not shown). Few exceptions were most likely due to the small sample size and relatively high standard deviations.

3.1. Evolution of headspace gas concentrations (O_2/CO_2)

Headspace gas concentrations were significantly affected by atmosphere, storage time and their interaction in both experiments (Table 1). These results indicate differences in the evolution of gas concentrations under different MAs. Under high-O_2 conditions (A), decrease of O_2 concentration could be observed by the end of storage, coupled with increase in CO_2 concentration (Table 2). Under common anoxic conditions (B-C), residual O_2 concentrations between 0.1 - 1.2 % were detected until late days of storage, whereas O_2 absorbers were able to eliminate these residual levels under enhanced anoxic conditions (D) during the entire storage time (Tables 2-3). Few significant differences suggesting some increase in CO_2 levels over storage time were observed under conditions A, B and D (Tables 2-3).

158 The impact of different MAs on headspace gas concentrations was analyzed by comparing
159 means of a given day of storage. O_2 concentration was significantly different between
160 atmospheres A and B throughout storage time because of the selected gas compositions (80 %
161 vs. 0 % O_2), whereas differences in CO_2 concentration were only observed on few days of
162 storage (Table 2). O_2 concentration was higher under common anoxic (C) than under enhanced
163 anoxic conditions (D) on days 3 and 8: at later stages of storage, residual O_2 was depleted from
164 the C headspace (Table 3). Respectively, CO_2 concentration was higher under atmosphere C
165 from day 8 on, possibly due to the CO_2 absorption capability of the O_2 absorber. Iron-based O_2
166 scavengers have been reported to absorb some CO_2 by allowing the reaction between CO_2 and
167 iron [38]. This could promote the decline of CO_2 concentration under enhanced anoxic
168 conditions when compared to common anoxic conditions and affect the headspace volume and/or
169 meat quality.

170 Headspace gas composition can be affected by microbial growth, respiration or gas absorption of
171 the product, or permeation of gas through the packaging materials [39]. O_2 is consumed and CO_2
172 produced because of microbial metabolism and muscle respiration [40, 41]. Muscle respiration
173 mostly occurs shortly after slaughter, whereas microbial metabolism increases during storage
174 time [42]. However, even though CO_2 increase observed in the present study was thus likely
175 affected by microbial activity, the results indicate that significant changes in headspace gas
176 composition were limited to the late days of storage. Analysis of gas concentrations over storage
177 time is thus not likely to be an efficient solution for meat quality monitoring during the whole
178 shelf life.

179 ***3.2. Evolution of surface pH***

Changes in meat pH have commonly been associated with the concentration of CO_2 and microbial growth. CO_2 is water-soluble and can dissolve into meat tissues at a rate of 960 mL/kg at 0 °C, pH 5.5 and 1 atm [43]. On the other hand, decrease in meat pH has been detected as the levels of lactic acid bacteria (LAB) that are typically associated with meat spoilage reach 7-7.5 $\log_{10}$ CFU g^{-1} [44, 45]. In the present study, atmosphere and storage time had no interactive effect on pH under the tested conditions (Table 1). However, the main effect of storage time was significant in both experiments and some decrease in pH could be observed towards the end of experiment. Under atmospheres A/B, significant differences were observed between means of different days of storage:

$$6/8^{a} > 1/3^{ab} > 10^{bc} > 13/15^{cd} > 17^{d} \quad (3)$$

where results have been averaged over the levels of atmosphere and arranged on the basis of highest (5.51 ± 0.06 on day 6) to lowest (5.26 ± 0.06 on day 17) pH values, different superscripts indicating significant difference. Under atmospheres C/D, respectively:

$$1/3/8/10/13/15^{a} > 6/17^{b} \quad (4)$$

where results have been arranged from highest (5.42 ± 0.09 on day 1) to lowest (5.22 ± 0.08 on day 17) pH values. Under atmospheres C/D, the effect of time was thus only caused by the relatively low pH values measured on days 6 and 17. On the other hand, significant main effect of atmosphere was only observed between atmospheres A and B when averaged over the levels of storage time (Table 1). Respectively, Viana *et al.* [29] observed little pH variation between pork loin samples packaged under different MAs and stored for up to 20 days at 5 ± 0.5 °C. However, standard deviations of means were commonly high in both experiments in the present study.

3.3. Evolution of color

Table 1 presents the effects of atmosphere, storage time, blooming time and their interactions on the studied CIELAB color variables. Lightness (L*) was significantly affected by storage time x blooming time interaction under atmospheres A/B and by atmosphere under C/D. Redness (a*) and yellowness (b*) were significantly affected by all studied variables and their interactions under atmospheres A/B. Under atmospheres C/D, redness was only affected by atmosphere and blooming time, whereas yellowness was affected by atmosphere, storage time, blooming time and atmosphere x blooming time. The mean values of redness and yellowness are presented in Tables 4-5 as a function of storage time. Initial post-blooming lightness and redness were higher and yellowness lower than the values determined by Brewer *et al.* [46] for fresh pork after ≥ 30 min blooming time with a respective measurement setup; this could be due to differences in meat properties, handling, processing and packaging.

Lightness remained highly constant under all tested atmospheres. Throughout storage time, lightness remained within 58.86 ± 2.69 (A), 59.41 ± 2.78 (B), 58.12 ± 2.57 (C) or 57.06 ± 1.59 (D), where results have been averaged over the levels of storage time and blooming time. The significant storage time x blooming time interaction under A/B was due to a single difference that was observed before and after blooming on day 17. These results correspond with previous studies indicating that lightness cannot be used for analyzing blooming [10, 46, 47]. However, varying results have previously been obtained regarding the evolution of lightness as a function of storage time under different MAs. Doherty and Allen [19] found that lightness of pork chops packaged in master packs under MAs (100 % CO_2 or 50 % CO_2 + 50 % N_2) enhanced with O_2 absorber did not change or differ during storage of 21 days at 0 °C. Krause *et al.* [48] found that lightness of pork loin chops packaged under different MAs increased during the first days of

storage at 0-2 °C and remained constant thereafter. Analogously as in the present study, chops stored under aerobic (air) or anoxic (20 % CO_2, 80 % N_2) conditions had highly similar lightness. Under 100 % CO_2, Viana *et al.* [29] observed decrease in pork loin lightness in contrast with the increase observed by Sørheim *et al.* [49], which was associated with possible differences in residual O_2 levels. Under high O_2 concentration (80 % O_2, 20 % CO_2), Spanos *et al.* [31] found that lightness of pork *Longissimus thoracis et lumborum* increased and *Semimembranosus* remained constant as a function of storage time. Lightness has also been observed to increase along with the amount of free water, which results from protein damage caused by decreasing pH [46]. In the present study, changes in lightness could not be associated with decreasing pH nor residual O_2.

Storage time had the most pronounced effect on the pre-blooming color of meat under high-O_2 conditions (A) (Table 4). Under these conditions, redness decreased during storage and lead to significant difference between days 1 and 13-17 as well as 1-6 and 15-17. Decreasing O_2 concentration (Table 2) possibly contributed to the content of oxymyoglobin in meat, thus allowing decrease in redness. Respective evolution of discoloration has also been observed in other studies under atmospheric air or high O_2 concentrations [29, 48, 50]. Sørheim *et al.* [50] found that the redness of pork chops decreased after three days of storage under 70 % O_2 and 30 % CO_2 at 4 °C. On the other hand, yellowness did not change significantly under atmosphere A over storage time in the present study (Table 4).

Storage time had an opposite impact on the pre-blooming color of meat under anoxic conditions (B-D) when compared with high-O_2 packaging (A). No significant effect on redness was observed under these conditions (Tables 1 and 4-5). This was most likely due to the maintenance of low O_2 levels (< 1.2 %) during storage and thus small changes in the oxymyoglobin content of

meat. On the other hand, some increase in yellowness was observed as a function of storage time under condition B (Table 4). Under conditions C/D, the main effect of time on yellowness was significant (Table 1): after averaging over the levels of atmosphere and blooming time, significantly lower values were observed on the earliest (1-3) days when compared to day 17. These results are in correspondence with Spanos *et al.* [31], even though they observed respective color evolution also under high O_2 concentration (80 % O_2, 20 % CO_2). Respectively, Doherty and Allen [19] found that pork chops stored with O_2 absorbers in master packs under 100 % CO_2 or 50 % CO_2 and 50 % N_2 were at least as red and generally more yellow than fresh chops during display after 7 or 21 days of storage. On the other hand, Sørheim *et al.* [49] found that residual O_2 concentrations (0.5 or 1 %) combined with CO_2 lead to decrease in redness and increase in yellowness during storage at 3 °C.

The impact of different MAs and blooming time on meat color could be attributed to the differences in O_2 concentrations. Before blooming, meat packaged under high-O_2 conditions (A) was more yellow than under common anoxic conditions (B) throughout the expected shelf life. Respectively, enhanced yellowness has been observed under aerobic conditions in previous studies [31, 48]. In the present study, however, difference in redness was only observed on day 6 of storage. Meat packaged under aerobic conditions has commonly been observed to be redder than under anoxic packaging [31, 48]. In the present study, the observed differences caused by the two atmospheres were likely interfered by the high standard deviations of the means. Blooming time had no effect on color under high-O_2 conditions (A), whereas under common anoxic conditions (B), significant increase was observed until day 13 in redness and throughout storage time in yellowness. This is in correspondence with Spanos *et al.* [31] who used visible spectroscopy for pork color analysis and suggested that the shift in O_2 concentration from

elevated (≥ 40 %) to atmospheric levels could have been insufficient to cause changes in the reflectance spectra. In the present study, initial color differences between samples stored under conditions A and B were thus reduced after blooming time so that no significant post-blooming color differences were observed after day 3.

Color differences between samples stored under common anoxic (C) and enhanced anoxic conditions (D) were less pronounced than between samples stored under high-O_2 (A) and common anoxic conditions (B) (Table 5). However, redness was higher under condition D (15.27 ± 1.18) than under condition C (13.51 ± 2.27) when averaged over the levels of storage time and blooming time. When averaged over the levels of storage time and atmosphere, increase in redness from 14.04 ± 1.83 to 14.72 ± 2.14 was observed during blooming. In yellowness, differences could be observed when averaged over the levels of storage time:

$$C_{after}^{a} > D_{after}^{ab} > C_{before}^{b} > D_{before}^{c} \quad (5)$$

where different superscripts indicate significant ($p < 0.05$) difference. These results are generally in line with Sørheim *et al.* [49] who found that both normal and pale, soft and exudative (PSE) pork stored at 3 °C was less red and more yellow under 0.5 and 1 % O_2 when compared to 0 % O_2. Difference in redness was detected after 7 days in PSE meat and after 14 days in normal meat, and difference in yellowness after 3 days in both meat types. On the other hand, Sørheim *et al.* [50] found that even though pork chops packaged under 60 % CO_2 and 40 % N_2 with an O_2 absorber at 4 °C were as red as chops under high-O_2 (70 % O_2, 30 % CO_2), discoloration occurred under anoxic conditions. In the present study, high standard deviations between individual packages promoted the statistically significant similarity typically observed on a given day of storage.

Advantages of anoxic atmospheres in meat packaging can be overruled by the unfavorable impact of low O_2 content on the red color of meat. High O_2 concentration is thus commonly applied despite its several potential deteriorative effects on meat quality. In the present study, however, color of meat was highly similar under the different MAs, especially after blooming. This could support the use of anoxic packaging for pork, as it might result in similar color compared to the use of high O_2 concentrations and a potentially lower undesirable impact on the oxidative status. Extended blooming time might further enhance this color development. In vacuum packaged pork, most of the color changes have been observed within 10-30 min after exposure to air [46, 47, 51].

Interference of residual O_2 concentrations with meat color and/or blooming has often been observed [11, 14, 15, 49]. Respectively, the results of the present study suggest that even though the impact of blooming time on redness was not different between common anoxic (C) and enhanced anoxic (D) conditions, redder and less yellow meat could be expected in the presence of O_2 absorbers. Since redness is highly associated with freshness, the presence of O_2 absorbers could support a more favorable meat color in comparison with common anoxic conditions.

In the present study, individual sirloin cutlets were analyzed on each day of storage due to the destructive nature of the analyses, meaning that a large number of samples was needed. Considerable variation was observed in the measured values of simultaneously studied packages. Possible factors affecting the color of pork include genetics, living conditions, diet and slaughtering procedures [11]. Several studies also show that the position of loin cut in whole loin affects the properties of the cut [52]. In order to reduce the interference of sample variation in future studies, methods for analyzing the same packages as a function of storage time would be beneficial.

4. Conclusions

In the present study, insights into the evolution of headspace gases, surface pH and color of pork sirloin packaged under MAs were obtained. The obtained results show that atmosphere, storage time, blooming time and/or their interactions affected the tested physicochemical variables. Many of the studied variables remained highly constant during the expected shelf life under a certain MA, which limits their potential in pork quality analysis. Even though some color differences could be observed during the early days of storage between samples stored under different atmospheres, these became soon negligible, especially after blooming. These results suggest that pork sirloin could benefit from anoxic packaging. Furthermore, O_2 absorbers could have a favorable additional impact on meat color. In future studies, reduction of sample variation could enhance the analysis of dependent variables over time or between different packaging conditions.

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References

[1] Blakistone BA. *10 Meats and poultry.* In: Blakistone BA (ed.). Principles and Applications of Modified Atmosphere Packaging of Foods. Springer US: 1999;240-290. DOI: 10.1007/978-1-4615-6097-5_10.

335 [2] Adams KR, Niebuhr SE, Dickson JS. Dissolved carbon dioxide and oxygen concentrations in purge of
336 vacuum-packaged pork chops and the relationship to shelf life and models for estimating microbial
337 populations. *Meat Science* 2015; 110(0), pp. 1-8. DOI: http://dx.doi.org/10.1016/j.meatsci.2015.06.003.

338 [3] De Santos F, Rojas M, Lockhorn G, Brewer MS. Effect of carbon monoxide in modified atmosphere
339 packaging, storage time and endpoint cooking temperature on the internal color of enhanced pork.
340 *Meat Science* 2007; 77(4), pp. 520-528. DOI: http://dx.doi.org/10.1016/j.meatsci.2007.04.031.

341 [4] Kapetanakou AE, Agathaggelou EI, Skandamis PN. Storage of pork meat under modified atmospheres
342 containing vapors from commercial alcoholic beverages. *International Journal of Food Microbiology*
343 2014; 178(0), pp. 65-75. DOI: http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.021.

344 [5] Lund MN, Heinonen M, Baron CP, Estévez M. Protein oxidation in muscle foods: A review. *Molecular*
345 *Nutrition & Food Research* 2011; 55(1), pp. 83-95. DOI: 10.1002/mnfr.201000453.

346 [6] Buys EM. Colour changes and consumer acceptability of bulk packaged pork retail cuts stored under
347 O2, CO2 and N2. *Meat Science* 2004; 68(4), pp. 641-647. DOI:
348 http://dx.doi.org/10.1016/j.meatsci.2004.05.017.

349 [7] Ngapo TM, Martin J-, Dransfield E. Consumer choices of pork chops: results from three panels in
350 France. *Food Quality and Preference* 2004; 15(4), pp. 349-359. DOI: http://dx.doi.org/10.1016/S0950-
351 3293(03)00082-X.

352 [8] O'Sullivan MG, Byrne DV, Martens H, Gidskehaug LH, Andersen HJ, Martens M. Evaluation of pork
353 colour: prediction of visual sensory quality of meat from instrumental and computer vision methods of
354 colour analysis. *Meat Science* 2003; 65(2), pp. 909-918. DOI: http://dx.doi.org/10.1016/S0309-
355 1740(02)00298-X.

356 [9] Zhang M, Sundar S. Effect of oxygen concentration on the shelf-life of fresh pork packed in a
357 modified atmosphere. *Packaging Technology and Science* 2005; 18(4), pp. 217-222. DOI:
358 10.1002/pts.693.

359 [10] Brewer MS, Novakofski J, Freise K. Instrumental evaluation of pH effects on ability of pork chops to
360 bloom. *Meat Science* 2006; 72(4), pp. 596-602. DOI: http://dx.doi.org/10.1016/j.meatsci.2005.09.009.

361 [11] Mancini RA, Hunt MC. Current research in meat color. *Meat Science* 2005; 71(1), pp. 100-121. DOI:
362 http://dx.doi.org/10.1016/j.meatsci.2005.03.003.

363 [12] McMillin KW. Where is MAP Going? A review and future potential of modified atmosphere
364 packaging for meat. *Meat Science* 2008; 80(1), pp. 43-65. DOI:
365 http://dx.doi.org/10.1016/j.meatsci.2008.05.028.

366 [13] Coma V. Bioactive packaging technologies for extended shelf life of meat-based products. *Meat*
367 *Science* 2008; 78(1–2), pp. 90-103. DOI: http://dx.doi.org/10.1016/j.meatsci.2007.07.035.

368 [14] Beggan M, Allen P, Butler F. Shelf-life of retail beef muscles following storage in a low oxygen
369 environment. *Journal of Muscle Foods* 2004; 15(4), pp. 269-285. DOI: 10.1111/j.1745-
370 4573.2004.07003.x.

371 [15] Venturini AC, Contreras CJC, Sarantópoulos CIGL, Villanueva NDM. The Effects of Residual Oxygen
372 on the Storage Life of Retail-Ready Fresh Beef Steaks Masterpackaged Under a CO2 Atmosphere. *Journal*
373 *of Food Science* 2006; 71(7), pp. S560-S566. DOI: 10.1111/j.1750-3841.2006.00126.x.

374 [16] Kerry JP, O'Grady MN, Hogan SA. Past, current and potential utilisation of active and intelligent
375 packaging systems for meat and muscle-based products: A review. *Meat Science* 2006; 74(1), pp. 113-
376 130. DOI: http://dx.doi.org/10.1016/j.meatsci.2006.04.024.

377 [17] Vermeiren L, Devlieghere F, van Beest M, de Kruijf N, Debevere J. Developments in the active
378 packaging of foods. *Trends in Food Science & Technology* 1999; 10(3), pp. 77-86. DOI:
379 http://dx.doi.org/10.1016/S0924-2244(99)00032-1.

380 [18] Mohan CO, Ravishankar CN, Srinivasagopal TK. Effect of O2 scavenger on the shelf-life of catfish
381 (Pangasius sutchi) steaks during chilled storage. *Journal of the Science of Food and Agriculture* 2008;
382 88(3), pp. 442-448. DOI: 10.1002/jsfa.3105.

383 [19] Doherty AM, Allen P. The effect of oxygen scavengers on the color stability and shelf-life of CO,
384 master packaged pork. *Journal of Muscle Foods* 1998; 9(4), pp. 351-363. DOI: 10.1111/j.1745-
385 4573.1998.tb00740.x.

386 [20] Limbo S, Uboldi E, Adobati A, Iametti S, Bonomi F, Mascheroni E, Santagostino S, Powers TH,
387 Franzetti L, Piergiovanni L. Shelf life of case-ready beef steaks (Semitendinosus muscle) stored in
388 oxygen-depleted master bag system with oxygen scavengers and CO2/N2 modified atmosphere
389 packaging. *Meat Science* 2013; 93(3), pp. 477-484. DOI:
390 http://dx.doi.org/10.1016/j.meatsci.2012.10.009.

391 [21] Martínez L, Djenane D, Cilla I, Beltrán JA, Roncalés P. Effect of varying oxygen concentrations on the
392 shelf-life of fresh pork sausages packaged in modified atmosphere. *Food Chemistry* 2006; 94(2), pp. 219-
393 225. DOI: http://dx.doi.org/10.1016/j.foodchem.2004.11.007.

394 [22] Mexis SF, Chouliara E, Kontominas MG. Combined effect of an oxygen absorber and oregano
395 essential oil on shelf life extension of rainbow trout fillets stored at 4 °C. *Food Microbiology* 2009; 26(6),
396 pp. 598-605. DOI: http://dx.doi.org/10.1016/j.fm.2009.04.002.

397 [23] Remya S, Mohan CO, Venkateshwarlu G, Sivaraman GK, Ravishankar CN. Combined effect of O2
398 scavenger and antimicrobial film on shelf life of fresh cobia (Rachycentron canadum) fish steaks stored
399 at 2 °C. *Food Control* 2017; 71, pp. 71-78. DOI: http://dx.doi.org/10.1016/j.foodcont.2016.05.038.

400 [24] Charles F, Sanchez J, Gontard N. Active Modified Atmosphere Packaging of Fresh Fruits and
401 Vegetables: Modeling with Tomatoes and Oxygen Absorber. *Journal of Food Science* 2003; 68(5), pp.
402 1736-1742. DOI: 10.1111/j.1365-2621.2003.tb12321.x.

403 [25] Lagerstedt Å, Lundström K, Lindahl G. Influence of vacuum or high-oxygen modified atmosphere
404 packaging on quality of beef M. longissimus dorsi steaks after different ageing times. *Meat Science* 2011;
405 87(2), pp. 101-106. DOI: http://dx.doi.org/10.1016/j.meatsci.2010.08.010.

406 [26] Resconi VC, Escudero A, Beltrán JA, Olleta JL, Sañudo C, Mar Campo Md. Color, Lipid Oxidation,
407 Sensory Quality, and Aroma Compounds of Beef Steaks Displayed under Different Levels of Oxygen in a
408 Modified Atmosphere Package. *Journal of Food Science* 2012; 77(1), pp. S10-S18. DOI: 10.1111/j.1750-
409 3841.2011.02506.x.

410 [27] Li X, Lindahl G, Zamaratskaia G, Lundström K. Influence of vacuum skin packaging on color stability
411 of beef longissimus lumborum compared with vacuum and high-oxygen modified atmosphere
412 packaging. *Meat Science* 2012; 92(4), pp. 604-609. DOI:
413 http://dx.doi.org/10.1016/j.meatsci.2012.06.006.

414 [28] Lindahl G. Colour stability of steaks from large beef cuts aged under vacuum or high oxygen
415 modified atmosphere. *Meat Science* 2011; 87(4), pp. 428-435. DOI:
416 http://dx.doi.org/10.1016/j.meatsci.2010.10.023.

417 [29] Viana ES, Gomide LAM, Vanetti MCD. Effect of modified atmospheres on microbiological, color and
418 sensory properties of refrigerated pork. *Meat Science* 2005; 71(4), pp. 696-705. DOI:
419 http://dx.doi.org/10.1016/j.meatsci.2005.05.013.

420 [30] Spanos D, Tørngren MA, Christensen M, Baron CP. Effect of oxygen level on the oxidative stability of
421 two different retail pork products stored using modified atmosphere packaging (MAP). *Meat Science*
422 2016; 113, pp. 162-169. DOI: http://dx.doi.org/10.1016/j.meatsci.2015.11.021.

423 [31] Spanos D, Christensen M, Tørngren MA, Baron CP. Visible spectroscopy as a tool for the assessment
424 of storage conditions of fresh pork packaged in modified atmosphere. *Meat Science* 2016; 113, pp. 154-
425 161. DOI: http://dx.doi.org/10.1016/j.meatsci.2015.11.019.

426 [32] R Core Team. The Comprehensive R Archive Network. 2018; Available at: https://cran.r-
427 project.org/index.html. Accessed 08/02/2018.

428 [33] Fox J, Weisberg S. An {R} Companion to Applied Regression. Second Edition 2011; Sage, Thousand
429 Oaks (CA).

430 [34] R Core Team. R: A language and environment for statistical computing. 2013; R Foundation for
431 Statistical Computing, Vienna, Austria.

432 [35] Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. *nlme: Linear and Nonlinear Mixed Effects*
433 *Models*. R package version 3.1-128 2016; .

434 [36] Lenth RV. Least-Squares Means: The R Package lsmeans. *Journal of Statistical Software* 2016; 69(1),
435 pp. 1-33. DOI: 10.18637/jss.v069.i01.

436 [37] Parry RT. *Introduction.* In: Parry RT (ed.). *Principles and Applications of Modified Atmosphere*
437 *Packaging of Foods.* Springer Science & Business Media, B.V: 2012;1-18.

438 [38] Sivertsvik M. *18 - Active packaging in practice: fish.* In: Ahvenainen R (ed.). *Novel Food Packaging*
439 *Techniques.* Woodhead Publishing: 2003;384-400. DOI:
440 http://dx.doi.org/10.1533/9781855737020.3.384.

441 [39] Esmer OK, Irkin R, Degirmencioglu N, Degirmencioglu A. The effects of modified atmosphere gas
442 composition on microbiological criteria, color and oxidation values of minced beef meat. *Meat Science*
443 2011; 88(2), pp. 221-226. DOI: http://dx.doi.org/10.1016/j.meatsci.2010.12.021.

444 [40] Gill CO. Extending the storage life of raw chilled meats. *Meat Science* 1996; 43, Supplement 1, pp.
445 99-109. DOI: http://dx.doi.org/10.1016/0309-1740(96)00058-7.

446 [41] Jakobsen M, Bertelsen G. The use of CO2 in packaging of fresh red meats and its effect on chemical
447 quality changes in the meat: a review. *Journal of Muscle Foods* 2002; 13(2), pp. 143-168. DOI:
448 10.1111/j.1745-4573.2002.tb00326.x.

449 [42] Jakobsen M, Bertelsen G. Colour stability and lipid oxidation of fresh beef. Development of a
450 response surface model for predicting the effects of temperature, storage time, and modified
451 atmosphere composition. *Meat Science* 2000; 54(1), pp. 49-57. DOI: http://dx.doi.org/10.1016/S0309-
452 1740(99)00069-8.

453 [43] Gill CO. The solubility of carbon dioxide in meat. *Meat Science* 1988; 22(1), pp. 65-71. DOI:
454 http://dx.doi.org/10.1016/0309-1740(88)90027-7.

455 [44] Hu P, Zhou G, Xu X, Li C, Han Y. Characterization of the predominant spoilage bacteria in sliced
456 vacuum-packed cooked ham based on 16S rDNA-DGGE. *Food Control* 2009; 20(2), pp. 99-104. DOI:
457 http://dx.doi.org/10.1016/j.foodcont.2008.02.007.

458 [45] Kreyenschmidt J, Hubner A, Beierle E, Chonsch L, Scherer A, Petersen B. Determination of the shelf
459 life of sliced cooked ham based on the growth of lactic acid bacteria in different steps of the chain.

460 *Journal of Applied Microbiology* 2010; 108(2), pp. 510-520. DOI: 10.1111/j.1365-2672.2009.04451.x
461 [doi].

462 [46] Brewer MS, Zhu LG, Bidner B, Meisinger DJ, McKeith FK. Measuring pork color: effects of bloom
463 time, muscle, pH and relationship to instrumental parameters. *Meat Science* 2001; 57(2), pp. 169-176.
464 DOI: http://dx.doi.org/10.1016/S0309-1740(00)00089-9.

465 [47] Zhu LG, Bidner B, Brewer MS. Postmortem pH, Muscle, and Refrigerated Storage Effects on Ability
466 of Vacuum-Packaged Pork to Bloom. *Journal of Food Science* 2001; 66(8), pp. 1230-1235. DOI:
467 10.1111/j.1365-2621.2001.tb16110.x.

468 [48] Krause TR, Sebranek JG, Rust RE, Honeyman MS. Use of Carbon Monoxide Packaging for Improving
469 the Shelf Life of Pork. *Journal of Food Science* 2003; 68(8), pp. 2596-2603. DOI: 10.1111/j.1365-
470 2621.2003.tb07067.x.

471 [49] Sørheim O, Erlandsen T, Nissen H, Lea P, Høyem T. Effects of modified atmosphere storage on
472 colour and microbiological shelf life of normal and pale, soft and exudative pork. *Meat Science* 1997;
473 47(1–2), pp. 147-155. DOI: http://dx.doi.org/10.1016/S0309-1740(97)00050-8.

474 [50] Sørheim O, Nissen H, Nesbakken T. The storage life of beef and pork packaged in an atmosphere
475 with low carbon monoxide and high carbon dioxide. *Meat Science* 1999; 52(2), pp. 157-164. DOI:
476 http://dx.doi.org/10.1016/S0309-1740(98)00163-6.

477 [51] Lindahl G, Karlsson AH, Lundström K, Andersen HJ. Significance of storage time on degree of
478 blooming and colour stability of pork loin from different crossbreeds. *Meat Science* 2006; 72(4), pp. 603-
479 612. DOI: http://dx.doi.org/10.1016/j.meatsci.2005.09.018.

480 [52] Norman JL, Berg EP, Ellersieck MR, Lorenzen CL. Prediction of color and pH measurement
481 throughout boneless center-cut pork loins. *Meat Science* 2004; 66(2), pp. 273-278. DOI:
482 http://dx.doi.org/10.1016/S0309-1740(03)00099-8.

Table 1. Statistical significance (p-values) of modified atmosphere (MA), storage time (T), blooming time (B) and their interactions on the studied variables, determined by a two or three-way mixed ANOVA. Statistical significance is observed at $p < 0.05$ and modified atmospheres ($\%O_2/CO_2/N_2$) A (80/20/0), B/C (0/20/80) and D (0/20/80 + oxygen absorber) were tested.

	O_2		**CO_2**		**pH**		**L***		**a***		**b***	
	A/B	C/D	A/B	C/D	A/B	C/D	A/B	C/D	A/B	C/D	A/B	C/D
MA	<0.0001	<0.0001	<0.0001	<0.0001	0.0259	0.9518	0.4274	0.0496	<0.0001	0.0001	<0.0001	<0.0001
T	0.0032	0.0032	<0.0001	0.0002	<0.0001	<0.0001	0.2709	0.2689	<0.0001	0.1398	0.0199	0.0147
B	-	-	-	-	-	-	0.6088	0.0514	<0.0001	<0.0001	<0.0001	<0.0001
MA x T	0.0050	0.0123	0.0027	0.0055	0.3225	0.2483	0.5211	0.3505	0.0017	0.1491	0.0003	0.2412
MA x B	-	-	-	-	-	-	0.3807	0.0970	<0.0001	0.1210	<0.0001	<0.0001
T x B	-	-	-	-	-	-	0.0018	0.5434	<0.0001	0.7261	0.0012	0.1040
MA x T x B	-	-	-	-	-	-	0.6017	0.6359	<0.0001	0.4490	0.0070	0.1600

Table 2. Evolution of headspace gas concentrations (%) under modified atmospheres (%O_2/CO_2/N_2) A (80/20/0) and B (0/20/80). Significant difference ($p < 0.05$) between means was determined by Tukey's post hoc test following a two-way mixed ANOVA and is indicated by superscripts within a variable (O_2 or CO_2). Significant difference between time points is indicated by different superscripts a-g within a column. At a certain time point, significant difference between the two packaging conditions is denoted with asterisks (*).

Time (d)	**O_2 (%)**		**CO_2 (%)**	
	A	B	A	B
1	$75.7 \pm 1.3^{ab*}$	$0.2 \pm 0.2^{*}$	$18.9 \pm 0.7^{a*}$	$23.7 \pm 1.0^{ab*}$
3	$73.5 \pm 8.1^{abc*}$	$0.2 \pm 0.1^{*}$	19.1 ± 1.1^{a}	21.4 ± 1.6^{ab}
6	$76.0 \pm 0.7^{a*}$	$0.4 \pm 0.2^{*}$	$18.8 \pm 0.5^{a*}$	$23.5 \pm 1.5^{ab*}$
8	$70.3 \pm 9.6^{abcd*}$	$0.3 \pm 0.1^{*}$	19.7 ± 1.3^{a}	20.9 ± 2.1^{a}
10	$73.6 \pm 1.4^{abc*}$	$0.3 \pm 0.2^{*}$	18.9 ± 0.6^{a}	22.2 ± 2.4^{ab}
13	$62.2 \pm 8.4^{cd*}$	$0.1 \pm 0.2^{*}$	$21.0 \pm 1.9^{a*}$	$25.6 \pm 2.4^{b*}$
15	$63.8 \pm 6.1^{bcd*}$	$0 \pm 0.1^{*}$	22.8 ± 0.2^{ab}	22.3 ± 1.9^{ab}
17	$61.1 \pm 9.4^{d*}$	$0 \pm 0.1^{*}$	26.1 ± 2.3^{b}	25.5 ± 2.9^{b}

Table 3. Evolution of headspace gas concentrations (%) under modified atmospheres (%O_2/CO_2/N_2) C (0/20/80) and D (0/20/80 + oxygen absorber). Significant difference ($p < 0.05$) between means was determined by Tukey's post hoc test following a two-way mixed ANOVA and is indicated by superscripts within a variable (O_2 or CO_2). Significant difference between time points is indicated by different superscripts a-g within a column. At a certain time point, significant difference between the two packaging conditions is denoted with asterisks (*).

Time (d)	**O_2 (%)**		**CO_2 (%)**	
	C	D	C	D
1	0.2 ± 0.2^{abc}	0 ± 0	23.8 ± 1.2	22.2 ± 0.8^{a}
3	$0.4 \pm 0.4^{bc*}$	$0 \pm 0^{*}$	22.5 ± 1.5	20.1 ± 1.5^{ab}
6	0.4 ± 0.2^{bc}	0.1 ± 0.1	22.0 ± 1.5	18.8 ± 0.4^{abc}
8	$0.5 \pm 0.3^{c*}$	$0 \pm 0^{*}$	$22.8 \pm 1.8^{*}$	$14.3 \pm 5.1^{c*}$
10	0.3 ± 0.3^{abc}	0 ± 0	$23.2 \pm 0.6^{*}$	$17.9 \pm 1.0^{bc*}$
13	0.1 ± 0.1^{ab}	0 ± 0	$23.4 \pm 1.1^{*}$	$18.5 \pm 2.9^{abc*}$
15	0.1 ± 0.3^{ab}	0 ± 0	$25.1 \pm 1.1^{*}$	$19.0 \pm 1.3^{ab*}$
17	0 ± 0^{a}	0 ± 0	$25.6 \pm 1.3^{*}$	$20.2 \pm 1.0^{ab*}$

Table 4. Redness (a) and yellowness (b*) under modified atmospheres (%O_2/CO_2/N_2) A (80/20/0) and B (80/20/0) before and after blooming (20 min). Significant difference ($p < 0.05$) between means was determined by Tukey's post hoc test following a three-way mixed ANOVA and is indicated by superscripts within a variable (a* or b*). Significant difference between time points is indicated by different superscripts a-g within a column. At a certain time point and stage of blooming, asterisk (*) indicates significant difference between atmospheres. At a certain time point and atmosphere, superscript S indicates significant difference between stages of blooming.*

Day	**a***				**b***			
	A		B		A		B	
	Before	After	Before	After	Before	After	Before	After
1	18.58 ± 1.13^{a}	18.56 ± 1.05^{a}	14.04 ± 0.80^{S}	15.26 ± 0.93^{S}	$10.80 \pm 0.32^{*}$	$10.80 \pm 0.23^{*}$	$4.18 \pm 0.44^{a*S}$	$7.23 \pm 0.36^{a*S}$
3	17.19 ± 2.31^{ab}	17.30 ± 2.28^{ab}	13.96 ± 2.40^{S}	15.14 ± 2.45^{S}	$10.87 \pm 0.49^{*}$	$10.98 \pm 0.49^{*}$	$5.45 \pm 1.41^{ab*S}$	$8.07 \pm 0.84^{ab*S}$
6	$16.87 \pm 0.51^{ab*}$	16.86 ± 0.58^{ab}	$12.09 \pm 0.92^{*S}$	12.93 ± 1.14^{S}	$10.58 \pm 0.51^{*}$	10.53 ± 0.52	$7.10 \pm 0.83^{bc*S}$	8.50 ± 0.57^{abS}
8	15.55 ± 1.18^{abc}	15.51 ± 1.16^{abc}	11.50 ± 1.00^{S}	12.37 ± 1.15^{S}	$10.32 \pm 0.57^{*}$	10.27 ± 0.60	$7.71 \pm 0.90^{bc*}$	8.75 ± 0.36^{ab}
10	14.57 ± 1.40^{abc}	14.70 ± 1.53^{abc}	10.62 ± 0.74^{S}	11.22 ± 1.09^{S}	10.35 ± 0.42	10.43 ± 0.59	9.05 ± 0.84^{c}	9.81 ± 0.62^{b}
13	13.11 ± 1.30^{bc}	13.24 ± 1.46^{bc}	12.66 ± 2.99^{S}	13.31 ± 3.20^{S}	$10.48 \pm 0.24^{*}$	10.78 ± 0.19	$6.36 \pm 2.54^{ab*S}$	8.57 ± 1.17^{abS}
15	11.71 ± 1.30^{c}	11.84 ± 1.38^{c}	12.12 ± 1.79	12.62 ± 2.22	10.13 ± 0.68	10.26 ± 0.53	7.65 ± 2.07^{bcS}	9.06 ± 1.12^{abS}
17	12.00 ± 0.53^{c}	12.02 ± 0.53^{c}	13.65 ± 2.19	13.29 ± 2.02	9.97 ± 0.40	10.05 ± 0.40	7.46 ± 1.47^{bcS}	9.44 ± 0.80^{abS}

Table 5. Redness (a) and yellowness (b*) under modified atmospheres (%O_2/CO_2/N_2) C (0/20/80) and D (0/20/80 + oxygen absorber) before and after blooming (20 min). Three-way mixed ANOVA showed non-significant ($p < 0.05$) atmosphere x storage time x blooming time interaction for both dependent variables.*

Day	**a***				**b***			
	C		D		C		D	
	Before	After	Before	After	Before	After	Before	After
1	13.23 ± 0.50	14.69 ± 0.48	16.14 ± 0.91	17.16 ± 0.88	5.16 ± 1.04	7.56 ± 0.58	3.20 ± 0.31	6.75 ± 0.39
3	14.51 ± 1.08	15.24 ± 1.69	14.94 ± 0.66	16.06 ± 0.55	5.11 ± 0.85	7.38 ± 0.68	3.57 ± 0.43	7.42 ± 0.59
6	13.54 ± 2.29	13.87 ± 2.97	15.46 ± 1.14	16.02 ± 0.76	5.87 ± 2.57	7.78 ± 0.97	3.86 ± 0.56	7.15 ± 0.35
8	12.02 ± 1.00	12.02 ± 0.98	14.03 ± 0.62	13.94 ± 2.26	7.66 ± 1.85	8.55 ± 1.24	3.18 ± 0.66	6.25 ± 0.61
10	11.27 ± 2.40	11.81 ± 3.36	15.03 ± 0.24	15.68 ± 0.47	7.92 ± 1.12	8.62 ± 0.35	3.83 ± 1.12	6.88 ± 0.68
13	13.80 ± 1.58	13.39 ± 2.35	13.63 ± 1.77	15.33 ± 1.88	6.39 ± 1.85	8.09 ± 0.79	4.79 ± 3.01	7.76 ± 1.77
15	12.37 ± 2.92	12.72 ± 2.97	14.97 ± 0.73	15.55 ± 0.75	6.98 ± 2.03	8.36 ± 1.42	4.92 ± 1.58	8.10 ± 1.16
17	15.22 ± 1.71	15.22 ± 2.44	14.26 ± 0.45	14.63 ± 0.12	6.99 ± 1.23	9.00 ± 0.39	5.22 ± 2.06	8.25 ± 1.48

P3

MICROBIOLOGICAL, CHEMICAL AND SENSORY SPOILAGE ANALYSIS OF RAW ATLANTIC COD (*GADUS MORHUA*) STORED UNDER MODIFIED ATMOSPHERES

by

Kuuliala, L., Al Hage, Y., Ioannidis, A.-G., Sader, M., Kerckhof, F.-M., Vanderroost, M., Boon, N., De Baets, B., De Meulenaer, B., Ragaert, P. & Devlieghere, F., 2018

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Microbiological, chemical and sensory spoilage analysis of raw Atlantic cod (*Gadus morhua*) stored under modified atmospheres

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ABSTRACT

During fish spoilage, microbial metabolism leads to the production of volatile organic compounds (VOCs), characteristic off-odors and eventual consumer rejection. The aim of the present study was to contribute to the development of intelligent packaging technologies by identifying and quantifying VOCs that indicate spoilage of raw Atlantic cod (*Gadus morhua*) under atmospheres (%v/v $CO_2/O_2/N_2$) 60/40/0, 60/5/35 and air. Spoilage was examined by microbiological, chemical and sensory analyses over storage time at 4 or 8 °C. Selected-ion flow-tube mass spectrometry (SIFT-MS) was used for quantifying selected VOCs and amplicon sequencing of the 16S rRNA gene was used for the characterization of the cod microbiota. OTUs classified within the *Photobacterium* genus increased in relative abundance over time under all storage conditions, suggesting that *Photobacterium* contributed to spoilage and VOC production. The onset of exponential VOC concentration increase and sensory rejection occurred at high total plate counts (7–7.5 log). Monitoring of early spoilage thus calls for sensitivity for low VOC concentrations.

1. Introduction

Raw fish is highly perishable due to the intrinsic properties of the product and inevitable microbial activity. Spoilage of fish is primarily caused by microbial growth and metabolism and is characterized by changes in the sensory properties that lead to unacceptable product quality (Gram and Huss, 1996; Gram and Dalgaard, 2002; Gram et al., 2002). Shelf life of fish is affected by several factors, including storage temperature, fish species, initial microbial contamination and packaging conditions (Sivertsvik et al., 2002). Even though 10^7 CFU/g has generally been considered as a maximum acceptable microbial load for fish (Stannard, 1997), sensory rejection has typically been found at microbial levels between 10^6-10^9 CFU/g (Dalgaard et al., 1997a; Mikš-Krajnik et al., 2016; Nuin et al., 2008; Parlapani et al., 2014, 2015b).

Specific spoilage organisms (SSOs) typically constitute a fraction of the initial microbiota and their outgrowth eventually leads to unacceptable changes in the product quality (Gram and Dalgaard, 2002). The microbiota of fresh marine fish generally consists of psychrotrophic Gram-negative rod-shaped bacteria along with Gram-positive microbes (Gram and Huss, 1996). In marine fish stored under refrigerated aerobic conditions, *Pseudomonas* and *Shewanella* spp. have been observed to be dominating (Gram et al., 1987; Gram and Huss, 1996; Gram and Dalgaard, 2002; Vogel et al., 2005), whereas *Photobacterium phosphoreum* has been identified as an SSO of Atlantic cod (*Gadus morhua*) under different modified atmosphere packaging (MAP) conditions (Dalgaard et al., 1997a; Dalgaard, 1995; Debevere and Boskou, 1996).

Odor is one of the most important quality determinants for fish

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freshness (Olafsdottir et al., 2005). As a result of microbial metabolism, volatile organic compounds (VOCs) are often produced, which leads to the production of characteristic off-odors and off-flavors. Typical compounds associated with fish spoilage include acids, alcohols, aldehydes, amines, ketones and sulfides (Gram and Dalgaard, 2002). The spoilage potential of SSOs is characterized by their qualitative ability to produce off-odors, whereas spoilage activity refers to the quantitative ability to produce spoilage metabolites (Gram and Dalgaard, 2002; Gram et al., 2002). Thus, evolution of spoilage-related VOCs could be used for fish quality evaluation during storage. Different approaches for characterizing the VOC profile have been applied to marine fish species such as cod (Fernández-Segovia et al., 2006; Noseda et al., 2010), salmon (Jónsdóttir et al., 2008; Jørgensen et al., 2001; Macé et al., 2013; Mikš-Krajnik et al., 2016), sea bream (Parlapani et al., 2014, 2015b; Soncin et al., 2009), sea bass (Parlapani et al., 2015a), hake (Baixas-Nogueras et al., 2001), mackerel (Alfaro et al., 2013) and turbot (Xu et al., 2014).

Intelligent packaging technologies aim at improving the quality and safety of the packaged product and/or informing about its status by detecting, sensing, communicating, recording or applying another intelligent function (Yam et al., 2005). Among these technologies, sensors that convert physical or chemical information into an informative signal have been considered to have high potential for future applications (Ghaani et al., 2016; Kerry et al., 2006; Vanderroost et al., 2014). The use of sensor technologies for monitoring VOCs indicating fish spoilage could enhance the detection of spoilage in individual packages, thus improving quality evaluation and reducing food and packaging material waste throughout the supply chain. Even though different applications for sensor-based quality monitoring of fish have been examined (Bhadra et al., 2015; Chung et al., 2017; Efremenko and Mirsky, 2017; García et al., 2017; Morsy et al., 2016; Pacquit et al., 2006, 2007; Perera et al., 2010), there is still a limited number of studies focusing on direct and real-time quantification of the VOC profile produced in the package headspace during storage time, aiming at the development of intelligent packaging technologies.

Efficient quality monitoring of fish spoilage calls for fast, non-destructive and sensitive methods. However, conventional quality analyses of fish packaged under modified atmospheres (MAs) are commonly destructive and time consuming, such as the determination of total volatile basic nitrogen (TVB-N) by steam distillation (Pacquit et al., 2006) or plate counts. Several technologies have been used for rapid and accurate characterization of VOCs, including gas chromatography-mass spectrometry (GC-MS) (Béné et al., 2001; Duflos et al., 2010; Edirisinghe et al., 2007; Fernández-Segovia et al., 2006; Grimm et al., 2000; Jaffrès et al., 2011; Leduc et al., 2012; Mikš-Krajnik et al., 2016; Zhang et al., 2010) and electronic noses (Natale et al., 2001; Olafsdottir et al., 2005; Zaragozá et al., 2014). On the other hand, selective-ion flow-tube mass spectrometry (SIFT-MS) can be used for non-destructive and sensitive real-time quantification of VOCs from the package headspace. The technology is based on reactions between precursor ions (H_3O^+, NO^+, O^+) and target compounds, followed by the quantification of the resulting product ions on the basis of their mass to charge (m/z) ratio. SIFT-MS has previously been validated for fish metabolite research (Noseda et al., 2010) and used for VOC analysis of different food products, including seafood (Noseda et al., 2012), meat (Carrapiso et al., 2015; Olivares et al., 2012), fruit (Zhang et al., 2013a, 2013b, 2014) and cheese (Castada et al., 2014; Castada et al., 2015; Langford et al., 2012).

Identification and quantification of VOCs related to spoilage is of high importance for the development of food quality monitoring. Establishing a relation between VOC production, microbial growth (both in total amount and in specific microorganisms) and sensorial quality is needed as the basis for the development of intelligent packaging solutions. In the present study, spoilage of Atlantic cod packaged under modified atmospheres was analyzed by following microbial growth, VOC concentrations and sensory quality during refrigerated storage. SIFT-MS was used for the real-time quantification of VOCs from the package headspace and amplicon (NGS) sequencing of the 16S rRNA gene was used for the characterization of the cod microbiota at different stages of storage. The results of the present study contribute to the development of intelligent packaging technologies within the CheckPack project (VLAIO grant number 130036).

2. Materials and methods

2.1. Raw material

For each individual storage experiment, Atlantic cod (minimum body weight ca. 4.5 kg) was caught in the North Atlantic Ocean (FAO zone 27), gutted, filleted and stored under ice. The fish was transported to Belgium by air and delivered to the Laboratory of Food Microbiology and Food Preservation (LFMFP) in polystyrene boxes under ice.

2.2. Packaging and storage

Cod fillet portions (217 ± 5 g) were packaged under different atmospheres with a gas-product ratio 2:1 using a tray sealer MECA 900 (DecaTechnic, Herentals, Belgium), multilayer packaging trays (PP/EVOH/PP, oxygen transmission rate 0.03 cm^3/tray*24 h at 23 °C and 50% R.H.) and top film (PA/EVOH/PA/PP, oxygen transmission rate 6.57 cm^3/m^2*24 h*atm at 23 °C, 50% R.H. and 1 atm). Three different atmospheres and two storage temperatures were applied (Table 1): independent batches of fish were used for each of the five storage experiments. In the present study, the storage experiments are referred to as H4, H8, L4, L8 and Air, where the notation of the MA conditions indicates high (H) or low (L) oxygen content and temperature in Celsius degrees (4 or 8). For the determination of background concentrations possibly originating from the packaging materials and/or heat sealing, sample-free packages (blanks) with similar gas atmospheres were prepared. The packages were stored at (4.0 ± 0.7) or (8.0 ± 0.4) °C until the day of analysis. On a regular basis, three randomly selected packages were analyzed. After sampling, the remaining fish portion was packaged under vacuum using high barrier film bags (oxygen transmission rate < 2.7 cm^3/m^2*24 h*bar at 23 °C and 0% R.H.) and stored at −32 °C for no longer than 120 days (sensory evaluation) or one year (amplicon sequencing).

2.3. Microbiological analysis

For microbiological analysis, 30 ± 0.1 g of individual fillet was aseptically weighed into a sterile stomacher bag and diluted ten times in physiological saline peptone solution (PPS; 0.85% m/v NaCl, 0.1% m/v peptone). The samples were homogenized in Stomacher Lab Blender (LED Techno, Heusden-Zolder, Belgium) for one minute and appropriate decimal dilutions were prepared in PPS. The total psychrotrophic count (TPC) was determined on Marine Agar (MA; Difco Le Pont de Claix, France) by spread plating, lactic acid bacteria (LAB) on Man Rogosa Sharpe Agar (MRS; Oxoid, Hamsphire, UK) or modified MRS (mMRS; yeast extract 4.0 g/L, Lab-Lemco powder 8.0 g/L, peptone 10.0 g/L, sorbitan mono-oleate (Tween 80) 1 ml/L, dipotassium hydrogen phosphate 2 g/L, sodium acetate 5 g/L, triammonium citrate 2 g/L, magnesium sulphate 0.2 g/L, manganese sulphate 0.05 g/L; pH 8.6 at 25 °C; 20% glucose solution 100 mL/L after autoclaving) by pour plating, hydrogen sulfide (H_2S)

Table 1
Packaging and storage conditions used in the study. Samples from days denoted with (*) were studied by amplicon sequencing.

	H4	H8	L4	L8	Air
Headspace gases (% $CO_2/O_2/N_2$)	60/40/0	60/40/0	60/5/35	60/5/35	air
Temperature (°C)	4	8	4	8	4
Days of analysis	0*,4,5*,6,7,8,11,13*	0*,3,4*,5,6,7*	0*,4,5*,6,7,11,13*	0*,3,4*,5,6,7*	0*,1,2*,3*

producers on Iron Agar Lyngby (IAL; Oxoid) supplemented with L-cysteine (Fluka, Steinheim, Germany) by pour plating, Pseudomonads on *Pseudomonas* Agar (PA; Oxoid) supplemented with *Pseudomonas* CFC supplement SR 103E (Oxoid) by spread plating and *Brochothrix thermosphacta* on Streptomycin Sulfate Thallous Acetate Actidione Agar (STAA; Oxoid) supplemented with selective supplement SR 151E (Oxoid) by spread plating. Plates were incubated at 22 °C for 2 (PA and STAA), 3 (MRS and IAL) or 5 days (MA).

2.4. Quantification of spoilage related VOCs by SIFT-MS

A selected-ion flow-tube mass spectrometer (Voice 200, Syft Technologies™, Christchurch, New Zealand) was used for quantifying a predefined set of VOCs in the package headspace. Principles of the instrument have been described elsewhere (Noseda et al., 2010). The compounds (Table 2) were selected on the basis of previous research and literature survey. The package headspace was sampled with a flow rate of 32 ml/min during 60 s (preparation 10s, sample 50s) through a septum inserted on the package lid and the VOC concentrations were averaged over eleven data points. Two consecutive gas samples per package were analyzed. During sampling, the headspace was connected to atmospheric air with a needle inlet in order to avoid package collapse and subsequent change in the internal conditions of the package. Respectively, empty packages (blanks, n = 9–14) from each headspace-temperature combination were randomly analyzed throughout the storage time and used for determining the limit of quantification (LOQ) of each compound and for background subtraction. Concentrations of the VOCs were determined with the LabSyft software (Syft Technologies™).

The relative standard deviation ($SD_{\%}$) of each VOC concentration during a SIFT-MS measurement was calculated as

Table 2
Product ions of volatile organic compounds (VOCs) quantified with SIFT-MS, respective mass to charge ratios (m/z), branching ratios (b) and reaction rate coefficients (k).

VOC	Precursor ion	m/z	b (%)	k	Production
Acids					
Acetic acid	H_3O^+	61	100	2.6 E −09	$CH_3COOH_2^+$
	NO^+	90	100	9.0 E −10	$NO^+ \cdot CH_3COOH$
	O_2^+	60	50	2.3 E −09	CH_3COOH^+
Alcohols					
Ethanol	H_3O^+	47	100	2.7 E −09	$C_2H_7O^+$
	H_3O^+	65			$C_2H_7O^+ \cdot H_2O$
	H_3O^+	83			$C_2H_7O^+ \cdot (H_2O)_2$
2,3-butanediol	NO^+	89	100	2.3 E −09	$C_4H_9O_2^+$
3-methyl-1-butanol	H_3O^+	71	100	2.8 E −09	$C_5H_{11}^+$
	NO^+	87	85	2.3 E −09	$C_5H_{11}O^+$
isobutyl alcohol	NO^+	73	95	2.4 E −09	$C_4H_9O^+$
	O_2^+	33	50	2.5 E −09	CH_5O^+
Aldehydes					
2-methylpropanal	O_2^+	72	70	3.0 E −09	$C_4H_8O^+$
3-methylbutanal	NO^+	85	100	2.4 E −09	$C_5H_9O^+$
Ketones					
Acetone	H_3O^+	59	100	3.9 E −09	C_3H_7O+
	NO^+	88	100	1.2 E −09	$NO^+ \cdot C_3H_6O$
Acetoin	O_2^+	88	20	2.5 E −09	$C_4H_8O_2^+$
2-pentanone	NO^+	116	100	3.1 E −09	$NO^+ \cdot C_5H_{10}O^+$
Sulfur compounds					
Hydrogen sulfide	H_3O^+	35	100	1.6 E −09	H_3S^+
	O_2^+	34	100	1.4 E −09	H_2S^+
Methyl mercaptan	H_3O^+	49	100	1.8 E −09	$CH_4S \cdot H^+$
Dimethyl sulfide	H_3O^+	63	100	2.5 E −09	$(CH_3)_2S.H^+$
	NO^+	62	100	2.2 E −09	$(CH_3)_2S^+$
Dimethyl disulfide	H_3O^+	95	100	2.6 E −09	$(CH_3)_2S_2.H^+$
	NO^+	94	100	2.4 E −09	$(CH_3)_2S_2^+$
	O_2^+	94	80	2.3 E −09	$(CH_3)_2S_2^+$
Dimethyl trisulfide	H_3O^+	127	100	2.8 E −09	$C_2H_6S_3H^+$
	NO^+	126		1.9 E −09	$C_2H_6S_3^+$
Esters					
Ethyl acetate	NO^+	118	90	2.1 E −09	$NO^+ \cdot CH_3COOC_2H_5$
Ethyl propanoate	H_3O^+	103	95	2.9 E −09	$C_2H_5COOC_2H_5.H^+$
	NO^+	132	60	2.5 E −09	$NO^+ \cdot C_2H_5COOC_2H_5$
Amines					
Ammonia	H_3O^+	18	100	2.6 E −09	NH_4^+
	O_2^+	17	100	2.4 E −09	NH_3^+
Dimethylamine	H_3O^+	46	100	2.1 E −09	$(CH_3)_2N \cdot H^+$
Trimethylamine	H_3O^+	60	90	2.0 E −09	$(CH_3)_3N \cdot H^+$
	NO^+	59	100	1.6 E −09	$(CH_3)_3N^+$

$$SD_{\%} = SD_m/x_m{}^* 100\% \quad (1)$$

where x_m is the average and SD_m the standard deviation of a single SIFT-MS measurement (n = 11). VOCs with concentrations exceeding 25% average relative standard deviation during the entire storage time within a certain packaging condition were excluded from further analysis.

The Limit of Quantification (LOQ) was calculated with the International Union of Pure and Applied Chemistry (IUPAC) equation (Mocak et al., 1997):

$$LOQ = x_{bl} + 6^*SD_{bl} \quad (2)$$

where x_{bl} is the total average and SD_{bl} the standard deviation of the blanks. Background was subtracted from the measured concentrations that exceeded the LOQ: the reported results are measured concentrations minus x_{bl}.

2.5. Headspace composition (% CO_2/O_2), pH and color measurements

The headspace gas composition (% v/v CO_2/O_2) was analyzed with a gas analyzer (CheckMate® 9900 CO_2/O_2, Dansensor A/S, Ringsted, Denmark). pH was determined as an average of three consecutive measurements from randomly selected spots in individual fillets within 30 min after opening the package using a pH electrode (Lab® 427, Mettler Toledo GmbH, Schwerzenbach, Switzerland) connected to a pH meter (SevenEasy, Mettler Toledo GmbH). The product color was determined as an average of ten measurements from randomly selected fillet spots by a spectrophotometer (CM 2500d, Konica Minolta Sensing Inc., New Jersey, USA) and related SpectraMagic™ NX color data software. Color was measured through a small Petri dish (diameter 230 mm) using the CIE L^* a^* b^* color space with a standard 10° observer and Illuminant D65.

2.6. Sensory evaluation

Sensory evaluation was based on olfactory evaluation and performed in individual booths under red light (UGent Sensolab). A panel of 8–12 persons having experience in sensory evaluation of fish was formed from the laboratory staff at LFMFP. One out of three daily replicates (A-C) was randomly selected and used per testing session. The samples were thawed at 2 °C overnight, cut to 5.0 ± 0.1 g portions and presented to the panelists at 4 °C in odor-free, transparent plastic cups (diameter 67 mm; AVA, Temse, Belgium), closed with lids (AVA) and labelled with three-digit random codes generated with Excel 2013 (Windows).

Ranking tests (ISO, 2006) were used to determine if significant differences occurred between different stages of storage within a certain packaging condition. Four samples were presented to the panelists to be ranked from least fresh (1) to most fresh (4). For conditions H4, H8, and L4, a second ranking test was performed for the critical days identified by the first test. The collected data was subjected to a Friedman test followed by a Least Significant Difference test (Excel 2013 for Windows) in order to determine whether significant differences occurred between samples from different days of storage.

Acceptance tests were used to determine the quality of cod samples from different stages of storage within a certain packaging condition. Four samples were presented to the panelists along with a fresh reference (day 0) from the same lot. A five-point scale (very good, good, satisfactory, marginal, spoiled) was used for the evaluation.

2.7. Amplicon sequencing

16s rRNA gene amplicon sequencing analysis was used for the characterization of the cod microbiota over storage time. Three samples stored at −32 °C were selected to represent early, intermediate and late stages of storage (Table 1). One randomly selected sample out of three daily replicates (A-C) was used for the analysis.

A phenol/chloroform extraction procedure with mechanical disruption using a FastPrep device (Vilchez-Vargas et al., 2013) was used for the extraction of DNA. Bacterial cells were aseptically collected from the frozen sample surface by swabbing. An individual swab was placed in an Eppendorf tube with 200 mg glass beads and 1000 μl of lysis buffer (100 mM Tris; 100 mM EDTA; 100 mM NaCl; 1 wt/vol % polyvinylpyrrolidone; 2 wt/vol % sodium dodecyl sulphate; 50 ml water; pH 8). The tube was transferred to the FastPrep-24 instrument (MP Biomedicals, Santa Ana, California, USA) and disrupted twice at 1400 rpm for 60 s. After centrifuging at maximum speed for 5 min, phenol-chloroform-isoamilic alcohol (500 μl; pH 7) was added to the supernatant and the solution was thoroughly vortexed and centrifuged at maximum speed for 60 s. Chloroform (700 μl) was added to the supernatant, mixed by vortexing and centrifuged at maximum speed for one minute. The resulting upper phase was divided into two Eppendorf tubes (450 μl per tube) where sodium acetate (3M; 45 μl) was added, followed by mixing and addition of isopropyl alcohol (−20 °C; 500 μl). The solution was mixed by inverting, stored for one hour at −20 °C and centrifuged at maximum speed for one minute at 4 °C. The resulting pellet was dried and dissolved into T10E1 (100 μl).

Library preparation and sequencing was carried out at LGC Genomics (Germany) according to the procedure presented by De Vrieze et al. (2016). The PCR mix contained 1 ng of DNA extract and PCRs showing low yields were further amplified for 5 additional cycles if needed. Sequencing was done on an Illumina MiSeq platform using v3 Chemistry (Illumina, San Diego, California, USA) along with a mock community that was included in triplicate in the sequencing run to assess the sequencing quality. The mock community consisted of the genomic DNA of 12 species from 10 different phyla and was pooled to an equimolar concentration of 16S rRNA gene copies based on Q-PCR with the Illumina primers.

The mothur software package v. 1.38.0 (Schloss et al., 2009) and guidelines developed by P. Schloss (Miseq sop, 2016; Kozich et al., 2013) were used for processing the amplicon sequencing data. From the total number of forward and reverse reads, contigs with lengths outside of the 2.5–97.5% quantiles or sequences with ambiguous base calls were removed. Remaining unique sequences were aligned to the mother-reconstructed SILVA Seed alignment v. 123 (Pruesse et al., 2007). Unique sequences were pre-clustered within a distance of 1/100 nucleotides and chimeras were screened with UCHIME (Edgar et al., 2011). Next, sequences were classified using RDP v. 14 (Cole et al., 2009) and Wang's algorithm.

Non-bacterial or unidentified sequences were removed and the remaining OTUs were clustered using average linkage and 97% sequence identity. Single-read OTUs were considered as likely errors and discarded from further analyses. The alpha diversity was examined by rarefaction curves and community richness estimators Chao (1984), Chao and Bunge (2002) and ACE-1 (Chao and Lee, 1992), diversity estimators Shannon and Weaver (1949), Simpson (1949) and inverse Simpson, and evenness estimator Pielou (1966).

3. Results

3.1. Headspace composition (% CO_2/O_2)

The development of headspace gas concentrations (CO_2/O_2) is

Table 3
Headspace gases (O_2, CO_2), pH and color (L*, a*, b*) as a function of time under conditions H4 (60% CO_2/40 & O_2/0% N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and Air (air 4 °C).

	Series	Time of storage (d)										
		0	1	2	3	4	5	6	7	8	11	13
O2	H4	41.17 ± 0.15				51.9 ± 1.6	51.77 ± 2.12	49.8 ± 1.9	49.33 ± 1.9	48.5 ± 1.48	43.97 ± 1.9	37.77 ± 2.98
	H8	42.47 ± 0.81			49.63 ± 1.27	39.57 ± 18.8	47.57 ± 0.58	46.17 ± 1.53	46.7 ± 0.78			
	L4	5.09 ± 0.12				4.26 ± 0.18	2.87 ± 0.44	2.41 ± 0.79	1.67 ± 0.52	0.25 ± 0.3	0.01 ± 0	0 ± 0
	L8	4.47 ± 0.51			4.36 ± 0.09	2.81 ± 0.87	0.73 ± 0.16	0.07 ± 0.11	0 ± 0.01			
	Air	20.73 ± 0.06	20.3 ± 0.1	19.67 ± 0.06	16.53 ± 1.4							
CO2	H4	55.83 ± 0.25				44.07 ± 0.65	43.43 ± 1.97	43.47 ± 0.83	46.17 ± 1.57	47 ± 1.9	52.27 ± 1.45	53.7 ± 12.38
	H8	55.43 ± 2.12			47 ± 1.04	37.5 ± 13.6	49.33 ± 0.38	50.17 ± 1.86	49.77 ± 0.45			
	L4	56.27 ± 1.02				42.87 ± 0.51	41.1 ± 2.17	44.17 ± 2.27	41.07 ± 0.55	42.47 ± 2.08	40.53 ± 1.79	44.83 ± 4.47
	L8	56.47 ± 0.51			45.13 ± 1	43.77 ± 2.4	47.87 ± 4.11	45.53 ± 1.61	48.47 ± 2.93			
	Air	0.27 ± 0.06	1.37 ± 0.21	1.3 ± 0	4.77 ± 1.31							
pH	H4	6.34 ± 0.16				6.2 ± 0.11	6.36 ± 0.13	6.34 ± 0.15	6.33 ± 0.09	6.41 ± 0.08	6.23 ± 0.04	6.46 ± 0.08
	H8	6.42 ± 0.04			6.41 ± 0.13	6.66 ± 0.21	6.6 ± 0.01	6.54 ± 0.1	6.78 ± 0.04			
	L4	6.68 ± 0.24				6.3 ± 0.1	6.4 ± 0.08	6.38 ± 0.04	6.62 ± 0.14	6.59 ± 0.06	6.67 ± 0.09	6.57 ± 0.06
	L8	6.88 ± 0.07			6.56 ± 0.04	6.71 ± 0.02	6.48 ± 0.17	6.71 ± 0.1	6.75 ± 0.06			
	Air	6.68 ± 0.07	6.72 ± 0.02	6.68 ± 0.08	6.7 ± 0.05							
L*	H4	59.08 ± 1.77				62.36 ± 5.41	60.3 ± 1.59	58.01 ± 2.22	64.15 ± 2.09	61.2 ± 2.57	66.12 ± 2.06	61.03 ± 2.3
	H8	58.15 ± 2.29			58.37 ± 1.15	60.63 ± 1.32	60.49 ± 0.41	56.9 ± 2.06	58.63 ± 0.82			
	L4	56.33 ± 1.68				61.1 ± 1.81	59.94 ± 2.66	59.94 ± 1.25	59.32 ± 1.09	60.97 ± 1.16	60.63 ± 0.69	62.53 ± 1.3
	L8	61.8 ± 2.37			59.94 ± 2.47	59.97 ± 1.7	62.13 ± 1.64	62.84 ± 0.35	60.86 ± 0.62			
	Air	55.7 ± 1.06	58.71 ± 1.33	58.71 ± 1.54	56.71 ± 1.46							
a*	H4	−2.5 ± 0.18				−2.7 ± 0.07	−2.85 ± 0.31	−2.61 ± 0.09	−3 ± 0.05	−2.91 ± 0.28	−2.97 ± 0.16	−2.63 ± 0.3
	H8	−2.43 ± 0.11			−2.78 ± 0.28	−2.88 ± 0.05	−3.09 ± 0.21	−3.14 ± 0.2	−2.99 ± 0.28			
	L4	−2.6 ± 0.45				−3.11 ± 0.12	−2.64 ± 0.23	−2.95 ± 0.14	−3.05 ± 0.3	−3.23 ± 0.46	−3.06 ± 0.06	−3.41 ± 0.3
	L8	−3.07 ± 0.37			−3.21 ± 0.48	−3.07 ± 0.44	−3.59 ± 0.16	−3.35 ± 0.23	−3.55 ± 0.12			
	Air	−2.55 ± 0.33	−2.65 ± 0.16	−2.5 ± 0.17	−2.98 ± 0.07							
*b**	H4	−1.64 ± 1.45				0.28 ± 1.05	−1.03 ± 0.16	−2.41 ± 1.11	−0.03 ± 0.96	0.49 ± 0.53	1.66 ± 2.9	2.68 ± 1.88
	H8	−1.09 ± 1.78			0.97 ± 1.72	−0.99 ± 1.14	0.61 ± 1.42	0.64 ± 0.16	−1.27 ± 1.52			
	L4	−1.15 ± 0.88				−0.06 ± 0.98	−0.14 ± 1.86	−1.21 ± 0.61	0.54 ± 2.32	0.05 ± 1.36	−0.06 ± 1.96	−0.42 ± 1.03
	L8	−2.97 ± 0.37			0.48 ± 1.13	0.13 ± 0.59	−0.32 ± 1.8	0.01 ± 0.65	−1 ± 0.88			
	Air	−2.97 ± 1.29	−1.73 ± 2.38	−3.2 ± 0.73	−2.06 ± 0.52							

presented in Table 3. Under high O_2 concentrations (H4 and H8), simultaneously, an initial increase in oxygen levels and a decrease in carbon dioxide levels were observed, the latter likely due to the dissolution of carbon dioxide into the food product. Under all tested conditions, oxygen content decreased and carbon dioxide content subsequently increased after several days of storage at the time of progressing microbial growth (see 3.3.).

3.2. pH and color

The evolution of pH and color variables L*, a* and b* is presented in Table 3. Throughout storage time, pH was 6.33 ± 0.12, 6.57 ± 0.16, 6.53 ± 0.17, 6.68 ± 0.15 and 6.70 ± 0.05 under the conditions H4, H8, L4, L8 and Air, respectively. In addition to some increase in yellowness (b*) as a function of time under MAP conditions, differences in color values were mostly not detected over time or between different storage conditions.

3.3. Microbiological analysis

Results of the microbiological analysis are presented in Fig. 1. Generally, more rapid growth was observed on all tested media under air when compared to the MAP conditions at the respective storage temperature (4 °C). Initially (day 0), high TPC (Fig. 1A) were typically enumerated on MA. The limit of 7.0 log CFU/g was exceeded after 2 days under air storage at 4 °C, whereas at both MAP conditions this limit was reached within 2 days at 8 °C and 4 days at 4 °C. Under low O_2 concentrations (L4 and L8), stationary phase was reached after 4 days at 8 °C and 8 days at 4 °C, which closely coincides with the total depletion of oxygen from the package headspace (Table 3). TPC of cod packaged under MAP was typically 0.5–1 log higher on MA than on IAL (Fig. 1A–B).

LAB enumerated on MRS (Fig. 1C) were able to grow especially well at 8 °C. Oxygen concentration had little effect on LAB growth under MAs until complete depletion from the headspace. Respective enumerations were obtained on modified MRS (results not shown). On the other hand, growth of H_2S producers (Fig. 1D) was promoted by low oxygen concentrations. Their growth was highly similar to LAB under low oxygen concentrations (L4 and L8), whereas stationary growth was observed after 4 days under H8 and six days under H4. Under Air, H_2S producers reached higher levels than LAB.

Pseudomonas spp. growth (Fig. 1E) was favored by storage under air and effectively inhibited by elevated carbon dioxide concentrations (60%). The initial level of *B. thermosphacta* (Fig. 1F) was between 2.5 and 4 log CFU/g and increased by at least 2.5 log CFU/g during storage under all tested conditions.

3.4. Quantification of VOCs

The VOC concentrations determined by SIFT-MS exceeding the LOQ and having relative standard deviation below 25% (Supplementary Table 1) are presented in Figs. 2–4 as a function of TPC enumerated on MA. In addition to these compounds, acetone exceeded the LOQ under L8 (104 μg/m^3 on day 7) and ammonia under L4 (9.0 μg/m^3 on day 13). When the LOQ was not exceeded, concentration was marked as 0 in Figs. 2–4.

The differences between the blank averages and the LOQ (Supplementary Table 1) could be attributed to the deviation between blanks. In most cases, concentration of a certain VOC was constant or slightly increasing throughout storage in the blanks. However, ethanol concentration increased in the MAP blanks by a

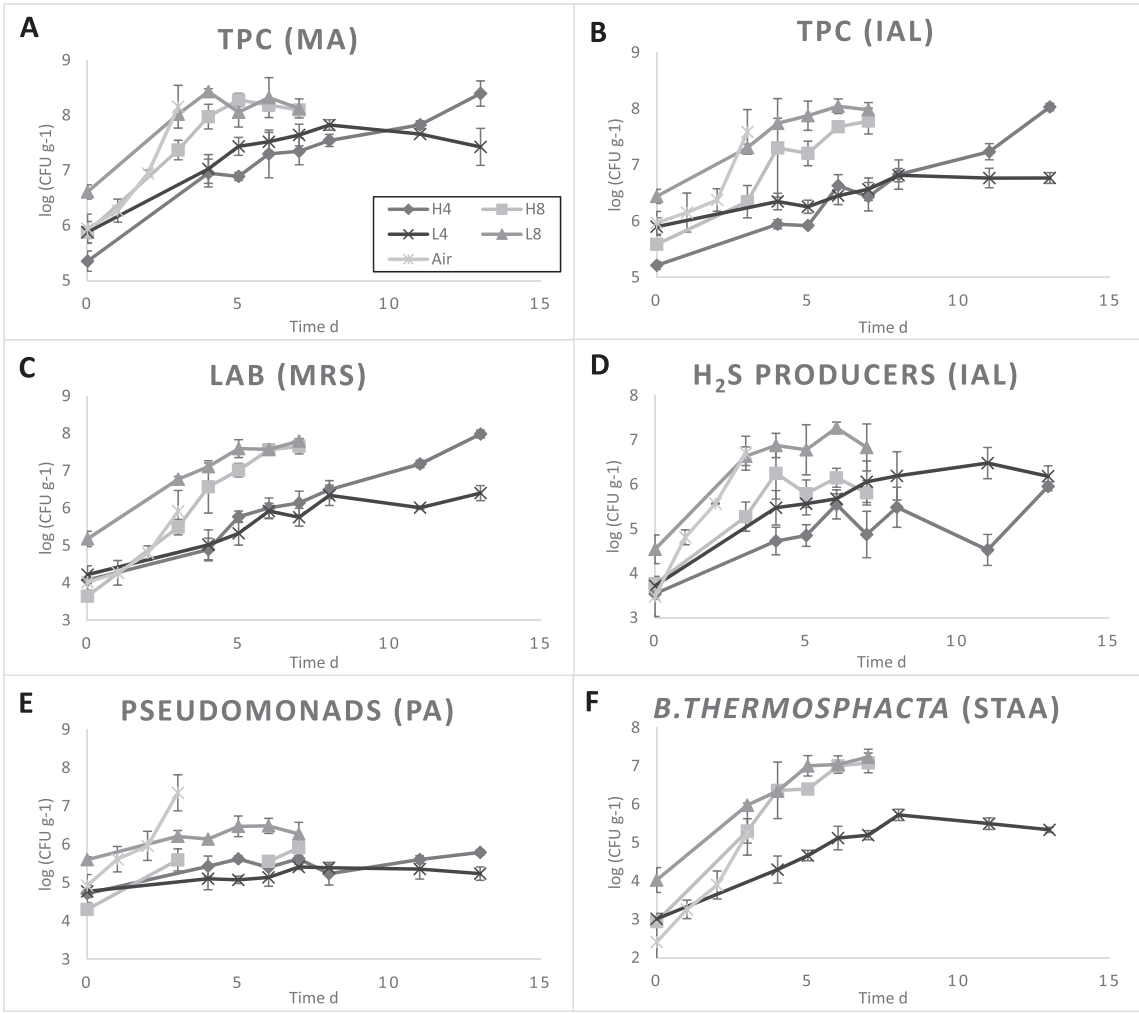

Fig. 1. Counts of total viable psychrotrophic bacteria (A–B), lactic acid bacteria (C), H_2S producers (D), pseudomonads (E) and *Brochothrix thermosphacta* (F) in Atlantic cod fillet portions stored under conditions H4 (60% CO_2/40 & O_2/0% N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C).

factor of 1000 or more by the end of storage.

3.4.1. Alcohols

Levels of alcohols in the package headspace are presented in Fig. 2. Ethanol, 3-methyl-1-butanol, isobutyl alcohol and 2,3-butanediol eventually exceeded the LOQ under most of the tested conditions. Ethanol yielded higher concentrations than the other studied compounds. However, a high initial ethanol concentration and increasing trend as a function of storage time were typically also detected in the blanks, leading to high LOQs that were only exceeded under high O_2 conditions. Concentrations of 3-methyl-1-butanol and 2,3-butanediol started to increase as 7.0 log CFU g^{-1} TPC was exceeded and reached up to 500 μg m^{-3}. Evolution of these compounds was similar under all tested MAP conditions, whereas lower quantities were produced under air. On the other hand, isobutyl alcohol was produced in low quantities and primarily under L4.

3.4.2. Ketones, esters and acids

During refrigerated storage, two ketones (2-pentanone and acetoin), two esters (ethyl acetate and ethyl propanoate) and one acid (acetic acid) were analyzed. Due to high relative standard deviations and/or LOQs, only ethyl acetate, acetic acid and 2-pentanone were quantified (Fig. 3). Increase of ethyl acetate concentration followed a similar trend under every tested condition, whereas other compounds did not exceed the LOQ under all conditions and remained below 200 μg m^{-3} throughout storage. Under air storage, only ethyl acetate exceeded the LOQ, whereas acetic acid was primarily quantified under low O_2 concentrations.

3.4.3. Amine compounds

Of all tested amine compounds, only trimethylamine (TMA) concentrations increased above the LOQ during storage (Fig. 4). At a certain level of microbial growth, higher concentrations of TMA

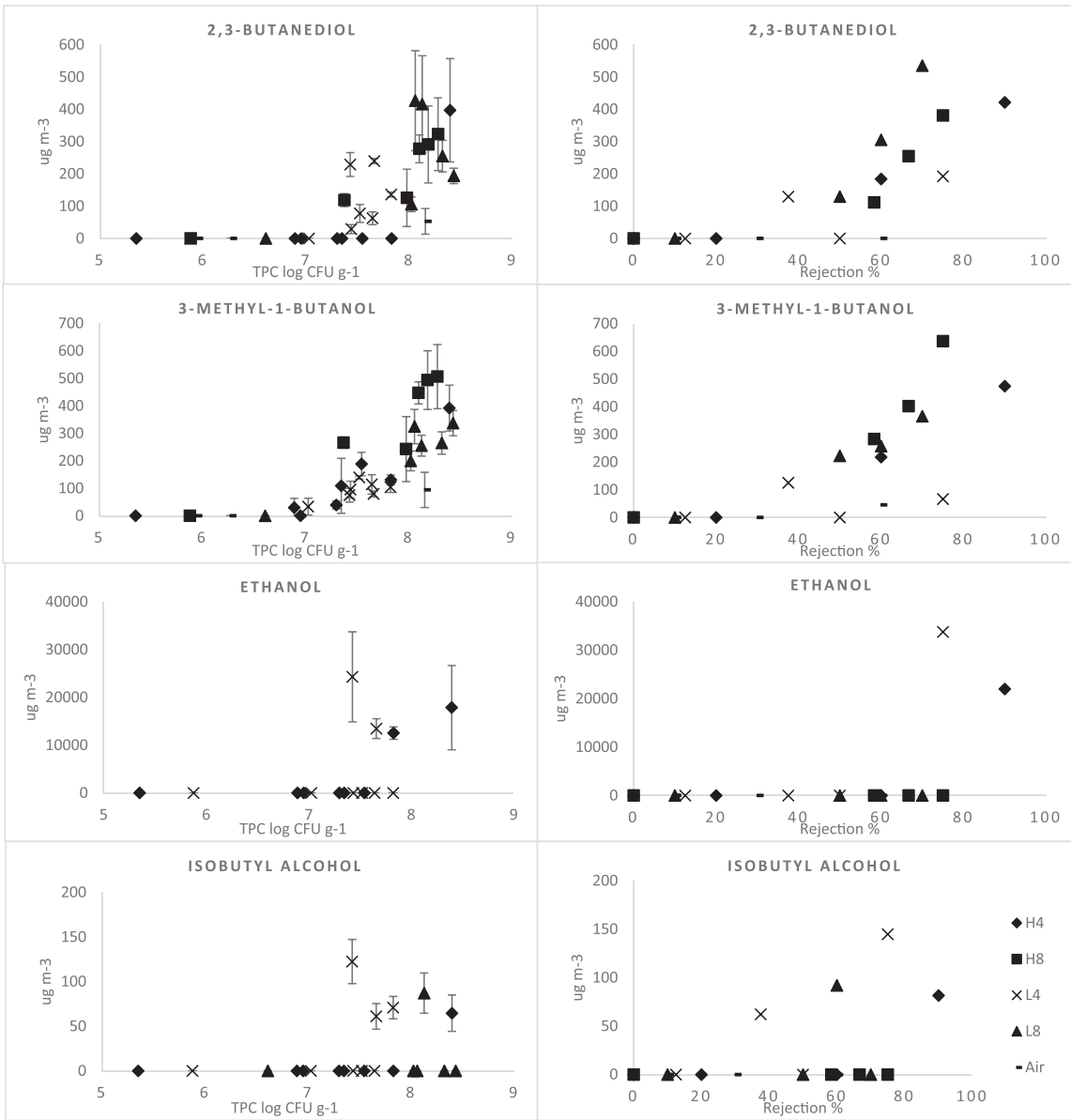

Fig. 2. Concentrations (μg m^{-3}) of alcohols quantified by SIFT-MS as a function of total viable psychrotrophic counts (TPC) or sensory rejection % in Atlantic cod fillet portions stored under conditions H4 (60% CO_2/40 & O_2/0% N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C).

were produced under low O_2 concentrations than under high O_2 or air. Under low O_2 concentrations at 4 °C (L4), some high concentrations were quantified at relatively low microbial levels. This happened during the late days of storage when TPC was decreasing.

3.4.4. Sulfur compounds

The time evolution of the sulfur compounds is presented in Fig. 4. Dimethyl disulfide and hydrogen sulfide had a relative standard deviation over 25%. Relative standard deviation of methyl mercaptan was below 25% only under condition L8. Dimethyl sulfide (DMS) was typically quantified at low microbial levels (TPC < 7 log CFU g^{-1}) and was the only sulfuric compound to exceed LOQ under air. Concentrations of DMS did often remain relatively stable throughout storage. Under air or low O_2 MAP, higher concentrations were detected than under high O_2 MAP at a respective level of microbial growth.

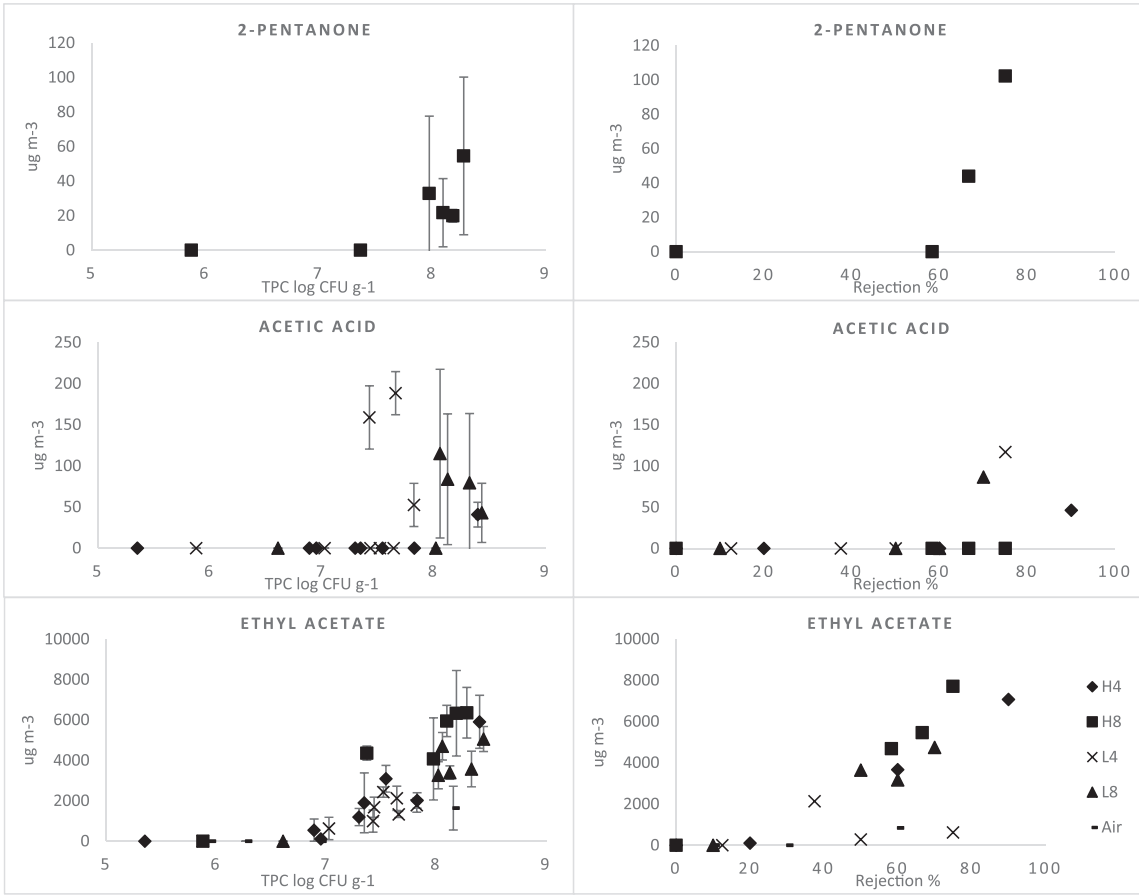

Fig. 3. Concentrations (μg m^{-3}) of ketones, esters and acids quantified by SIFT-MS as a function of total viable psychrotrophic counts (TPC) or sensory rejection % in Atlantic cod fillet portions stored under conditions H4 (60% CO_2/40 & O_2/0% N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C).

3.5. Sensory evaluation

Figs. 2–4 present the individual VOC concentrations as a function of sensory rejection (%). A sample was considered rejected if labelled as marginal or spoiled. The onset of VOC concentration increase typically coincided with approximately 25% rejection, irrespective of the identity of the VOC. At ≥ 50% rejection, TPC enumerated on MA was generally over 7.5 log.

Friedman and LSD tests were used for analyzing significant differences among the ranking data. The Friedman test indicated no significant differences ($\alpha = 0.05$) between samples from different days of storage under Air or in the second test of L4, which is why subsequent LSD tests were not carried out. On the basis of LSD tests (Fig. 5), significant differences ($\alpha = 0.05$) between samples indicated perceivable change in product quality. Under conditions H4, H8, L4 and L8, a change in olfactory quality was observed approximately between days 6–8, 3–5, 4–8 and 3–5, respectively. These changes closely coincide with 50% rejection (Figs. 2–4).

3.6. 16S rRNA gene sequencing

Rarefaction curves of samples from intermediate to late days of storage commonly showed trends to level off (Supplementary Fig. 1), indicating appropriate sampling depth for most of these samples. Even though relatively high species diversity was estimated in samples from early stages of storage (Supplementary Table 2), the low read counts were likely insufficient for appropriate sampling of diversity. Alpha diversity analysis indicated that ACE-1 (Chao and Lee, 1992) was the only stable richness estimator for the studied dataset (Supplementary Table 3). Under modified atmospheres, the ACE-1 index suggested that community richness increased during the early days of storage and decreased during the late days, respectively. However, within 95% confidence intervals, this was only observed under condition H8. On the other hand, diversity indices showed that community diversity was highest in the beginning of storage (day 0) under all tested conditions (Supplementary Table 4). Diversity was lowest during intermediate storage under MA conditions and in the end of storage under air.

After data processing, 503 OTUs were retained at the 97% sequence identity threshold and a high variation in the number of reads was observed between samples (Supplementary Table 2). The relative distribution of the eight most abundant genera is presented in Fig. 6. Initial microbiota (day 0) were generally diverse under all tested conditions. Even though *Acinetobacter*, *Flavobacterium*,

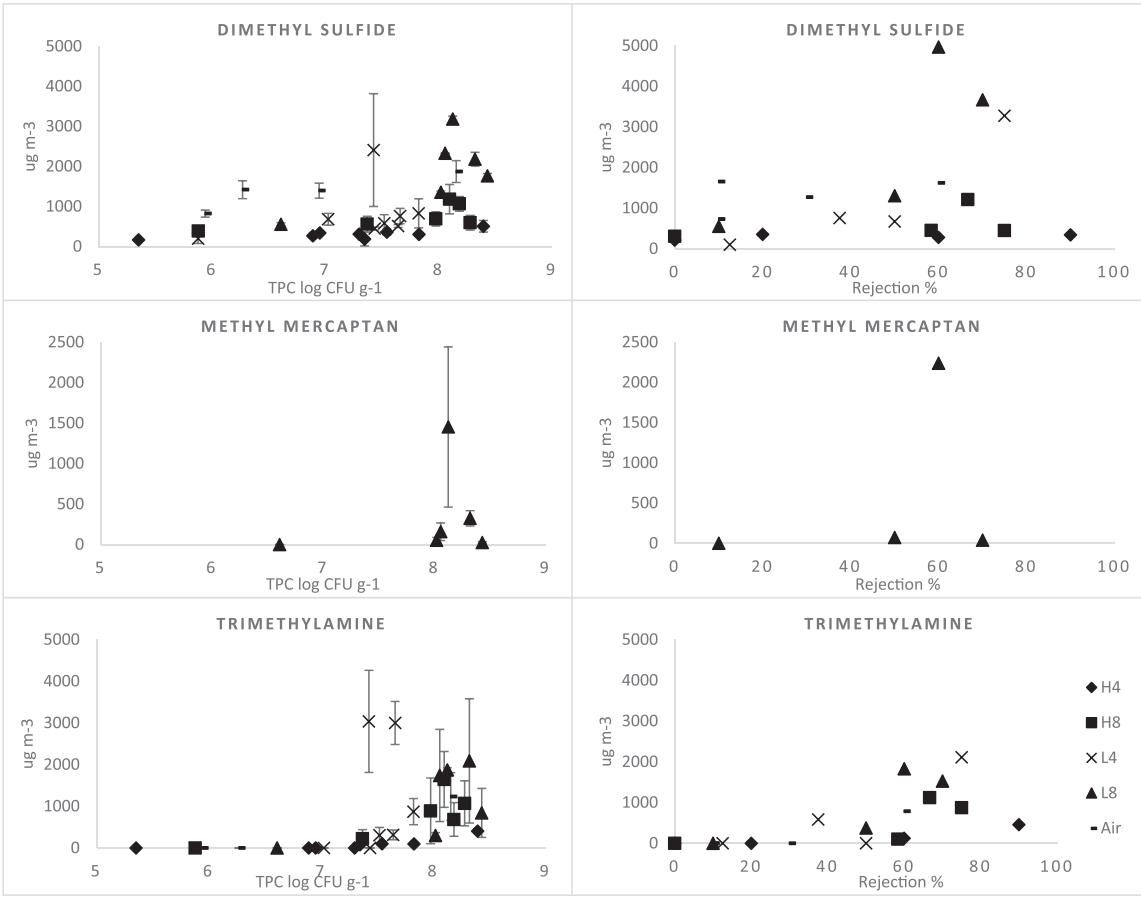

Fig. 4. Concentrations (μg m^{-3}) of amines and sulfur compounds quantified by SIFT-MS as a function of total viable psychrotrophic counts (TPC) or sensory rejection % in Atlantic cod fillet portions stored under conditions H4 (60% CO_2/40 & O_2/0% N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C).

Photobacterium, *Pseudomonas* and *Psychrobacter* were the most abundant genera on day 0, their proportion of the total microbiota was relatively small. However, under condition L8, *Psychrobacter* and *Flavobacterium* were dominating and a relatively high proportion of *Photobacterium* was detected under H8.

The *Photobacterium* genus became dominant in relative abundance over storage time under all tested conditions. Initially, *Photobacterium* formed ca. 30% of the total microbiota under H8 and less than 15% of under H4, L4, L8 and Air. On the later days of storage, over 88% was detected under both MAP conditions at 4 °C. Under MA conditions, the relative abundance of *Photobacterium* was highest during intermediate storage and decreased to some extent by the end of storage, thus increasing community diversity (Supplementary Table 4). At higher storage temperature (8 °C), *Photobacterium* decreased from 78 to 60% under H8 and 65 to 46% under L8: under these conditions, *Acinetobacter*, *Brochothrix* and *Carnobacterium* were also able to grow. However, a lower number of reads was also obtained from day 7 samples when compared to day 4 samples under these conditions. Under air, 70 and 86% of *Photobacterium* was detected on days 2 and 3 of storage.

4. Discussion

Growth of SSOs is dependent on the packaging and storage conditions. *Photobacterium phosphoreum* has been identified as an SSO of marine fish under elevated CO_2 concentrations (Dalgaard et al., 1997a, 1997b; Gram and Dalgaard, 2002; Leroi, 2010). In the present study, the *Photobacterium* genus became indeed dominating under all tested MAP conditions. Since *P. phosphoreum* and *P. iliopiscicarium* are able to grow on MA (Broekaert et al., 2011), the results suggest that TPC enumerated on MA reflects the growth of this genus. Furthermore, it was observed that ca. 7 log CFU/g is needed for the onset of exponential VOC increase and ca. 7.5 log CFU/g for 50% rejection. The results are in line with Dalgaard et al. (1997a) for cod fillets stored under 60/40/0 and 60/0/40 (CO_2/O_2/N_2) at 0 °C. The results thus suggest that representatives of the *Photobacterium* genus contribute to the increase in VOC concentrations and sensory rejection and that the onset of exponential VOC increase can be observed at relatively high microbial levels.

When stored under air, *Pseudomonas* and *Shewanella* spp. have commonly been considered as SSOs of refrigerated or iced marine fish (Gram and Huss, 1996; Gram and Dalgaard, 2002). Parlapani et al. (2015a) observed *Pseudomonas* and H_2S producers to be

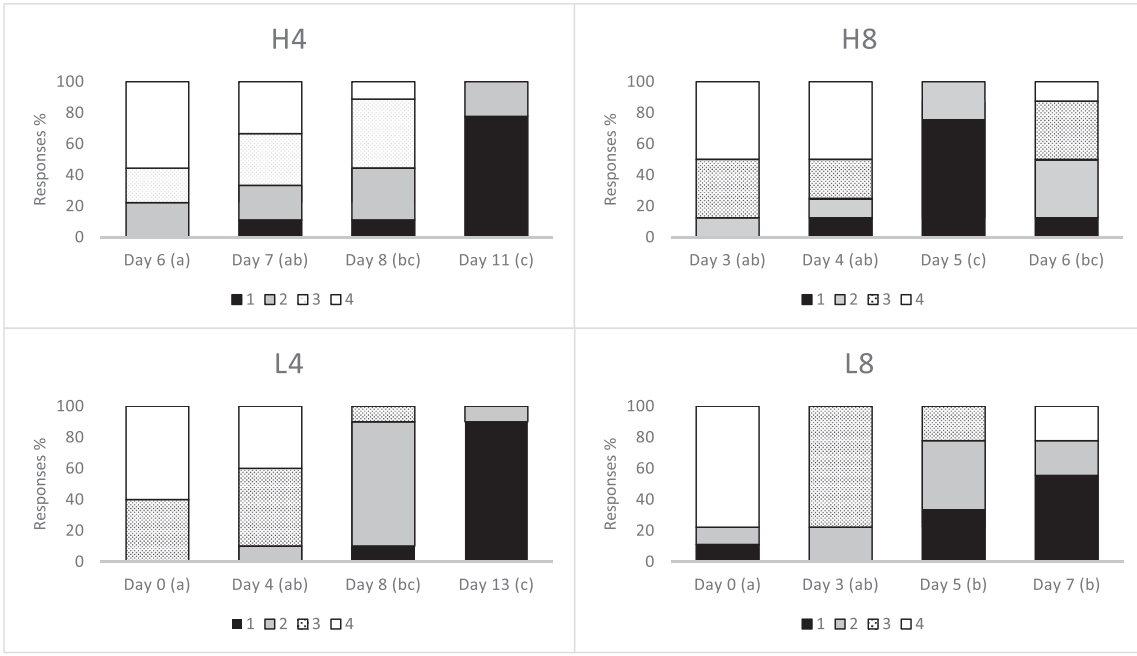

Fig. 5. Ranks (1 = least fresh, 4 = most fresh) assigned to cod fillet samples from four different days of storage under conditions H4 (60% CO_2/40 & O_2/0% N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C) and L8 (60/5/35 8 °C). Storage days with different postscripts (a–c) within a condition are significantly different ($p < 0.05$).

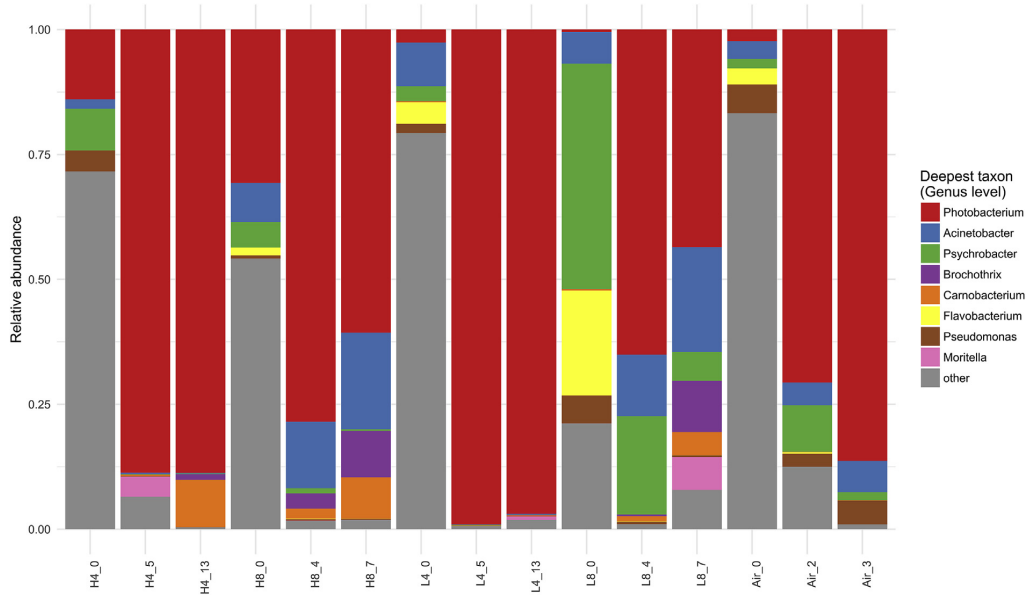

Fig. 6. Composition of microbiota in Atlantic cod fillet portions stored under conditions H4 (60% CO_2/40 & O_2/0% N_2 at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C), determined by amplicon (NGS) sequencing of the 16S rRNA gene.

dominating in sea bass stored both under air and MAP (60/10/30% CO_2/O_2/N_2); under MAP, LAB and *B. thermosphacta* were observed to be co-dominating. Respectively, both enumeration (Fig. 1) and sequencing (Fig. 6) results of the present study indicate *Pseudomonas* growth under air and inhibition under MAP. Under MA conditions, high carbon dioxide concentrations are known to

inhibit pseudomonads (Gram and Huss, 1996).

Enumeration of *Photobacterium* can be affected by the properties of the growth media. Broekaert et al. (2011) identified MA to be more suitable for the enumeration of marine bacteria than IAL. In the present study, the difference typically observed between TPC enumerated on MA and IAL likely reflects the dominance of the *Photobacterium* genus. In the beginning of storage (day 0), highly similar results are obtained on both media, whereas higher counts are generally enumerated on MA on later days of storage (Fig. 1). According to the oligotyping results (Fig. 6), respectively, the *Photobacterium* genus typically forms a small fraction of the initial microbiota and majority at later stages of storage. Since *P. phosphoreum* is sensitive to heat, pour plating temperatures (<50 °C) have been suggested to lead into underestimation of its growth (Dalgaard et al., 1997a). Incubation temperature of 23–25 °C or higher has also been suggested to inhibit *P. phosphoreum* growth (Dalgaard et al., 1997a); however, similar enumeration results were obtained in the present study on MA incubated at 22 or 15 °C (results not shown).

Elevated CO_2 concentrations have been reported to favor the growth of CO_2 tolerant LAB (Gram and Dalgaard, 2002; Leroi, 2010). Analogously, the present enumeration results on MRS suggests that facultative anaerobic LAB were able to grow under both MAP conditions. Even though acetate-containing MRS has been reported to inhibit certain LAB such as carnobacteria (Leroi, 2010), comparative enumeration on MRS and mMRS resulted in highly similar CFU levels (results not shown). According to the sequencing results (Fig. 6), the relative abundance of carnobacteria was higher under MA conditions when compared to storage under air. High CO_2 concentration also had an inhibitive effect on H_2S producers under MAP when compared to air storage, especially under high O_2 conditions. An additional inhibitive effect of O_2 was also observed by López-Caballero et al. (2001), which was suggested to be due to synergistic effect between the gases.

An increase in concentrations of several alcohols was detected in the present study. Respectively, ethanol, 3-methyl-1-butanol and 2,3-butanediol have frequently been identified as potential spoilage indicators of marine fish under air and/or MAP in several studies (Duflos et al., 2006; Mikš-Krajnik et al., 2016; Olafsdottir et al., 2005; Parlapani et al., 2014, 2015a, 2015b). Olafsdottir et al. (2005) observed increasing concentrations of ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 2,3-butanediol for aerobically stored cod fillets at 0.5 °C. Ethanol and 2-methyl-1-propanol were suggested to have importance in early detection of spoilage despite non-continuous increase. Duflos et al. (2006) found several alcohols including ethanol, 3-methyl-1-butanol and 2,3-butanediol to increase in cod, mackerel and whiting stored under vacuum at 4 °C for ten days. Production of different alcohols has been associated with several microbial species among LAB, *Shewanella*, *Pseudomonas*, *P. phosphoreum* and *B. thermosphacta* (Casaburi et al., 2015; Hernández-Macedo et al., 2012; Noseda et al., 2012). In the present study, the dominance of *Photobacterium* suggests that the production of alcohols could be largely attributed to this genus.

The H_2S concentrations remained low under all tested conditions. Low H_2S production by *P. phosphoreum* has been observed in other studies (Dalgaard et al., 1993). In Danish marine fish, *Shewanella baltica* has been identified as the main H_2S producer (Vogel et al., 2005). Even though *S. putrefaciens* has high spoilage potential due to the production of intensive off-odors, high levels (8 log CFU/g) are needed for off-odor production (Dalgaard, 1995). The present results thus support the conclusion that significant VOC production can only be observed at relatively high microbial levels.

TMA is produced by bacteria that utilize trimethylamine oxide (TMAO) for anaerobic respiration and results in ammonia-like or “fishy” odors characteristic for spoiled marine fish (Gram and Dalgaard, 2002). Oxygen has been observed to inhibit the reduction of TMAO into TMA as well as to reduce the growth of TMA-producing *P. phosphoreum* (Boskou and Debevere, 1997; Dalgaard et al., 1997a). This is in line with the results of the present study. Since TMA concentration was notably higher at 50% rejection than its human olfactory threshold (OT) 6 μg m^{-3} (Devos et al., 1990), TMA was likely to contribute to the rejection of the samples.

Even though VOCs are often produced in low quantities, their effect on the perceived quality of the fish can be significant if they have low OTs. Alcohols have generally high OTs, whereas sulfur and amine compounds often become detectable at very low quantities (Devos et al., 1990). However, OTs are commonly determined for single compounds from a continuous airflow. Furthermore, OT values indicate the lowest quantity of a VOC that can be perceived by the panelists, instead of indicating whether it is considered acceptable. Acceptance of an odor may depend on cultural, social and economic aspects, as well as the characteristics of the food product. Since olfactory evaluation of fish freshness is based on the overall smell, OTs and acceptability of VOCs are likely dependent on the composition of the whole VOC profile. Instead of using single compounds for quality and spoilage evaluation of fish, multiple-compound quality indices have shown promising potential (Jørgensen et al., 2001).

In the present study, the concentration of several VOCs increased as a function of microbial growth. Under most of the tested conditions, increase in 2,3-butanediol, ethanol, ethyl acetate, 3-methyl-1-butanol and trimethylamine were observed. All these compounds have been recognized as fish spoilage metabolites (Duflos et al., 2006; Olafsdóttir et al., 1997). For example, ethanol, ethyl acetate and/or 3-methyl-1-butanol have also been associated with the spoilage of several non-seafood products packaged under modified atmospheres (Casaburi et al., 2015; Nieminen et al., 2016; Zhang et al., 2013a, 2013b): monitoring of such compounds could enhance the applicability of an intelligent packaging solution into a wider range of food products. Since the *Photobacterium* genus was highly abundant under all storage conditions, differences in its metabolism could contribute to the observed differences in the VOC profiles between the tested storage conditions. Onset of exponential concentration increase was typically observed between TPC 7–7.5 log CFU/g and 25–50% rejection. Respectively, late increase of VOC concentrations in relation to microbial growth has also been detected in other studies (Olafsdottir et al., 2005). Detection of early spoilage thus requires that low concentrations of relevant VOCs can be detected.

5. Conclusions

Different packaging and storage conditions affect the evolution of fish microbiota and the generated VOCs in the package headspace. In the present study, the SIFT-MS technology allowed the real-time quantification of VOCs directly from the package headspace. This approach eliminated the need of sample preparation procedures, while allowing fast and sensitive analysis of the VOC profile over storage time. The obtained results directly represent the quality deterioration of fish and thus the reality that a sensor needs to be able to respond to during storage.

Packaging and storage conditions affect the evolution of the VOC profile and should be considered in the selection of spoilage indicators. In the present study, increase in 2,3-butanediol, ethanol, ethyl acetate, 3-methyl-1-butanol and trimethylamine concentrations during storage suggests that these compounds could be used in detecting spoilage of raw Atlantic cod. However, since VOC concentrations typically remain at low quantities even at the late stage of storage, detection of early spoilage calls for sensitivity for low concentration ranges.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2017.10.011.

References

Alfaro, B., Hernández, I., Baliño-Zuazo, L., Barranco, A., 2013. Quality changes of atlantic horse mackerel fillets (trachurus trachurus) packed in a modified atmosphere at different storage temperatures. J. Sci. Food Agric. 93 (9), 2179–2187. https://doi.org/10.1002/jsfa.6025.

Baixas-Nogueras, S., Bover-Cid, S., Vidal-Carou, M., Veciana-Nogués, M.T., Mariné-Font, A., 2001. Trimethylamine and total volatile basic nitrogen determination by flow Injection/Gas diffusion in mediterranean hake (merluccius merluccius). J. Agric. Food Chem. 49 (4), 1681–1686. https://doi.org/10.1021/jf000649n.

Béné, A., Hayman, A., Reynard, E., Luisier, J.L., Villettaz, J.C., 2001. A new method for the rapid determination of volatile substances: the SPME-direct method: Part II. determination of the freshness of fish. Sensors Actuators B Chem. 72 (3), 204–207. https://doi.org/10.1016/S0925-4005(00)00652-3.

Bhadra, S., Narvaez, C., Thomson, D.J., Bridges, G.E., 2015. Non-destructive detection of fish spoilage using a wireless basic volatile sensor. Talanta 134, 718–723. http://doi.org/10.1016/j.talanta.2014.12.017.

Boskou, G., Debevere, J., 1997. Reduction of trimethylamine oxide byShewanellaspp. under modified atmosphieresin vitro. Food Microbiol. 14 (6), 543–553. https://doi.org/10.1006/fmic.1997.0132.

Broekaert, K., Heyndrickx, M., Herman, L., Devlieghere, F., Vlaemynck, G., 2011. Seafood quality analysis: molecular identification of dominant microbiota after ice storage on several general growth media. Food Microbiol. 28 (6), 1162–1169. https://doi.org/10.1016/j.fm.2011.03.009.

Carrapiso, A.I., Noseda, B., García, C., Reina, R., Sánchez del Pulgar, J., Devlieghere, F., 2015. SIFT-MS analysis of iberian hams from pigs reared under different conditions. Meat Sci. 104, 8–13. https://doi.org/10.1016/j.meatsci.2015.01.012.

Casaburi, A., Piombino, P., Nychas, G., Villani, F., Ercolini, D., 2015. Bacterial populations and the volatilome associated to meat spoilage. Food Microbiol. 45 (Part A), 83–102. https://doi.org/10.1016/j.fm.2014.02.002.

Castada, H.Z., Wick, C., Harper, W.J., Barringer, S., 2015. Headspace quantification of pure and aqueous solutions of binary mixtures of key volatile organic compounds in swiss cheeses using selected ion flow tube mass spectrometry. Rapid Commun. Mass Spectrom. RCM 29 (1), 81–90. https://doi.org/10.1002/rcm.7089.

Castada, H.Z., Wick, C., Taylor, K., Harper, W.J., 2014. Analysis of selected volatile organic compounds in split and nonsplit swiss cheese samples using selected-ion flow tube mass spectrometry (SIFT-MS). J. Food Sci. 79 (4), C489–C498. https://doi.org/10.1111/1750-3841.12418.

Chao, A., 1984. Nonparametric estimation of the number of classes in a population. Scand. J. Statistics 11 (4), 265–270.

Chao, A., Bunge, J., 2002. Estimating the number of species in a stochastic abundance model. Biometrics 58 (3), 531–539. https://doi.org/10.1111/j.0006-341X.2002.00531.x.

Chao, A., Lee, S., 1992. Estimating the number of classes via sample coverage. J. Am. Stat. Assoc. 87 (417), 210–217. https://doi.org/10.1080/01621459.1992.10475194.

Chung, W., Le, G.T., Tran, T.V., Nguyen, N.H., 2017. Novel proximal fish freshness monitoring using batteryless smart sensor tag. Sensors Actuators B Chem. http://doi.org/10.1016/j.snb.2017.01.134.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., et al., 2009. The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37 (Suppl. 1), D141–D145. https://doi.org/10.1093/nar/gkn879.

Dalgaard, P., Mejlholm, O., Christiansen, T.J., Huss, H.H., 1997a. Importance of photobacterium phosphoreum in relation to spoilage of modified atmosphere-packed fish products. Lett. Appl. Microbiol. 24 (5), 373–378. https://doi.org/10.1046/j.1472-765X.1997.00152.x.

Dalgaard, P., 1995. Qualitative and quantitative characterization of spoilage bacteria from packed fish. Int. J. Food Microbiol. 26 (3), 319–333. https://doi.org/10.1016/0168-1605(94)00137-U.

Dalgaard, P., Gram, L., Huss, H.H., 1993. Spoilage and shelf-life of cod fillets packed in vacuum or modified atmospheres. Int. J. Food Microbiol. 19 (4), 283–294. https://doi.org/10.1016/0168-1605(93)90020-H.

Dalgaard, P., Mejlholm, O., Huss, H.H., 1997b. Application of an iterative approach for development of a microbial model predicting the shelf-life of packed fish. Int. J. Food Microbiol. 38 (2–3), 169–179. https://doi.org/10.1016/S0168-1605(97)00101-3.

Debevere, J., Boskou, G., 1996. Effect of modified atmosphere packaging on the TVB/TMA-producing microflora of cod fillets. Int. J. Food Microbiol. 31 (1–3), 221–229. https://doi.org/10.1016/0168-1605(96)01001-X.

Devos, M., Patte, F., Rouault, J., Laffort, P., Van Gemert, L.J., 1990. In: Devos, M., Patte, F., Rouault, J., Laffort, P., Van Gemert, L.J. (Eds.), Standardized Human Olfactory Thresholds. Oxford University Press, New York, US.

De Vrieze, J., Coma, M., Debeuckelaere, M., Van der Meeren, P., Rabaey, K., 2016. High salinity in molasses wastewaters shifts anaerobic digestion to carboxylate production. Water Res. 98, 293–301. https://doi.org/10.1016/j.watres.2016.04.035.

Duflos, G., Leduc, F., N'Guessan, A., Krzewinski, F., Kol, O., Malle, P., 2010. Freshness characterisation of whiting (merlangius merlangus) using an SPME/GC/MS method and a statistical multivariate approach. J. Sci. Food Agric. 90 (15), 2568–2575. https://doi.org/10.1002/jsfa.4122.

Duflos, G., Coin, V.M., Cornu, M., Antinelli, J., Malle, P., 2006. Determination of volatile compounds to characterize fish spoilage using headspace/mass spectrometry and solid-phase microextraction/gas chromatography/mass spectrometry. J. Sci. Food Agric. 86 (4), 600–611. https://doi.org/10.1002/jsfa.2386.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27 (16), 2194–2200. https://doi.org/10.1093/bioinformatics/btr381.

Edirisinghe, R.K.B., Graffham, A.J., Taylor, S.J., 2007. Characterisation of the volatiles of yellowfin tuna (thunnus albacares) during storage by solid phase microextraction and GC?MS and their relationship to fish quality parameters. Int. J. Food Sci. Technol. 42 (10), 1139–1147. https://doi.org/10.1111/j.1365-2621.2006.01224.x.

Efremenko, Y., Mirsky, V.M., 2017. Virtual sensor array consisting of a single sensor element with variable affinity: an application for analysis of fish freshness. Sensors Actuators B Chem. 241, 652–657. http://doi.org/10.1016/j.snb.2016.10.126.

Fernández-Segovia, I., Escriche, I., Gómez-Sintes, M., Fuentes, A., Serra, J.A., 2006. Influence of different preservation treatments on the volatile fraction of desalted cod. Food Chem. 98 (3), 473–482. https://doi.org/10.1016/j.foodchem.2005.06.021.

García, M.R., Cabo, M.L., Herrera, J.R., Ramilo-Fernández, G., Alonso, A.A., Balsa-Canto, E., 2017. Smart sensor to predict retail fresh fish quality under ice storage. J. Food Eng. 197, 87–97. http://doi.org/10.1016/j.jfoodeng.2016.11.006.

Ghaani, M., Cozzolino, C.A., Castelli, G., Farris, S., 2016. An overview of the intelligent packaging technologies in the food sector. Trends Food Sci. Technol. 51, 1–11. http://doi.org/10.1016/j.tifs.2016.02.008.

Gram, L., Dalgaard, P., 2002. Fish spoilage bacteria – problems and solutions. Curr. Opin. Biotechnol. 13 (3), 262–266. https://doi.org/10.1016/S0958-1669(02)00309-9.

Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products. Int. J. Food Microbiol. 33 (1), 121–137. https://doi.org/10.1016/0168-1605(96)01134-8.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M., 2002. Food spoilage—interactions between food spoilage bacteria. Int. J. Food Microbiol. 78 (1–2), 79–97. https://doi.org/10.1016/S0168-1605(02)00233-7.

Gram, L., Trolle, G., Huss, H.H., 1987. Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. Int. J. Food Microbiol. 4 (1), 65–72. https://doi.org/10.1016/0168-1605(87)90060-2.

Grimm, C.C., Lloyd, S.W., Batista, R., Zimba, P.V., 2000. Using microwave distillation-solid-phase Microextraction—gas Chromatography—mass spectrometry for analyzing fish tissue. J. Chromatogr. Sci. 38 (7), 289–296. https://doi.org/10.1093/chromsci/38.7.289.

Hernández-Macedo, M.L., Contreras-Castillo, C.J., Tsai, S.M., Da Cruz, S.H., Sarantopoulos, C.I.G.L., Padula, M., Dias, C.T.S., 2012. Gases and volatile compounds associated with micro-organisms in blown pack spoilage of brazilian vacuum-packed beef. Lett. Appl. Microbiol. 55 (6), 467–475. https://doi.org/10.1111/lam.12004.

ISO, 2006. Sensory Analysis-methodology-ranking (ISO Standard No. 8587:2006).

Jaffrès, E., Lalanne, V., Macé, S., Cornet, J., Cardinal, M., Sérot, T., et al., 2011. Sensory characteristics of spoilage and volatile compounds associated with bacteria isolated from cooked and peeled tropical shrimps using SPME–GC–MS analysis. Int. J. Food Microbiol. 147 (3), 195–202. https://doi.org/10.1016/j.ijfoodmicro.2011.04.008.

Jónsdóttir, R., Ólafsdóttir, G., Chanie, E., Haugen, J., 2008. Volatile compounds suitable for rapid detection as quality indicators of cold smoked salmon (salmo salar). Food Chem. 109 (1), 184–195. https://doi.org/10.1016/j.foodchem.2007.12.006.

Jørgensen, L.V., Huss, H.H., Dalgaard, P., 2001. Significance of volatile compounds produced by spoilage bacteria in vacuum-packed cold-smoked salmon (salmo salar) analyzed by GC-MS and multivariate regression. J. Agric. Food Chem. 49 (5), 2376–2381. https://doi.org/10.1021/jf0009908.

Kerry, J.P., O'Grady, M.N., Hogan, S.A., 2006. Past, current and potential utilisation of

active and intelligent packaging systems for meat and muscle-based products: a review. Meat Sci. 74 (1), 113–130. https://doi.org/10.1016/j.meatsci.2006.04.024.
Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120. https://doi.org/10.1128/AEM.01043-13.
Langford, V.S., Reed, C.J., Milligan, D.B., McEwan, M.J., Barringer, S.A., Harper, W.J., 2012. Headspace analysis of Italian and New Zealand parmesan cheeses. J. Food Sci. 77 (6), C719–C726. https://doi.org/10.1111/j.1750-3841.2012.02730.x.
Leduc, F., Krzewinski, F., Le Fur, B., N'Guessan, A., Malle, P., Kol, O., Duflos, G., 2012. Differentiation of fresh and frozen/thawed fish, european sea bass (dicentrarchus labrax), gilthead seabream (sparus aurata), cod (gadus morhua) and salmon (salmo salar), using volatile compounds by SPME/GC/MS. J. Sci. Food Agric. 92 (12), 2560–2568. https://doi.org/10.1002/jsfa.5673.
Leroi, F., 2010. Occurrence and role of lactic acid bacteria in seafood products. Food Microbiol. 27 (6), 698–709. https://doi.org/10.1016/j.fm.2010.05.016.
López-Caballero, M.E., Sánchez-Fernández, J.A., Moral, A., 2001. Growth and metabolic activity of shewanella putrefaciens maintained under different CO_2 and O_2 concentrations. Int. J. Food Microbiol. 64 (3), 277–287. https://doi.org/10.1016/S0168-1605(00)00473-6.
Macé, S., Joffraud, J., Cardinal, M., Malcheva, M., Cornet, J., Lalanne, V., et al., 2013. Evaluation of the spoilage potential of bacteria isolated from spoiled raw salmon (salmo salar) fillets stored under modified atmosphere packaging. Int. J. Food Microbiol. 160 (3), 227–238. https://doi.org/10.1016/j.ijfoodmicro.2012.10.013.
Mikš-Krajnik, M., Yoon, Y., Ukuku, D.O., Yuk, H., 2016. Volatile chemical spoilage indexes of raw atlantic salmon (salmo salar) stored under aerobic condition in relation to microbiological and sensory shelf lives. Food Microbiol. 53 (Part B), 182–191. https://doi.org/10.1016/j.fm.2015.10.001.
Miseq sop. (12th October 2016). Retrieved from http://www.mothur.org/wiki/MiSeq_SOP.
Mocak, J., Bond, A.M., Mitchell, S., Scollary, G., 1997. A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of detection and quantification: application to voltammetric and stripping techniques (technical report). Pure Appl. Chem. 69 (2), 297–328. https://doi.org/10.1351/pac199769020297.
Morsy, M.K., Zór, K., Kostesha, N., Alstrøm, T.S., Heiskanen, A., El-Tanahi, H., et al., 2016. Development and validation of a colorimetric sensor array for fish spoilage monitoring. Food Control 60, 346–352. https://doi.org/10.1016/j.foodcont.2015.07.038.
Natale, C.D., Olafsdottir, G., Einarsson, S., Martinelli, E., Paolesse, R., D'Amico, A., 2001. Comparison and integration of different electronic noses for freshness evaluation of cod-fish fillets. Sensors Actuators B Chem. 77 (1–2), 572–578. https://doi.org/10.1016/S0925-4005(01)00692-X.
Nieminen, T.T., Dalgaard, P., Björkroth, J., 2016. Volatile organic compounds and photobacterium phosphoreum associated with spoilage of modified-atmosphere-packaged raw pork. Int. J. Food Microbiol. 218, 86–95. https://doi.org/10.1016/j.ijfoodmicro.2015.11.003.
Noseda, B., Islam, M.T., Eriksson, M., Heyndrickx, M., De Reu, K., Van Langenhove, H., Devlieghere, F., 2012. Microbiological spoilage of vacuum and modified atmosphere packaged vietnamese pangasius hypophthalmus fillets. Food Microbiol. 30 (2), 408–419. https://doi.org/10.1016/j.fm.2011.12.025.
Noseda, B., Ragaert, P., Pauwels, D., Anthierens, T., Van Langenhove, H., Dewulf, J., Devlieghere, F., 2010. Validation of selective ion flow tube mass spectrometry for fast quantification of volatile bases produced on atlantic cod (gadus morhua). J. Agric. Food Chem. 58 (9), 5213–5219. https://doi.org/10.1021/jf904129j.
Nuin, M., Alfaro, B., Cruz, Z., Argarate, N., George, S., Le Marc, Y., et al., 2008. Modelling spoilage of fresh turbot and evaluation of a time–temperature integrator (TTI) label under fluctuating temperature. Int. J. Food Microbiol. 127 (3), 193–199. https://doi.org/10.1016/j.ijfoodmicro.2008.04.010.
Olafsdóttir, G., Martinsdóttir, E., Oehlenschläger, J., Dalgaard, P., Jensen, B., Undeland, I., et al., 1997. Methods to evaluate fish freshness in research and industry. Trends Food Sci. Technol. 8 (8), 258–265. https://doi.org/10.1016/S0924-2244(97)01049-2.
Olafsdottir, G., Jonsdottir, R., Lauzon, H.L., Luten, J., Kristbergsson, K., 2005. Characterization of volatile compounds in chilled cod (gadus morhua) fillets by gas chromatography and detection of quality indicators by an electronic nose. J. Agric. Food Chem. 53 (26), 10140–10147. https://doi.org/10.1021/jf0517804.
Olivares, A., Dryahina, K., Španěl, P., Flores, M., 2012. Rapid detection of lipid oxidation in beef muscle packed under modified atmosphere by measuring volatile organic compounds using SIFT-MS. Food Chem. 135 (3), 1801–1808. https://doi.org/10.1016/j.foodchem.2012.06.075.
Pacquit, A., Frisby, J., Diamond, D., Lau, K.T., Farrell, A., Quilty, B., Diamond, D., 2007. Development of a smart packaging for the monitoring of fish spoilage. Food Chem. 102 (2), 466–470. https://doi.org/10.1016/j.foodchem.2006.05.052.
Pacquit, A., Lau, K.T., McLaughlin, H., Frisby, J., Quilty, B., Diamond, D., 2006. Development of a volatile amine sensor for the monitoring of fish spoilage. Talanta 69 (2), 515–520. https://doi.org/10.1016/j.talanta.2005.10.046.
Parlapani, F.F., Haroutounian, S.A., Nychas, G.E., Boziaris, I.S., 2015a. Microbiological spoilage and volatiles production of gutted european sea bass stored under air and commercial modified atmosphere package at 2 °C. Food Microbiol. 50, 44–53. https://doi.org/10.1016/j.fm.2015.03.006.
Parlapani, F.F., Mallouchos, A., Haroutounian, S.A., Boziaris, I.S., 2014. Microbiological spoilage and investigation of volatile profile during storage of sea bream fillets under various conditions. Int. J. Food Microbiol. 189, 153–163. https://doi.org/10.1016/j.ijfoodmicro.2014.08.006.
Parlapani, F.F., Verdos, G.I., Haroutounian, S.A., Boziaris, I.S., 2015b. The dynamics of pseudomonas and volatilome during the spoilage of gutted sea bream stored at 2 °C. Food Control 55, 257–265. https://doi.org/10.1016/j.foodcont.2015.03.004.
Perera, A., Pardo, A., Barrettino, D., Hierlermann, A., Marco, S., 2010. Evaluation of fish spoilage by means of a single metal oxide sensor under temperature modulation. Sensors Actuators B Chem. 146 (2), 477–482. http://doi.org/10.1016/j.snb.2009.11.037.
Pielou, E.C., 1966. The measurement of diversity in different types of biological collections. J. Theor. Biol. 13, 131–144. https://doi.org/10.1016/0022-5193(66)90013-0.
Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35 (21), 7188–7196. https://doi.org/10.1093/nar/gkm864.
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75 (23), 7537–7541. https://doi.org/10.1128/AEM.01541-09.
Shannon, C.E., Weaver, W., 1949. The Mathematical Theory of Communication. The University of Illinois Press, Urbana.
Simpson, E.H., 1949. Measurement of diversity. Nature 163, 688, 688.
Sivertsvik, M., Jeksrud, W.K., Rosnes, J.T., 2002. A review of modified atmosphere packaging of fish and fishery products ? significance of microbial growth, activities and safety. Int. J. Food Sci. Technol. 37 (2), 107–127. https://doi.org/10.1046/j.1365-2621.2002.00548.x.
Soncin, S., Chiesa, L.M., Panseri, S., Biondi, P., Cantoni, C., 2009. Determination of volatile compounds of precooked prawn (penaeus vannamei) and cultured gilthead sea bream (sparus aurata) stored in ice as possible spoilage markers using solid phase microextraction and gas chromatography/mass spectrometry. J. Sci. Food Agric. 89 (3), 436–442. https://doi.org/10.1002/jsfa.3466.
Stannard, C., 1997. Development and use of microbiological criteria for foods. Food Sci. Technol. Today 11 (3), 137–176.
Vanderroost, M., Ragaert, P., Devlieghere, F., De Meulenaer, B., 2014. Intelligent food packaging: the next generation. Trends Food Sci. Technol. 39 (1), 47–62. https://doi.org/10.1016/j.tifs.2014.06.009.
Vilchez-Vargas, R., Geffers, R., Suárez-Diez, M., Conte, I., Waliczek, A., Kaser, V.S., et al., 2013. Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system. Environ. Microbiol. 15 (4), 1016–1039. https://doi.org/10.1111/j.1462-2920.2012.02752.x.
Vogel, B.F., Venkateswaran, K., Satomi, M., Gram, L., 2005. Identification of shewanella baltica as the most important H2S-producing species during iced storage of Danish marine fish. Appl. Environ. Microbiol. 71 (11), 6689–6697 doi:71/11/6689 [pii].
Xu, Y., Liu, Y., Jiang, C., Zhang, C., Li, X., Zhu, D., Li, J., 2014. Determination of volatile compounds in turbot (psetta maxima) during refrigerated storage by headspace solid-phase microextraction and gas chromatography?mass spectrometry. J. Sci. Food Agric. 94 (12), 2464–2471. https://doi.org/10.1002/jsfa.6581.
Yam, K.L., Takhistov, P.T., Miltz, J., 2005. Intelligent packaging: concepts and applications. J. Food Sci. 70 (1), R1–R10. https://doi.org/10.1111/j.1365-2621.2005.tb09052.x.
Zaragozá, P., Fernández-Segovia, I., Fuentes, A., Vivancos, J., Ros-Lis, J.V., Barat, J.M., Martínez-Máñez, R., 2014. Monitorization of atlantic salmon (salmo salar) spoilage using an optoelectronic nose. Sensors Actuators B Chem. 195, 478–485. https://doi.org/10.1016/j.snb.2014.01.017.
Zhang, B., Samapundo, S., Pothakos, V., de Baenst, I., Sürengil, G., Noseda, B., Devlieghere, F., 2013a. Effect of atmospheres combining high oxygen and carbon dioxide levels on microbial spoilage and sensory quality of fresh-cut pineapple. Postharvest Biol. Technol. 86, 73–84. https://doi.org/10.1016/j.postharvbio.2013.06.019.
Zhang, B., Samapundo, S., Pothakos, V., Sürengil, G., Devlieghere, F., 2013b. Effect of high oxygen and high carbon dioxide atmosphere packaging on the microbial spoilage and shelf-life of fresh-cut honeydew melon. Int. J. Food Microbiol. 166 (3), 378–390. https://doi.org/10.1016/j.ijfoodmicro.2013.08.002.
Zhang, B., Samapundo, S., Rademaker, M., Noseda, B., Denon, Q., de Baenst, I., et al., 2014. Effect of initial headspace oxygen level on growth and volatile metabolite production by the specific spoilage microorganisms of fresh-cut pineapple. LWT - Food Sci. Technol. 55 (1), 224–231. https://doi.org/10.1016/j.lwt.2013.08.018.
Zhang, Z., Li, G., Luo, L., Chen, G., 2010. Study on seafood volatile profile characteristics during storage and its potential use for freshness evaluation by headspace solid phase microextraction coupled with gas chromatography–mass spectrometry. Anal. Chim. Acta 659 (1–2), 151–158. https://doi.org/10.1016/j.aca.2009.11.024.

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MULTIVARIATE STATISTICAL ANALYSIS FOR THE IDENTIFICATION OF POTENTIAL SEAFOOD SPOILAGE INDICATORS

by

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Multivariate statistical analysis for the identification of potential seafood spoilage indicators

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ABSTRACT

Volatile organic compounds (VOCs) characterize the spoilage of seafood packaged under modified atmospheres (MAs) and could thus be used for quality monitoring. However, the VOC profile typically contains numerous multicollinear compounds and depends on the product and storage conditions. Identification of potential spoilage indicators thus calls for multivariate statistics. The aim of the present study was to define suitable statistical methods for this purpose (exploratory analysis) and to consequently characterize the spoilage of brown shrimp (*Crangon crangon*) and Atlantic cod (*Gadus morhua*) stored under different conditions (selective analysis). Hierarchical cluster analysis (HCA), principal components analysis (PCA) and partial least squares regression analysis (PLS) were applied as exploratory techniques (brown shrimp, 4 °C, 50%CO_2/50%N_2) and PLS was further selected for spoilage marker identification. Evolution of acetic acid, 2,3-butanediol, isobutyl alcohol, 3-methyl-1-butanol, dimethyl sulfide, ethyl acetate and trimethylamine was frequently in correspondence with changes in the microbiological quality or sensory rejection. Analysis of these VOCs could thus enhance the detection of seafood spoilage and the development of intelligent packaging technologies.

1. Introduction

Modified atmosphere packaging (MAP) is commonly used for perishable food products such as seafood in order to inhibit or delay microbial growth and thus to extend the shelf life and quality of the packaged product. During microbiological spoilage of foodstuffs, decomposition of available nutrients by microbial activity can lead to the generation of volatile organic compounds (VOCs) associated with both primary and secondary metabolism (Wang, Li, Yang, Ruan, & Sun, 2016). Growth of specific spoilage organisms (SSOs) and subsequent production of off-odors into the package headspace eventually causes consumer rejection (Gram & Dalgaard, 2002). Consequently, odor is considered as one of the most important seafood quality parameters (Olafsdottir, Jonsdottir, Lauzon, Luten, & Kristbergsson, 2005; Olafsdóttir et al., 1997).

Microbial spoilage of fish may manifest itself as sweet, fruity, ammonia-like, putrid and sulfuric off-odors. VOCs contributing to the odor of fish can be divided into three groups, specifying compounds associated with freshness (C_6-C_9 alcohols and carbonyl compounds), lipid oxidation (aldehydes) and microbiological spoilage (Olafsdóttir et al., 1997). According to Olafsdóttir et al. (1997), microbiological spoilage odor is generally due to compounds such as ammonia, ethanol, ethyl acetate, hydrogen sulfide, 3-methyl-1-butanol, methyl mercaptan and trimethylamine. However, the composition and the development of the VOC profile are affected by several factors, including food product, headspace gas composition, temperature, initial contaminating microbiota and microbial metabolism (Wang et al., 2016).

Brown shrimp (*Crangon crangon*) is highly susceptible to

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microbiological spoilage. Shrimp contains high amounts of free amino acids and other readily available nutrients for microbial growth (Zeng, Thorarinsdottir, & Olafsdottir, 2005). Unlike other crustaceans, shrimp cannot be kept alive for extended periods before processing (Adams & Moss, 2008). Currently, the shelf life of preservative-free cooked brown shrimp is maximally 4–6 days under refrigerated conditions (Broekaert, Heyndrickx, Herman, Devlieghere, & Vlaemynck, 2013).

Since microbial activity is the main cause of fish spoilage (Gram & Dalgaard, 2002), identification and quantification of VOCs produced during microbial metabolism under different packaging and storage conditions could enhance efficient quality analysis of the packaged product. Evolution of these spoilage indicators in relation to microbial growth and sensory rejection could be used for the development of intelligent packaging applications. Generally, concentrations of VOCs that indicate spoilage can be expected to increase as a function of storage time and progressing microbial growth. However, VOCs are produced and degraded as a result of several biological and chemical processes. Furthermore, certain odors may be considered as a part of natural odor in one foodstuff and rejected in another product (Gram & Dalgaard, 2002). Thus, the complexity of concentration evolution and acceptancy as well as the wide number of potential spoilage indicators calls for multivariate statistical analysis.

Different statistical methods have been applied to multivariate microbiological and chemical data, including hierarchical cluster analysis (HCA), principal components analysis (PCA) and partial least squares regression analysis (PLS). Previously, PCA has been applied to the comparison of different food products (Blixt & Borch, 2002), microbiota (Hierro et al., 2005; Verginer, Leitner, & Berg, 2010), treatments (Ciesa et al., 2013) or times of storage (Duflos et al., 2010; Fik, Surówka, Maciejaszek, Macura, & Michalczyk, 2012). PLS has been used for the analysis of progressing microbial growth on the basis of VOC concentrations (Jørgensen, Huss, & Dalgaard, 2001; Marín et al., 2007; Storer, Hibbard-Melles, Davis, & Scotter, 2011) and also applied along with HCA or PCA (Argyri, Doulgeraki, Blana, Panagou, & Nychas, 2011; Argyri, Mallouchos, Panagou, & Nychas, 2015; Blixt & Borch, 2002; Mataragas, Skandamis, Nychas, & Drosinos, 2007; Mikš-Krajnik, Yoon, Ukuku, & Yuk, 2016; Siroli et al., 2014; Vervoort et al., 2012; Wibowo, Grauwet, Gedefa, Hendrickx, & Van Loey, 2015).

The aims of the present study were to 1) determine suitable multivariate statistical methods for characterizing the VOC profile of seafood (exploratory analysis) and 2) consequently identify the most potential spoilage indicators of Atlantic cod (*Gadus morhua*) and brown shrimp stored under different modified atmosphere (MA) conditions (selective analysis). Firstly, HCA, PCA and PLS were applied as exploratory techniques to microbiological, chemical and/or sensory data. Comparison of the three techniques was carried out using a dataset collected during refrigerated storage of seafood (brown shrimp, 4 °C, 50%CO_2/50%N_2) where selected-ion flow-tube mass spectrometry (SIFT-MS) was used for the quantification of VOCs from the package headspace. On the basis of the exploratory analysis, PLS was chosen to be used in selective analysis. Independent PLS analyses were carried out for data collected during spoilage of Atlantic cod (Kuuliala et al. submitted manuscript) and brown shrimp under different packaging and storage conditions.

2. Materials and methods

2.1. Data collection

The datasets used in the study were collected during individual storage experiments of brown shrimp (2×) or Atlantic cod (5×) and used for exploratory (brown shrimp, 4 °C, 50%CO_2/50%N_2) or selective (all storage experiments) statistical analyses.

2.1.1. Brown shrimp

The two individual storage experiments of brown shrimp consisted of sample preparation and packaging, real-time quantification of VOCs with SIFT-MS, microbiological analysis and sensory evaluation.

2.1.1.1. Raw material. Brown shrimp were caught in the North Atlantic Ocean (FAO zone 27) in October and November 2015. The shrimp were sorted according to size and washed before cooking according to normal Belgian fishing practices. No additives or preservatives such as benzoic or sorbic acid were added during processing. After cooking, the shrimp were cooled and stored overnight in plastic bags under ice. The shrimp were brought onshore the following morning and directly transported to the Laboratory of Food Microbiology and Food Preservation (LFMFP, UGent) where the batch was hand peeled. During peeling, shrimp were kept on ice in plastic bags while avoiding direct contact between shrimp and ice. Shrimp portions of 150 ± 2 g were packaged at 2:1 headspace-product ratio with a tray sealer (MECA 900, DecaTechnic, Herentals, Belgium) using multilayer packaging trays (PP/EVOH/PP, oxygen transmission rate 0.03 cm^3/tray*24 h at 23 °C and 50% R.H.) and top film (PA/EVOH/PA/PP, oxygen transmission rate 6.57 cm^3/m^2*24 h*atm at 23 °C, 50% R.H. and 1 atm). Two individual batches of shrimp were independently packaged under modified atmospheres ($CO_2/O_2/N_2$%) 50/0/50 or 30/0/70 and stored at (4.0 ± 0.7) °C prior to analyses. Analyses were carried out on days 0 (day of packaging), 3, 5, 7, 10 and 12 for three randomly chosen packages (A-C). New replicates A-C were analyzed on each day of storage due to the destructive nature of the microbiological analyses. After sampling, the remaining shrimp was packaged under vacuum using high barrier film bags (oxygen transmission rate < 2.7 cm^3/m^2*24 h*bar at 23 °C and 0% R.H.) and stored at −32 °C for no longer than 70 days.

2.1.1.2. Quantification of spoilage related VOCs by SIFT-MS. The principles of selected-ion flow-tube mass spectrometry have been described in previous studies (Noseda et al., 2010). VOCs (Table 1) were selected on the basis of previous research and literature and quantified from the package headspace by a spectrometer (Voice 200, Syft Technologies™, Christchurch, New Zealand). Package headspace was sampled through a septum inserted on the package lid with a flow rate of 25.6 ml/min for 60 s (preparation 10 s, sample 50 s) and the concentrations were averaged over eleven data points. A certain package was sampled twice. During sampling, the headspace was connected to atmospheric air with a needle inlet in order to avoid collapse and changes in the internal conditions of the package.

The relative standard deviation ($SD_{\%}$) of each VOC concentration during an individual SIFT-MS scan was calculated as follows:

$$SD_{\%} = SD_m/x_m*100\% \quad (1)$$

where x_m is the average and SD_m the standard deviation of a single SIFT-MS scan (n = 11). VOCs with concentrations exceeding 25% average $SD_{\%}$ during the entire storage time within a certain packaging condition were considered not to allow sufficiently accurate quantification and were thus excluded from further analyses.

2.1.1.3. Microbiological analysis. Each shrimp sample of 30 ± 0.1 g was aseptically weighed into a sterile stomacher bag, diluted ten times in physiological saline peptone solution (PPS; 0.85% NaCl, 0.1% peptone) and homogenized in Stomacher Lab Blender (LED

Table 1
Product ions of volatile organic compounds (VOCs) quantified with SIFT-MS from the headspace of brown shrimp samples, respective mass to charge ratios (m/z), branching ratios (b) and reaction rate coefficients (k).

VOC	Code	Precursor ion	m/z	b (%)	k	Production
Acids						
Acetic acid	C1	NO^+	90	100	9.0 E-10	$NO^+ \cdot CH_3COOH$
		NO^+	108		9.0 E-10	$NO^+ \cdot CH_3COOH \cdot H_2O$
Alcohols						
2,3-butanediol	C2	H_3O^+	91	100	3.0 E-09	$C_4H_{10}O_2^+ \cdot H^+$
		NO^+	89	100	2.3 E-09	$C_4H_9O_2^+$
2-propanol	C3	H_3O^+	43	80	2.7 E-09	$C_3H_7^+$
3-methyl-1-butanol	C4	H_3O^+	71	100	2.8 E-09	$C_5H_{11}^+$
		NO^+	87	85	2.3 E-09	$C_5H_{11}O^+$
Ethanol	C5	H_3O^+	47	100	2.7 E-09	$C_2H_7O^+$
		H_3O^+	65			$C_2H_7O^+ \cdot H_2O$
		H_3O^+	83			$C_2H_7O^+ \cdot (H_2O)_2$
Isobutyl alcohol	C6	H_3O^+	57	100	2.7 E-09	$C_4H_9^+$
		NO^+	73	95	2.4 E-09	$C_4H_9O^+$
		O_2^+	33	50	2.5 E-09	CH_5O^+
Ketones						
Acetone	C7	H_3O^+	59	100	3.9 E-09	C_3H_7O+
		NO^+	88	100	1.2 E-09	$NO^+ \cdot C_3H_6O$
Acetoin	C8	O_2^+	88	20	2.5 E-09	$C_4H_8O_2^+$
Butanone	C9	NO^+	102	100	2.8 E-09	$NO^+ \cdot C_4H_8O$
2-pentanone	C10	H_3O^+	87	100	3.9 E-09	$C_5H_{11}O^+$
		H_3O^+	105		3.9 E-09	$C_5H_{11}O^+ \cdot H_2O$
		NO^+	116	100	3.1 E-09	$NO^+ \cdot C_5H_{10}O^+$
Sulfur compounds						
Hydrogen sulfide	C11	H_3O^+	35	100	1.6 E-09	H_3S^+
		H_3O^+	53		1.6 E-09	$H_3S^+ \cdot H_2O$
		O_2^+	34	100	1.4 E-09	H_2S^+
Carbon disulfide	C12	O_2^+	76	100	7.0 E-10	CS_2^+
Dimethyl sulfide	C13	NO^+	62	100	2.2 E-09	$(CH_3)_2S^+$
Dimethyl disulfide	C14	H_3O^+	95	100	2.6 E-09	$(CH_3)_2S_2 \cdot H^+$
		NO^+	94	100	2.4 E-09	$(CH_3)_2S_2^+$
Methyl mercaptan	C15	H_3O^+	49	100	1.8 E-09	$CH_4S \cdot H^+$
		H_3O^+	67		1.8 E-09	$CH_4S.H^+ \cdot H_2O$
Esters						
Ethyl acetate	C16	NO^+	118	90	2.1 E-09	$NO^+ \cdot CH_3COOC_2H_5$
		O_2^+	31	20	2.4 E-09	CH_3O^+
Amines						
Ammonia	C17	H_3O^+	18	100	2.6 E-09	NH_4^+
		H_3O^+	36		2.6 E-09	$NH_4^+ \cdot H_2O$
		O_2^+	17	100	2.4 E-09	NH_3^+
Dimethylamine	C18	H_3O^+	46	100	2.1 E-09	$(CH_3)_2N \cdot H^+$
Trimethylamine	C19	H_3O^+	58	10	2.0 E-09	$C_3H_8N^+$
		H_3O^+	60	90	2.0 E-09	$(CH_3)_3N \cdot H^+$
Others						
Ethylene oxide	C20	NO^+	74	100	1.0 E-10	$C_2H_4O \cdot NO^+$

Techno, Heusden-Zolder, Belgium) for 1 min. Appropriate decimal dilutions were prepared in PPS. Total psychrotrophic count (TPC) was determined on Marine Agar (MA; Difco Le Pont de Claix, France) spread plates after incubation at 22 °C for five days.

2.1.1.4. Sensory evaluation. Sensory evaluation was performed in individual booths under red light (UGent Sensolab). A panel having experience in sensory evaluation of fish was formed from the laboratory staff at LFMFP. For both independent shrimp batches, two testing sessions with eight to ten panelists were organized on consecutive days. During both sessions, four shrimp samples from different days of storage were evaluated. One out of three daily replicates (A-C) was randomly selected and used per testing session. Prior to evaluation, the frozen (−32 °C) samples were cut to 5.0 ± 0.1 g portions and stored overnight at 2 °C. The samples were presented to the assessors at 4 °C in odor-free, transparent plastic cups (diameter 67 mm; AVA, Temse, Belgium), closed with lids (AVA) and labelled with three-digit random codes, along with a fresh reference (day 0) from the same batch. A five-point scale (very good, good, satisfactory, marginal, spoiled) was used in the olfactory evaluation. Marginal or spoiled was considered as rejection.

2.1.2. Atlantic cod

Atlantic cod data collected during storage under modified atmospheres (% $CO_2/O_2/N_2$) 60/40/0 and 60/5/35 at (4.0 ± 0.7) or (8.0 ± 0.4) °C and air at (4.0 ± 0.7) (Kuuliala et al. submitted manuscript) was used in the study. The VOC data was processed correspondingly to brown shrimp (see 2.1.1.3). VOCs with concentrations exceeding 25% average relative standard deviation during the entire storage time within a certain packaging condition were excluded from further analyses.

2.2. Exploratory analysis

Exploratory analysis techniques were applied to data collected during the storage of brown shrimp under modified atmosphere 50/0/50 (% $CO_2/O_2/N_2$) at 4 °C.

2.2.1. Hierarchical cluster analysis (HCA)

Agglomerative HCA was used for the analysis of the VOC data. The method is based on the identification of groups among objects (samples or variables) on the basis of similarity in their properties. Samples are clustered on the basis of the similarity in their variable

profiles and variables on the basis of similarity between their patterns. In agglomerative clustering, each object initially represents an individual cluster. The most similar clusters are progressively joined together to larger clusters until one collective cluster is formed (Rendall et al., 2015). N objects are thus processed by N − 1 clustering steps (Almeida, Barbosa, Pais, & Formosinho, 2007). The process depends on how the similarity of objects is assessed (distance) and how new clusters are formed from subclusters (linkage). Euclidean or Manhattan distance measures are commonly used for continuous variables, whereas common linkage methods include single, complete, average, centroid and Ward (Smoliński, Walczak, & Einax, 2002).

HCA was carried out using Euclidean distance and average linkage. Individual replicate packages A-C of each day were treated as samples and individual VOCs as variables. Both measured concentrations (non-transformed values) as well as logarithmic and/or standardized (z-scores) concentrations (transformed values) of the VOCs were used in the analyses. R 3.3.1 (R Core Team, 2016) was used for producing heat maps (clustering of variables) with function pheatmap() from package **pheatmap** (Kolde, 2015) and dendrograms (clustering of samples) with function pvclust() from package **pvclust** (R. Suzuki & Shimodaira, 2015). Approximately unbiased (AU) p-values included in the dendrograms indicate how the clustering is supported by the data: the greater the p-value, the greater the reliability of the clustering (Shimodaira, 2002; Suzuki & Shimodaira, 2006).

2.2.2. Principal components analysis (PCA)

PCA was used for the characterization of VOCs and their evolution during storage. The method can be used for extracting the most important information from a dataset containing several intercorrelated variables by determining a series of new variables (Abdi & Williams, 2010). These principal components (PCs) are linear combinations of the original variables and uncorrelated with each other. The first PC retains most of the total variance of the data and following PCs retain most of each residual variance, respectively (Chen, Li, Ouyang, & Zhao, 2014). PCA can thus be used for simplifying the description of the dataset and for the determination of underlying variables, similarity among samples and correlation among variables (Abdi & Williams, 2010; Mataragas et al., 2007).

Logarithmic and standardized VOCs were used in the analysis. R 3.3.1 was used for producing biplots describing both samples (replicate packages) and variables (VOCs) with function prcomp() from package **stats** (R Core Team, 2013). Suitability for data reduction was analyzed with Bartlett's sphericity test using function bart_spher() and sampling adequacy with Kaiser-Meyer-Olkin (KMO) test (Kaiser, 1970) with function KMOS() from package **REdaS** (Hatzinger, Hornik, Nagel, & Maier, 2014, 2015). KMO test result gives the level of sampling adequacy as marvelous (>0.90), meritorious (>0.80), middling (>0.70), mediocre (>0.60), miserable (>0.50) or unacceptable (<0.50) (Kaiser, 1974).

2.2.3. Partial least squares regression (PLS)

Partial least squares regression analysis (PLS) can be used for modeling one or more response variables (Y) with several predictor variables (X) that can be noisy and highly collinear. On the basis of the original X-variables, new orthogonal variables are defined as linear combinations where the coefficients of the original X-variables are referred to as weights. The new variables are used for modeling the X variables and predicting the Y variables. The part of the data that is not explained by the model is referred to as residuals: high Y residuals indicate insufficient model performance (Wold, Sjöström, & Eriksson, 2001). The influence of an X-variable on the Y-response can be expressed with a Variable Importance in Projection (VIP) coefficient which gives the weighed sum of squares of the PLS weights. The VIP coefficients indicate which X-variables have highest importance in explaining the Y-variance (Farrés, Platikanov, Tsakovski, & Tauler, 2015). Even though high regression coefficients can also be used for determining predictor variables that have high importance on the response, the VIP coefficients summarize the importance of the variable for both Y and X matrices (Wold et al., 2001).

PLS was used for the analysis of predictor and response variables with JMP v. 12 using the NIPALS algorithm and leave-one-out cross validation. Logarithmic and standardized VOCs were used as predictor variables and time, TPC or sensory rejection % as the response variable. Logarithmic transformation of predictor variables was used in order to achieve linear relationship with all response variables. The number of factors was chosen so that the root mean predicted residual sum of squares (PRESS) was at its minimum. VIP values and regression coefficients were determined for all VOCs.

2.3. Selective statistics

Selective statistical analyses were applied to data collected during storage of Atlantic cod and brown shrimp under all tested conditions. On the basis of the exploratory analysis, PLS was chosen for the determination of most potential spoilage indicators. Logarithmic and standardized VOCs were used as predictor variables and TPC or rejection % as the response variable. When using TPC as the response variable, independent packaging conditions were separately analyzed and samples were excluded from the analysis if stationary or declining TPC had been reached. When using rejection % as the response variable, data from all independent packaging conditions per seafood product was used and VOCs were excluded from the analysis if over 25% relative standard deviation was observed under any of the tested conditions. Following selection criteria were used for the spoilage indicators: 1) positive correlation with the dependent variable, 2) VIP >1, and 3) positive regression coefficient. JMP v. 12 was used for all analyses.

3. Results and discussion

The majority of the VOCs had an average relative standard deviation below 25% and were included in the analyses. Six VOCs were excluded from the analyses of brown shrimp under 50% CO_2: 3-methyl-1-butanol, acetoin, 2-pentanone, dimethyl amine, dimethyl disulfide and hydrogen sulfide. Under 30% CO_2, additional excluded VOCs were acetic acid, 2,3-butanediol and isobutyl alcohol. The excluded VOCs correspond well to the compounds that were excluded from the cod data (Table 2). Fluctuation of the concentrations of these VOCs during a SIFT-MS scan did not allow sufficiently accurate quantification and thus excluded them from most potential spoilage indicators.

3.1. Exploratory analysis

3.1.1. Hierarchical cluster analysis (HCA)

Clustering of VOCs produced during the storage of brown shrimp under (% CO_2/O_2/N_2) 50/0/50 at 4 °C is presented as heat maps (Fig. 1) and clustering of samples as dendrograms (Fig. 2). Both Figs. 1 and 2 indicate the similarity of the objects (variables or samples) within the studied dataset as a tree structure. In the heat maps, VOC concentrations are expressed on a color scale representing measured concentrations (Fig. 1A) or their transformed values (Fig. 1B–D). Generally, similarity of objects in the same cluster decreases as smaller clusters are merged into larger ones since objects that are clustered together sooner are more similar than those clustered at a higher distance (Rendall et al., 2015).

Clustering of VOCs was affected by the applied data

Table 2
Most potential spoilage indicators of Atlantic cod (C) and brown shrimp (S) stored under different atmospheres (% $CO_2/O_2/N_2$), determined by PLS regression analysis. TPC or rejection % were used as the dependent variable and VOCs as independent variables.

	TPC							Rejection %	
	C 4 °C 60/40/0	C 8 °C 60/40/0	C 4 °C 60/5/35	C 8 °C 60/5/35	C 4 °C Air	S 4 °C 50/0/50	S 4 °C 30/0/70	C	S
2,3-butanediol	x	x	x	x	x	x	0	x	0
2-methylpropanal						–	–		–
2-pentanone	0	x	0	0	0	0	0	0	0
2-propanol	–	–	–	–	–			–	
3-methyl-1-butanol	x	x	x	x	x	0	0	x	0
3-methylbutanal						–	–		–
Acetic acid	x		x		0	x	0	0	0
Acetoin	0	0	0	0	0	0	0	0	0
Acetone	x				x				
Ammonia						x			
Butanone	–	–	–	–	–			–	
Carbon disulfide	–	–	–	–	–	x		–	
Dimethyl amine	0	0	0	0	0	0	0	0	0
Dimethyl disulfide	0	0	0	0	0	0	0	0	0
Dimethyl sulfide			x	x	x	x	x		x
Dimethyl trisulfide						–	–		–
Ethanol		x							x
Ethyl acetate	x	x	x	x	x	x	x	x	x
Ethyl propanoate	0	0	0	0	0	–	–	0	–
Ethylene oxide	–	–	–	–	–			–	
Hydrogen sulfide	0	0	0	0	0	0	0	0	0
Isobutyl alcohol	x	x		x			0	x	0
Methyl mercaptan	0	0	0		0	x		0	
Trimethyl amine	x	x	x	x	x	x	x	x	x

x: selection criteria (VIP > 1, regression coefficient > 0, positive correlation with dependent variable) were met.
-: VOC was not included in the SIFT-MS analysis.
0: relative standard deviation > 25%.

transformations. When non-logarithmic and non-standardized data was used (Fig. 1A), VOCs were clustered on the basis of their concentration ranges. This highlighted the high differences observed in initial concentration levels as well as in the production of different VOCs during storage time. Ethanol was the only VOC that exceeded 10^4 μg m^{-3}, which is why it dominated the color scale and the analysis of VOC evolution was thus not possible. Even though logarithmic conversion of non-standardized data (Fig. 1C) allowed better separation of VOCs, the variables were still clustered on the basis of concentration ranges and resulted in subclusters containing VOCs from highest (ethanol, ethyl acetate, ethylene oxide, trimethylamine) to lowest (2,3-butanediol, isobutyl alcohol, ammonia) concentrations. An overall increase in several VOC concentrations and separation between early (0–3) and remaining (5–12) days of storage could be observed. Respective average logarithmic TPC were 6.51 ± 0.53 and 7.62 ± 0.24 CFU g^{-1}. On the other hand, clustering of non-logarithmic and standardized data (Fig. 1B) allowed the comparison of VOC evolution since VOC concentrations were presented on the same scale. Several subclusters of VOCs were formed and three main types of VOC patterns were identified on their basis. Firstly, concentration of ten out of fourteen VOCs generally increased as a function of storage time. Secondly, three VOCs (ethyl acetate, ethylene oxide, trimethylamine) reached highest concentrations on days 5–7 and decreased thereafter. In addition, high initial concentrations of butanone led to the formation of a separate cluster that did not show any clear pattern. Respectively, clustering of logarithmic and standardized data (Fig. 1D) emphasized the evolution of VOCs during storage time.

Clustering of samples (Fig. 2) showed that replicate packages of a given day of storage commonly had a short distance and were clustered together at low heights, whereas samples from the earliest and latest days of storage were finally joined at a relatively high distance. The results thus indicate that the VOC profile was usually highly similar between samples from a given day of storage and the most different between samples from the early and late days of storage. AU values were typically high, indicating that the clustering was well supported by the data. Non-logarithmic and non-standardized data (Fig. 2A) separated days 0–7 from days 10–12. Samples from the early days of storage (0–3) were highly similar, which is in good correspondence with the VOC concentration patterns (Fig. 1). Otherwise (Fig. 2B–D), intermediate days (5–7) clustered together with late days (10–12) sooner than with the early days.

The choice of distances and linkages affects the clustering results and depends on the dataset and purpose of application. Different alternatives can be compared during exploratory analysis (Rendall et al., 2015; Smoliński et al., 2002). Euclidean distance or correlation coefficient are most commonly used as distance measures together with different linkages. In the present study, preliminary comparison of different linkages resulted in slightly different dendrograms, whereas highly similar results were obtained when comparing different distances (results not shown). Since different transformations were applied to the VOC data in order to examine the similarity both in terms of values and evolution, Euclidean distance and average linkage were chosen. When clustering is based on average linkage, distance of two objects from separate clusters can be either smaller or larger than the average distance of the clusters, which might lead into under- or overestimating the distance between two objects. Even though using single linkage might avoid this phenomenon, problems caused by outliers and cluster density differences limit the use of this linkage (Almeida et al., 2007).

Concentrations of VOCs that are produced as a result of microbial metabolism can be expected to increase exponentially during the log phase of microbial growth. The three main VOC patterns identified in the present study are analogous to VOC groups observed by Küntzel et al. (2016) during the *in vitro* growth of *Mycobacterium avium* ssp. *paratuberculosis*. VOCs that were

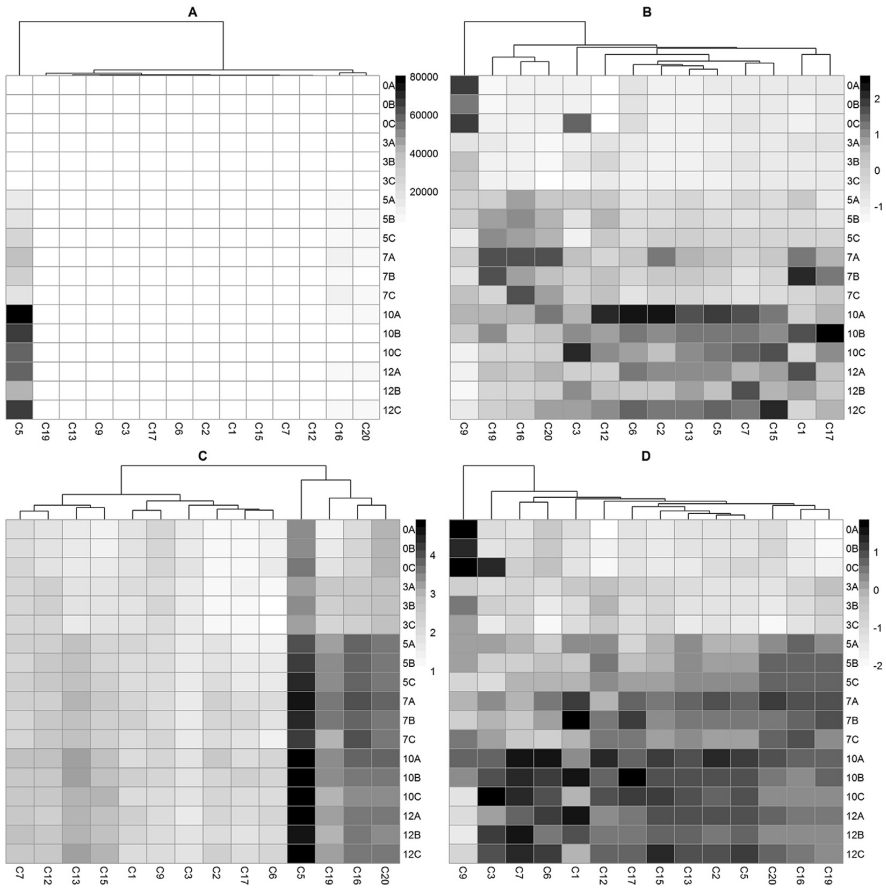

Fig. 1. Hierarchical cluster analysis (HCA) of volatile organic compounds (VOCs) produced during storage of brown shrimp under modified atmosphere (% $CO_2/O_2/N_2$) 50/0/50 at 4 °C. Euclidean distance and average linkage were used for building the heat maps. The columns represent individual VOCs (Table 1) and rows represent shrimp samples labelled with day of storage and replicate A-C. VOCs were analyzed as (A) non-logarithmic and non-standardized, (B) non-logarithmic and standardized, (C) logarithmic and non-standardized and (D) logarithmic and standardized data.

increasing throughout storage time were associated with microbial growth, whereas those that reached a peak during storage were suggested to be produced by microbes and decrease after a change in their metabolism (Küntzel et al., 2016). In the present study, logarithmic transformation supported the monitoring of microbiologically related changes in VOC concentrations and separated the samples below and beyond 7 log TPC, whereas non-logarithmic concentrations emphasized the differences in concentration magnitudes and thus separated the late days of storage from the rest. This indicated not only that exponential increase in VOC concentrations occurred after exceeding 7.0 log TPC, but also that the VOC concentrations were still low at this point when compared to the late days of storage. Development of food monitoring systems should thus be sensitive enough in order to detect the onset of exponential concentration increase.

HCA is often applied in the beginning of exploratory analysis in order to characterize the internal structures within a dataset (Smoliński et al., 2002). In the present study, HCA provided an overview of the VOC profile both in terms of concentration range and evolution. Most of the VOCs were increasing as a function of time and microbial growth, suggesting that these VOCs could be considered as potential spoilage indicators. However, since logarithmic transformation and scaling may emphasize small concentration changes and natural variation in the data, significance of the observed changes should be evaluated prior to statistical analyses.

3.1.2. Principal components analysis (PCA)

Fig. 3 presents the PCA scores and correlation loadings as a two-dimensional biplot where the scores represent samples (independent packages) and correlation loadings indicate the relationships between the individual VOCs. The first two latent variables PC1 and PC2 were linear combinations of the original variables (VOCs) and explained 86.5% of the total variance within the data. Result of the KMO test (0.62) indicated sufficient sampling adequacy and significance of the Bartlett's sphericity test ($p < 2.22^{*}10^{-16} < 0.05$) suitability for data reduction.

Separation between samples from different days of storage could be observed on the biplot (Fig. 3). The closer the samples are located on the biplot, the higher is the similarity between their VOC profiles (Vervoort et al., 2012). Four main groups of samples could be identified in good correspondence with the clustering results (Figs. 1D and 2D): day 0, day 3, days 5–7 and days 10–12. Butanone

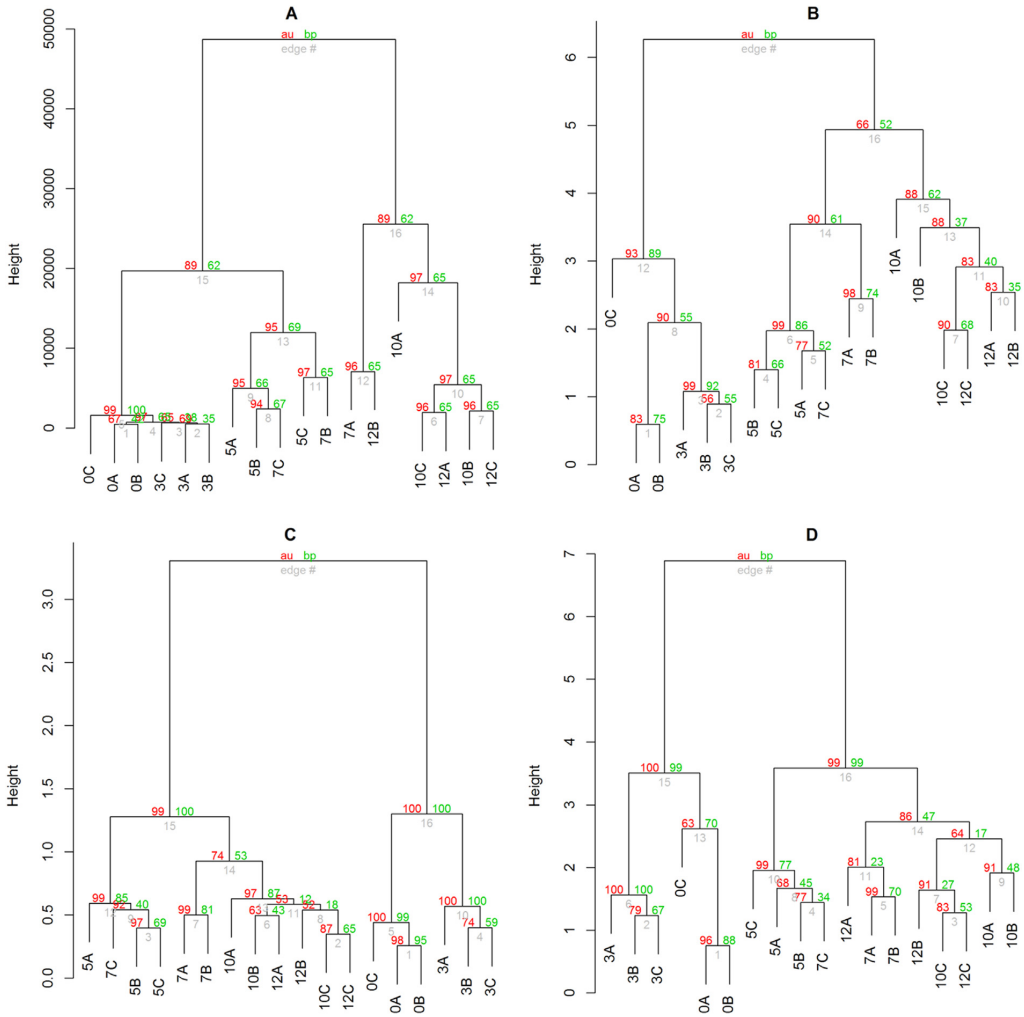

Fig. 2. Hierarchical cluster analysis (HCA) of brown shrimp samples stored under modified atmosphere (% $CO_2/O_2/N_2$) 50/0/50 at 4 °C. Euclidean distance and average linkage were used for building the dendrograms. Approximate unbiased (AU) and bootstrap probability (BP) values are given above the corresponding clusters. The shrimp samples are labelled with day of storage and replicate A-C.

was associated with fresh samples, whereas most of the VOCs were characteristic for late stages of storage. On the other hand, correlation loadings could be used for evaluating correlations between VOCs as well as their occurrence in different samples. Closely located VOCs are highly positively correlated, whereas projection in opposite directions indicates negative correlation. Respectively, VOCs that characterize a certain sample group are closely located to the respective scores (Vervoort et al., 2012). In the present study, the main VOC groups identified by PCA (Fig. 3) corresponded to those determined by hierarchical clustering (Fig. 1D). Most of the VOCs were highly positively correlated and characteristic to the late days of storage. Isobutyl alcohol, 2-propanol and acetone were most closely associated with late storage (days 10–12), whereas ethyl acetate, ethylene oxide and trimethylamine were characteristic to intermediate to late storage due to the decreasing concentrations after day 7. Butanone was negatively correlated with the other VOCs and associated with fresh samples.

The scores of Fig. 3 illustrated an arch-shaped trend. The "horseshoe" is formed when the second axis is distorted in relation to the first axis (Lewis & Menzies, 2015). In the present study, this phenomenon was most likely due to the effect of time. VOC concentrations increased as a function of time, which is why most of the observed variance within the data was caused by progressing time and thus VOC evolution. The first principal component was thus likely related to time, whereas the second principal component had no clear biological interpretation.

3.1.3. *Partial least squares regression (PLS)*

The PLS plots show the correlation between VOCs and time (Fig. 4A), TPC (Fig. 4B) or sensory rejection % (Fig. 4C) and describe both samples (scores) and VOCs (correlation loadings) along with the response variable. When variables are located between the 75

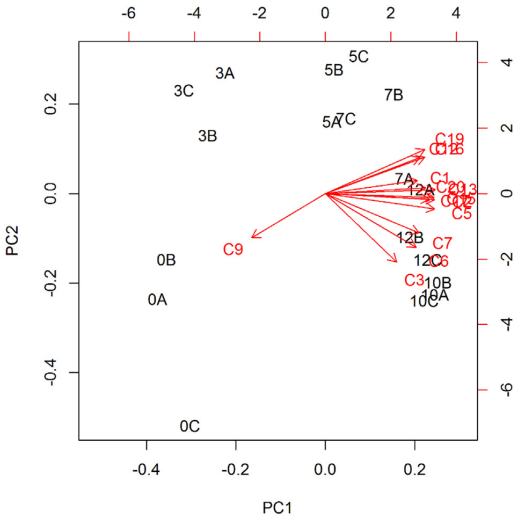

Fig. 3. Principal components analysis (PCA) biplot of brown shrimp stored under modified atmosphere (% $CO_2/O_2/N_2$) 50/0/50 at 4 °C. The shrimp samples (scores) are labelled with day of storage and replicate A-C. The correlation loadings represent individual VOCs.

and 100% circles, more than 75% of their variance is explained by the first two latent variables. The importance of a VOC in explaining the variance in the dataset decreases towards the origin of the biplot (Vervoort et al., 2012). VOCs that are projected away from the origin and towards the response variable are highly positively correlated with the response, whereas projection in opposite direction indicates negative correlation (Vervoort et al., 2012; Wibowo et al., 2015). The respective VIP vs. regression coefficient plots (Fig. 4D–F) show the impact of each VOC on the linear models. VOCs with a high VIP coefficient have high impact on the response variable and regression coefficient indicates whether the impact is positive or negative. In the present study, the PLS biplots were analyzed according to the principles presented for PCA (see 3.1.2).

Most of the analyzed VOCs were close to the time vector (Fig. 4A), indicating that the VOC concentrations were increasing as a function of time. Respectively as observed in the heatmaps (Fig. 1) and PCA biplot (Fig. 3), butanone was negatively correlated with time and associated with day 0 samples. Furthermore, in case of nine out of fourteen VOCs, 75–100% of variance was explained by the first two latent variables, indicating that these VOCs had a strong correlation with time. Acetone and methyl mercaptan were the most positively correlated VOCs with time. The VIP plot (Fig. 4D) identified six out of fourteen VOCs having VIP >1 and a positive regression coefficient: acetone, ammonia, 2,3-butanediol, dimethyl sulfide, ethanol and methyl mercaptan. These VOCs also had positive correlations with time.

Even though most of the studied VOCs were positively correlated with TPC (Fig. 4B), their correlations were typically less positive than between VOCs and time (Fig. 4A). Carbon disulfide, ethyl acetate and trimethylamine had strong positive correlation with TPC, whereas most of the other VOCs had slightly positive correlation with TPC. Respectively, four out of fourteen VOCs had VIP >1 and positive regression coefficients (Fig. 4E): carbon disulfide, dimethyl sulfide, ethyl acetate and trimethylamine. However, since TPC reached stationary phase after day 5, VOCs that showed decreasing concentrations during late storage were highlighted in the respective PLS model. The observed decrease in VOC concentration is likely affected by several reasons independent of microbial growth, such as degradation into other compounds. VOC concentrations cannot thus be directly related to microbial counts after the stationary phase has been reached.

Finally, three main groups of VOCs could be identified in the PLS model for rejection % (Fig. 4C). These VOC groups closely coincided with those observed in the respective heatmap (Fig. 1D). The concentrations of the majority of VOCs had strong positive correlations with panelist rejection. Seven out of fourteen VOCs had VIP >1 and positive regression coefficients: ammonia, 2,3-butanediol, dimethyl sulfide, ethanol, ethyl acetate, ethylene oxide and trimethylamine. Acetone, isobutyl alcohol and 2-propanol had less positive or weaker correlations and butanone a negative correlation with rejection %.

The VIP value 1 has generally been used as a cut-off limit in variable selection: variables exceeding this limit can be considered to be highly influential (Afanador, Tran, & Buydens, 2014; Zaragozá et al., 2014). However, since the VIP approach considers every studied variable, VOCs that have high importance in the model are not necessarily limited to those showing constant increase during storage. This can be observed in Fig. 4D–E where the VIP of butanone nearly exceeded 1 despite its negative correlation with time and TPC (Fig. 4A–B) and negative regression coefficients (Fig. 4D–E). Butanone concentration decreased from 170 to 100 μg m^{-3} during storage, indicating that it is not likely relevant for spoilage analysis. Excluding butanone from the analysis could allow other VOCs to exceed the chosen VIP limit. Selection of variables on the basis of VIP thus gives the most influential VOCs, irrespectively of their impact on the value of the response variable.

PLS is commonly used when numerous highly correlated predictor variables are present (Wold et al., 2001). A positive correlation between a VOC and the response indicates that increase in VOC concentration is associated with increase in the response. However, correlation does not necessarily indicate a relationship between the variables. In the present study, multicollinearity between VOCs could be expected because increase in VOC concentrations was related to microbial growth and likely to the same producer microbes (Kuuliala et al. submitted manuscript). Some VOCs might thus correlate with the response even though no direct relationship existed between them. Furthermore, since correlation does not consider the possible dependencies between VOCs, direct relationships between the variables and the response might be hidden because of suppression. This phenomenon could be due to e.g. degradation or consumption of a VOC during storage. For example, several VOCs had a relatively strong positive correlation with consumer rejection (Fig. 4C), although their regression coefficients were negative (Fig. 4F). This could suggest that increase in their concentrations may depend on another VOCs and/or that they do not contribute to unpleasant off-odors.

In the present study, storage time was extended beyond consumer rejection. After the moment of rejection (day 5), declining TPC and concentrations of certain VOCs were detected. During extended storage, evolution of VOCs produced during microbial metabolism does not necessarily correlate with TPC, which may interfere with the identification of potential spoilage indicators. Analysis of VOC evolution should thus focus on the log phase of microbial growth.

3.2. Selective statistics

The results of the exploratory analyses indicate that an overview of the evolution and relevance of VOCs can be obtained with all the analytical methods applied in the present study. However, especially HCA was also associated with demanding results interpretation. Systematic and facilitated determination of spoilage indicators

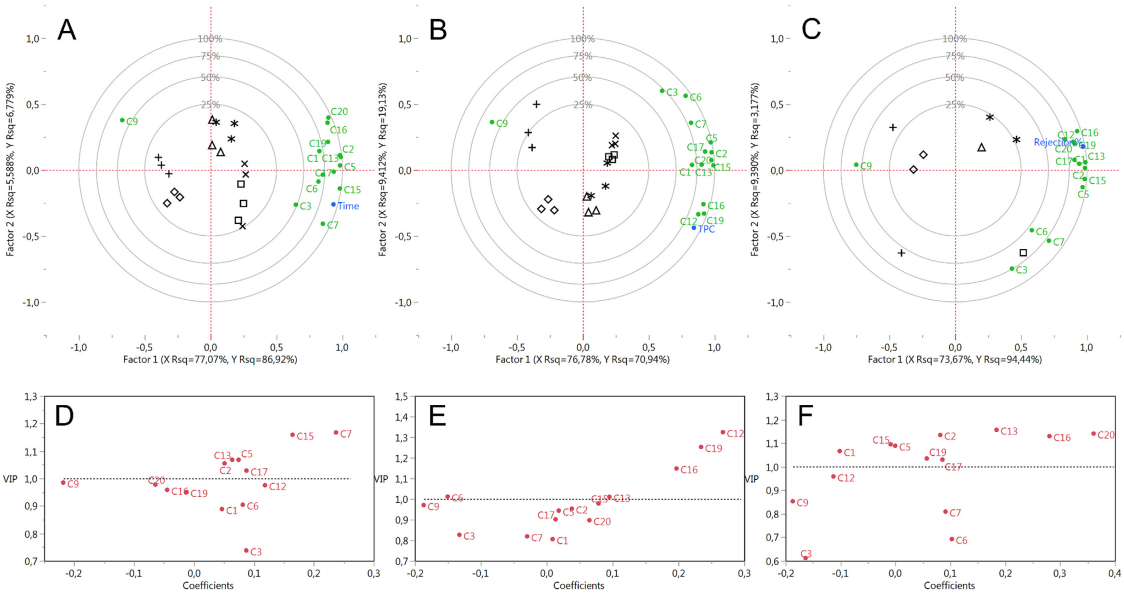

Fig. 4. Partial least squares (PLS) biplots (A–C) and VIP vs. regression coefficient plots (D–F) of brown shrimp stored under modified atmosphere (% $CO_2/O_2/N_2$) 50/0/50 at 4 °C. VOCs are treated as predictor variables and time (A and D), TPC (B and E) or rejection % (C–F) as response variables. The biplots present samples as scores (day 0: +; day 3: ◊; day 5: Δ; day 7:*; day 10: x; day 12: □) and VOCs as correlation loadings.

calls for cut-off values and correlation between VOCs and a dependent response variable. PLS regression was thus identified as the most systematic approach for selective analysis. Table 2 presents the most potential spoilage indicators of Atlantic cod and brown shrimp identified by PLS and the selection criteria: positive correlation with the response, VIP >1 and positive regression coefficient. The number of factors resulting in minimal root mean PRESS was in most cases between 2 and 7. In case of sensory rejection of Atlantic cod, the minimizing number was 1; two factors were selected on the basis of van der Voet test (van der Voet, 1994) indicating that the residuals of the model with two factors were not significantly larger than with one factor.

When considering TPC as a dependent variable, several VOCs could be identified for cod. Under at least three out of five storage conditions, 2,3-butanediol, dimethyl sulfide, ethyl acetate, 3-methyl-1-butanol, isobutyl alcohol and trimethylamine fulfilled the selection criteria. These VOCs could thus indicate spoilage under different storage conditions and could be related to the metabolism of representatives from the *Photobacterium* genus (Kuuliala et al. submitted manuscript). Dimethyl sulfide was associated with low oxygen MAP or air, whereas acetic acid was associated with MAP at lower storage temperature (4 °C). When rejection % was used as a dependent variable, the selected VOCs were well in correspondence with the TPC model.

Under the two atmospheres tested for brown shrimp, different VOC profiles were identified. Under 50% CO_2, most of the VOCs corresponded to the compounds identified for cod under low oxygen concentrations. In addition, carbon disulfide and methyl mercaptan fulfilled the selection criteria. Under 30% CO_2, only dimethyl sulfide, ethyl acetate and trimethylamine were identified. Respectively, few compounds were identified when rejection % was used as a dependent variable. The results are in good correspondence with VOCs detected in previous studies concerning crustaceans. Noseda et al. (2012) observed a significant increase in acetic acid, ammonia, dimethyl sulfide, dimethyl amine, ethanol, ethyl acetate and trimethylamine in brown shrimp stored under 50% CO_2 and 50% N_2. Production of hydrogen sulfide, carbon disulfide and methyl mercaptan was inhibited in the presence of carbon dioxide. Broekaert et al. (2013) observed the production of several respective VOCs in aerobically stored brown shrimp inoculated with *Pseudoalteromonas*. Respectively, increasing concentrations of several compounds including alcohols, aldehydes, ketones and trimethylamine have been observed with other crustaceans (Fall et al., 2012; Laursen, Leisner, & Dalgaard, 2006; Olafsdottir et al., 2005). The wet dog odor of Nordic shrimp has been attributed to the co-culture of *Carnobacterium maltaromaticum* and *Brochothrix thermosphacta* (Mejlholm, Bøknæs, & Dalgaard, 2005), particularly to the interaction of their metabolic products (Malcolm Love, 1979).

The potential spoilage indicators observed in the present study are produced during microbial metabolism (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015; Olafsdóttir et al., 1997). 3-methyl-1-butanol has frequently been observed during seafood spoilage (Duflos et al., 2010; Mikš-Krajnik et al., 2016; Parlapani, Mallouchos, Haroutounian, & Boziaris, 2014) and has been associated with cheesy or fruity off-odors (Montel, Masson, & Talon, 1998). In addition, both 3-methyl-1-butanol and 2,3-butanediol have been associated with fermented odor under vacuum (Casaburi et al., 2015). Ethyl esters such as ethyl acetate have been associated with fruity off-odors in meat (Ercolini et al., 2010). Production of dimethyl sulfide results in sulfurous and cabbage-like odors (Ercolini et al., 2010), whereas trimethylamine contributes to the characteristic smell of spoiled marine fish (Gram & Dalgaard, 2002).

The results of the present study indicate that several VOCs are produced during refrigerated storage of seafood. Even though some VOCs were identified as potential spoilage indicators under various conditions, single compounds have limited potential in quality analysis because their evolution is dependent on the storage conditions and subject to natural variation. The results are thus in line with previous studies suggesting that the use of multiple compound indices could enhance seafood quality analysis (Jørgensen et al., 2001; Leroi, Joffraud, Chevalier, & Cardinal, 2001; Ólafsdóttir, Högnadóttir, Martinsdóttir, & Jónsdóttir, 2000).

4. Conclusions

The identification of volatile organic compounds (VOCs) related to spoilage allows the analysis of seafood spoilage by following the concentrations of these compounds over storage time. Multivariate statistics provides analytical methods for the characterization and selection of relevant spoilage indicators. In the present study, acetic acid, 2,3-butanediol, isobutyl alcohol, 3-methyl-1-butanol, dimethyl sulfide, ethyl acetate and trimethylamine were most frequently identified as potential spoilage indicators of Atlantic cod and/or brown shrimp under different atmospheres. Due to the complex nature of microbiological spoilage and VOC evolution as well as the wide range of available packaging and storage conditions, seafood quality analysis could thus benefit from the analysis of multiple VOCs instead of single compounds over storage time.

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References

Abdi, H., & Williams, L. J. (2010). Principal component analysis. *Wiley Interdisciplinary Reviews: Computational Statistics, 2*(4), 433–459. http://dx.doi.org/10.1002/wics.101.

Adams, K. R., & Moss, M. O. (2008). *Food microbiology* (3rd ed.). Cambridge: Royal Society of Chemistry.

Afanador, N. L., Tran, T. N., & Buydens, L. M. C. (2014). An assessment of the jackknife and bootstrap procedures on uncertainty estimation in the variable importance in the projection metric. *Chemometrics and Intelligent Laboratory Systems, 137*, 162–172. http://dx.doi.org/10.1016/j.chemolab.2014.05.016.

Almeida, J. A. S., Barbosa, L. M. S., Pais, A. A. C. C., & Formosinho, S. J. (2007). Improving hierarchical cluster analysis: A new method with outlier detection and automatic clustering. *Chemometrics and Intelligent Laboratory Systems, 87*(2), 208–217. http://dx.doi.org/10.1016/j.chemolab.2007.01.005.

Argyri, A. A., Doulgeraki, A. I., Blana, V. A., Panagou, E. Z., & Nychas, G. E. (2011). Potential of a simple HPLC-based approach for the identification of the spoilage status of minced beef stored at various temperatures and packaging systems. *International Journal of Food Microbiology, 150*(1), 25–33. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.07.010.

Argyri, A. A., Mallouchos, A., Panagou, E. Z., & Nychas, G. E. (2015). The dynamics of the HS/SPME–GC/MS as a tool to assess the spoilage of minced beef stored under different packaging and temperature conditions. *International Journal of Food Microbiology, 193*, 51–58. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.09.020.

Blixt, Y., & Borch, E. (2002). Comparison of shelf life of vacuum-packed pork and beef. *Meat Science, 60*(4), 371–378. http://dx.doi.org/10.1016/S0309-1740(01)00145-0.

Broekaert, K., Heyndrickx, M., Herman, L., Devlieghere, F., & Vlaemynck, G. (2013). Molecular identification of the microbiota of peeled and unpeeled brown shrimp (crangon crangon) during storage on ice and at 7.5 °C. *Food Microbiology, 36*(2), 123–134. http://dx.doi.org/10.1016/j.fm.2013.04.009.

Casaburi, A., Piombino, P., Nychas, G., Villani, F., & Ercolini, D. (2015). Bacterial

populations and the volatilome associated to meat spoilage. *Food Microbiology, 45*(Part A), 83–102. http://dx.doi.org/10.1016/j.fm.2014.02.002.
Chen, Q., Li, H., Ouyang, Q., & Zhao, J. (2014). Identification of spoilage bacteria using a simple colorimetric sensor array. *Sensors and Actuators B: Chemical, 205*, 1–8. http://dx.doi.org/10.1016/j.snb.2014.08.025.
Ciesa, F., Dalla Via, J., Wisthaler, A., Zanella, A., Guerra, W., Mikoviny, T., et al. (2013). Discrimination of four different postharvest treatments of 'Red delicious' apples based on their volatile organic compound (VOC) emissions during shelf-life measured by proton transfer reaction mass spectrometry (PTR-MS). *Postharvest Biology and Technology, 86*, 329–336. http://dx.doi.org/10.1016/j.postharvbio.2013.06.036.
Duflos, G., Leduc, F., N'Guessan, A., Krzewinski, F., Kol, O., & Malle, P. (2010). Freshness characterisation of whiting (merlangius merlangus) using an SPME/GC/MS method and a statistical multivariate approach. *Journal of the Science of Food and Agriculture, 90*(15), 2568–2575. http://dx.doi.org/10.1002/jsfa.4122.
Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Ferranti, P., et al. (2010). Different molecular types of pseudomonas fragi have the same overall behaviour as meat spoilers. *International Journal of Food Microbiology, 142*(1–2), 120–131. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.06.012.
Fall, P. A., Pilet, M. F., Leduc, F., Cardinal, M., Duflos, G., Guérin, C., et al. (2012). Sensory and physicochemical evolution of tropical cooked peeled shrimp inoculated by brochothrix thermosphacta and lactococcus piscium CNCM I-4031 during storage at 8°C. *International Journal of Food Microbiology, 152*(3), 82–90. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.07.015.
Farrés, M., Platikanov, S., Tsakovski, S., & Tauler, R. (2015). Comparison of the variable importance in projection (VIP) and of the selectivity ratio (SR) methods for variable selection and interpretation. *Journal of Chemometrics, 29*(10), 528–536. http://dx.doi.org/10.1002/cem.2736.
Fik, M., Surówka, K., Maciejaszek, I., Macura, M., & Michalczyk, M. (2012). Quality and shelf life of calcium-enriched wholemeal bread stored in a modified atmosphere. *Journal of Cereal Science, 56*(2), 418–424. http://dx.doi.org/10.1016/j.jcs.2012.06.006.
Gram, L., & Dalgaard, P. (2002). Fish spoilage bacteria – problems and solutions. *Current Opinion in Biotechnology, 13*(3), 262–266. http://dx.doi.org/10.1016/S0958-1669(02)00309-9.
Hatzinger, R., Hornik, K., Nagel, H., & Maier, M. J. (2014). *R: Einführung durch angewandte statistik* (2nd ed.). München: Pearson Studium.
Hierro, E., Ordóñez, J. A., Bruna, J. M., Pin, C., Fernández, M., & de la Hoz, L. (2005). Volatile compound generation in dry fermented sausages by the surface inoculation of selected mould species. *European Food Research and Technology, 220*(5), 494–501. http://dx.doi.org/10.1007/s00217-004-1083-2.
Jørgensen, L. V., Huss, H. H., & Dalgaard, P. (2001). Significance of volatile compounds produced by spoilage bacteria in vacuum-packed cold-smoked salmon (salmo salar) analyzed by GC-MS and multivariate regression. *Journal of Agricultural and Food Chemistry, 49*(5), 2376–2381. http://dx.doi.org/10.1021/jf0009908.
Kaiser, H. F. (1970). A second generation little jiffy. *Psychometrika, 35*(4), 401–415. http://dx.doi.org/10.1007/BF02291817.
Kaiser, H. F. (1974). An index of factorial simplicity. *Psychometrika, 39*(1), 31–36. http://dx.doi.org/10.1007/BF02291575.
Kolde, R. (2015). *Pheatmap: Pretty heatmaps*. R package version 1.0.8.
Küntzel, A., Fischer, S., Bergmann, A., Oertel, P., Steffens, M., Trefz, P., et al. (2016). Effects of biological and methodological factors on volatile organic compound patterns during cultural growth of mycobacterium avium ssp. paratuberculosis. *Journal of Breath Research, 10*(3), 037103.
Kuuliala, L., Al Hage, Y., Ioannidis, A.-G., Sader, M., Kerckhof, F.-M., Vanderroost, M., Boon, N., De Baets, B., De Meulenaer, B., Ragaert, P., & Devlieghere, F.. Microbiological, chemical and sensory spoilage analysis of raw Atlantic cod (Gadus morhua) stored under modified atmospheres for the development of intelligent packaging technologies. Submitted 05/05/2017 in Food Microbiology.
Laursen, B. G., Leisner, J., & Dalgaard, P. (2006). Carnobacterium species: Effect of metabolic activity and interaction with brochothrix thermosphacta on sensory characteristics of modified atmosphere packed shrimp. *Journal of Agricultural and Food Chemistry, 54*(10), 3604–3611. http://dx.doi.org/10.1021/jf053017f.
Leroi, F., Joffraud, J. J., Chevalier, F., & Cardinal, M. (2001). Research of quality indices for cold-smoked salmon using a stepwise multiple regression of microbiological counts and physico-chemical parameters. *Journal of Applied Microbiology, 90*(4), 578–587. http://dx.doi.org/10.1046/j.1365-2672.2001.01283.x.
Lewis, P. D., & Menzies, G. E. (2015). Vibrational spectra, principal components analysis and the horseshoe effect. *Vibrational Spectroscopy, 81*, 62–67. http://dx.doi.org/10.1016/j.vibspec.2015.10.002.
Maier, M. J. (2015). *Companion package to the book "R: Einführung durch angewandte statistik"*. R package version 0.9.3.
Malcolm Love, R. (1979). The post-mortem ph of cod and haddock muscle and its seasonal variation. *Journal of the Science of Food and Agriculture, 30*(4), 433–438. http://dx.doi.org/10.1002/jsfa.2740300414.
Marín, S., Vinaixa, M., Brezmes, J., Llobet, E., Vilanova, X., Correig, X., et al. (2007). Use of a MS-electronic nose for prediction of early fungal spoilage of bakery products. *International Journal of Food Microbiology, 114*(1), 10–16. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.11.003.
Mataragas, M., Skandamis, P., Nychas, G. E., & Drosinos, E. H. (2007). Modeling and predicting spoilage of cooked, cured meat products by multivariate analysis. *Meat Science, 77*(3), 348–356. http://dx.doi.org/10.1016/j.meatsci.2007.03.023.
Mejlholm, O., Bøknæs, N., & Dalgaard, P. (2005). Shelf life and safety aspects of chilled cooked and peeled shrimps (pandalus borealis) in modified atmosphere packaging. *Journal of Applied Microbiology, 99*(1), 66–76. http://dx.doi.org/10.1111/j.1365-2672.2005.02582.x.
Mikš-Krajnik, M., Yoon, Y., Ukuku, D. O., & Yuk, H. (2016). Volatile chemical spoilage indexes of raw atlantic salmon (salmo salar) stored under aerobic condition in relation to microbiological and sensory shelf lives. *Food Microbiology, 53*(Part B), 182–191. http://dx.doi.org/10.1016/j.fm.2015.10.001.
Montel, M. C., Masson, F., & Talon, R. (1998). Bacterial role in flavour development. *Meat Science, 49*, S111–S123. http://dx.doi.org/10.1016/S0309-1740(98)90042-0.
Noseda, B., Goethals, J., De Smedt, L., Dewulf, J., Samapundo, S., Van Langenhove, H., et al. (2012). Effect of O2CO2 enriched atmospheres on microbiological growth and volatile metabolite production in packaged cooked peeled gray shrimp (crangon crangon). *International Journal of Food Microbiology, 160*(1), 65–75. http://dx.doi.org/10.1016/j.ijfoodmicro.2012.09.018.
Noseda, B., Ragaert, P., Pauwels, D., Anthierens, T., Van Langenhove, H., Dewulf, J., et al. (2010). Validation of selective ion flow tube mass spectrometry for fast quantification of volatile bases produced on atlantic cod (gadus morhua). *Journal of Agricultural and Food Chemistry, 58*(9), 5213–5219. http://dx.doi.org/10.1021/jf904129j.
Ólafsdóttir, G., Högnadóttir, Á., Martinsdóttir, E., & Jónsdóttir, H. (2000). Application of an electronic nose to predict total volatile bases in capelin (mallotus villosus) for fishmeal production. *Journal of Agricultural and Food Chemistry, 48*(6), 2353–2359. http://dx.doi.org/10.1021/jf990322q.
Olafsdottir, G., Jonsdottir, R., Lauzon, H. L., Luten, J., & Kristbergsson, K. (2005). Characterization of volatile compounds in chilled cod (gadus morhua) fillets by gas chromatography and detection of quality indicators by an electronic nose. *Journal of Agricultural and Food Chemistry, 53*(26), 10140–10147. http://dx.doi.org/10.1021/jf0517804.
Olafsdóttir, G., Martinsdóttir, E., Oehlenschläger, J., Dalgaard, P., Jensen, B., Undeland, I., et al. (1997). Methods to evaluate fish freshness in research and industry. *Trends in Food Science & Technology, 8*(8), 258–265. http://dx.doi.org/10.1016/S0924-2244(97)01049-2.
Parlapani, F. F., Mallouchos, A., Haroutounian, S. A., & Boziaris, I. S. (2014). Microbiological spoilage and investigation of volatile profile during storage of sea bream fillets under various conditions. *International Journal of Food Microbiology, 189*, 153–163. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.08.006.
R Core Team. (2013). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org/.
R Core Team. (2016). *The comprehensive R archive network*. Retrieved from: https://cran.r-project.org/index.html.
Rendall, R., Reis, M. S., Pereira, A. C., Pestana, C., Pereira, V., & Marques, J. C. (2015). Chemometric analysis of the volatile fraction evolution of Portuguese beer under shelf storage conditions. *Chemometrics and Intelligent Laboratory Systems, 142*, 131–142. http://dx.doi.org/10.1016/j.chemolab.2015.01.015.
Shimodaira, H. (2002). An approximately unbiased test of phylogenetic tree selection. *Systematic Biology, 51*(3), 492–508. http://dx.doi.org/10.1080/10635150290069913.
Siroli, L., Patrignani, F., Serrazanetti, D. I., Tabanelli, G., Montanari, C., Tappi, S., et al. (2014). Efficacy of natural antimicrobials to prolong the shelf-life of minimally processed apples packaged in modified atmosphere. *Food Control, 46*, 403–411. http://dx.doi.org/10.1016/j.foodcont.2014.05.049.
Smoliński, A., Walczak, B., & Einax, J. W. (2002). Hierarchical clustering extended with visual complements of environmental data set. *Chemometrics and Intelligent Laboratory Systems, 64*(1), 45–54. http://dx.doi.org/10.1016/S0169-7439(02)00049-7.
Storer, M. K., Hibbard-Melles, K., Davis, B., & Scotter, J. (2011). Detection of volatile compounds produced by microbial growth in urine by selected ion flow tube mass spectrometry (SIFT-MS). *Journal of Microbiological Methods, 87*(1), 111–113. http://dx.doi.org/10.1016/j.mimet.2011.06.012.
Suzuki, R., & Shimodaira, H. (2006). Pvclust: An R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics, 22*(12), 1540–1542. http://dx.doi.org/10.1093/bioinformatics/btl117.
Suzuki, R., & Shimodaira, H. (2015). *Pvclust: Hierarchical clustering with P-values via multiscale bootstrap resampling*. R package version 2.0-0.
Verginer, M., Leitner, E., & Berg, G. (2010). Production of volatile metabolites by grape-associated microorganisms. *Journal of Agricultural and Food Chemistry, 58*(14), 8344–8350. http://dx.doi.org/10.1021/jf100393w.
Vervoort, L., Grauwet, T., Kebede, B. T., Van der Plancken, I., Timmermans, R., Hendrickx, M., et al. (2012). Headspace fingerprinting as an untargeted approach to compare novel and traditional processing technologies: A case-study on orange juice pasteurisation. *Food Chemistry, 134*(4), 2303–2312. http://dx.doi.org/10.1016/j.foodchem.2012.03.096.
van der Voet, H. (1994). Comparing the predictive accuracy of models using a simple randomization test. *Chemometrics and Intelligent Laboratory Systems, 25*(2), 313–323. http://dx.doi.org/10.1016/0169-7439(94)85050-X.
Wang, Y., Li, Y., Yang, J., Ruan, J., & Sun, C. (2016). Microbial volatile organic compounds and their application in microorganism identification in foodstuff. *TrAC Trends in Analytical Chemistry, 78*, 1–16. http://dx.doi.org/10.1016/j.trac.2015.08.010.
Wibowo, S., Grauwet, T., Gedefa, G. B., Hendrickx, M., & Van Loey, A. (2015). Quality changes of pasteurised mango juice during storage. part I: Selecting shelf-life markers by integration of a targeted and untargeted multivariate approach. *Food Research International, 78*, 396–409. http://dx.doi.org/10.1016/j.foodres.2015.09.002.
Wold, S., Sjöström, M., & Eriksson, L. (2001). PLS-regression: A basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems, 58*(2), 109–130.

http://dx.doi.org/10.1016/S0169-7439(01)00155-1.
Zaragozá, P., Fernández-Segovia, I., Fuentes, A., Vivancos, J., Ros-Lis, J. V., Barat, J. M., et al. (2014). Monitorization of atlantic salmon (salmo salar) spoilage using an optoelectronic nose. *Sensors and Actuators B: Chemical, 195*, 478–485. http://dx.doi.org/10.1016/j.snb.2014.01.017.
Zeng, Q. Z., Thorarinsdottir, K. A., & Olafsdottir, G. (2005). Quality changes of shrimp (pandalus borealis) stored under different cooling conditions. *Journal of Food Science, 70*(7), s459–s466. http://dx.doi.org/10.1111/j.1365-2621.2005.tb11493.x.

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- Kuuliala, L.*, Al Hage, Y., Ioannidis, A.-G., Sader, M., Kerckhof, F.-M., Vanderroost, M., Boon, N., De Baets, B., De Meulenaer, B., Ragaert, P. & Devlieghere, F. (2018). Microbiological, chemical and sensory spoilage analysis of raw Atlantic cod (*Gadus morhua*) stored under modified atmospheres. Food Microbiology, 70, 232-244.
- Kuuliala, L.*, Abatih, E., Ioannidis, A.-G., Vanderroost, M., De Meulenaer, B., Ragaert, P., & Devlieghere, F. (2018). Multivariate statistical analysis for the identification of potential seafood spoilage indicators. Food Control 84, 49-60.
- Sader, M.*, Pérez-Fernández, R., Kuuliala, L., Devlieghere, F. & De Baets, B. A combined scoring and ranking approach for determining overall food quality. International Journal of Approximate Reasoning, 100, 161-176.

Unpublished manuscripts

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- Kuuliala, L.*, Ali-Löytty, S., Auvinen, S.-M., Kolppo, K. & Kuusipalo, J. Effect of high, residual and absent oxygen on the development of headspace gas concentrations, surface pH and color in pork sirloin packaged under modified atmospheres. Unpublished manuscript.
- Hindle, F.*, Kuuliala, L., Mouelhi, M., Cuisset, A., Bray, C., Vanwolleghem, M., Devlieghere, F., Mouret, G. & Bocquet, R. Monitoring of food spoilage by high resolution THz analysis. Unpublished manuscript.

Conference presentations

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- L. Kuuliala*, T. Nieminen^, S. Auvinen, K. Kolppo, J. Kuusipalo and J. Björkroth. Antibacterial activity of silver-containing packaging materials against lactic acid bacteria associated with meat spoilage. Poster presented at ICAR2012, 21.-23.11.2012, Lisbon, Portugal
- L. Kuuliala*^, E. Jääskeläinen, S. Auvinen, K. Kolppo. J. Björkroth and J. Kuusipalo. Testing of silver-containing packaging materials against meat spoilage related bacteria. Poster presented at RAFA2013, 5.-8.11.2013, Prague, Czech Republic
- L. Kuuliala*^, A.-G. Ioannidis, M. Sader, M. Vanderroost, B. De Meulenaer, F. Devlieghere. Production of volatile organic compounds during spoilage of raw Atlantic cod fillets in modified atmosphere packaging. 3-min talk at 20th National Symposium for Applied Biological Sciences, 31.01.2015, Louvain-la-Neuve, Belgium.
- L. Kuuliala*^, Y. Al Hage, A.-G. Ioannidis, M. Sader, M. Vanderroost, B. De Meulenaer, F. Devlieghere. Microbiological, chemical and sensory spoilage analysis of raw Atlantic cod (Gadus morhua) - towards an integrated optical sensor for monitoring food quality. Oral presentation at Innovations in Food Packaging, Shelf Life and Food Safety, 15.-17.09.2015, Erding, Munich, Germany.
- Ioannidis, A.-G.*^, Kuuliala, L., Vanderroost, M., Devlieghere, F., De Meulenaer, B. The development of an integrated optical sensor for modified atmosphere food packaging: A shelf-life study on meat and fish. Poster presented at Innovations in Food Packaging, Shelf Life and Food Safety – 15-17/09/2015, Erding, Germany.

Research visits

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