Fresh view in fish microbiology

Analysis of microbial changes in fish during storage, decontamination and curing of fish, using molecular detection and analysis methods.

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Specific molecular detection methodologies, fish microflora characterization during storage, decontamination of bacteria and isolation of novel species

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Abstract

The objective of this study was to develop and explore the potential use of new rapid molecular detection and analysis methods and to investigate the bacteriology of fish during storage as an alternative to the conventional methods of cultivation.

The project can be divided in three separate themes, were the first one deals with methodological developments and optimisation for the detection and quantification of microbes in food (papers I and II), second one with fish processing hygiene (paper III) and the third one with the bacteriological succession that occurs in the skin and flesh during storage of marine fishes in different storage conditions (papers IV-VI).

In the first theme, strategies to improve the sensitivity and efficiency of real-time PCR by selection of fluorescent dyes and suitable probe chemistries were addressed. Based on those experiences and findings, quantitative method developments for important spoilage bacteria in fish were developed to be used as spoilage markers for the industry. It was shown that quantification of *Pseudomonas* could be achieved in only 5 hours with high correlation to existing cultivation plate count methods.

In the second theme, efficiency of a typical washing protocol was determined by testing three critical parameters and their effects on bacterial decontamination using *in situ* bacterial flora from fish fillet. Two surface materials (plastic and stainless steel), water temperatures (7 and 25 °C) and detergent concentrations (2 and 4 %) were used for this purpose in combination with two types of detergents.

The third theme deals with questions regarding bacteriological succession during storage of fish, with examples from three different fish species. Cod and haddock represent teleosts (bony fishes) while skate represents elasmobranches (cartilaginous fishes). The teleosts species are important fish stocks for fisheries and have therefore received more attention when it comes to research on their spoilage process and bacteriology. The present study demonstrates *Photobacterium phosphoreum* as one of the most abundant fish spoilage bacterium both in cod and haddock under different conditions. The bacterial population structure in elasmobranches and the succession during storage is analysed by molecular techniques which brings to light new knowledge on the presence of previously undescribed bacterial species that progress during the conventional curing process of skate.

Ágrip á íslensku

Í þessu verkefni eru skemmdarferlar fiskafurða skoðaðir með notkun sameindalíffræðilegra aðferða sem einungis nýlega hefur verið beitt á þessu sviði. Verkefninu má skipta upp í þrjá hluta þar sem sá fyrsti beinist að bestun og þróun hraðvirkra greiningaraðferða á óæskilegum bakteríum í matvælum með notkun real-time PCR aðferðafræði (greinar I og II). Annar hlutinn tengist hreinlæti og þrifum í fiskvinnslu (grein III) og í þeim þriðja er framvinda bakteríusamfélaga við geymslu á fiski skoðuð þar sem kannað var hvort sameindalíffræðilegar aðferðir kasti nýju ljósi á þá þekkingu sem hefur fengist með hefðbundnum ræktunaraðferðum (greinar IV-VI).

Í fyrsta hlutanum voru leiðir kannaðar til að auka næmni og áreiðanleika real-time PCR greininga með hliðsjón af vali á flúrljómandi efnum og gerð þreifara. Í framhaldinu var hafist handa við að þróa magnbundnar greiningaraðferðir á mikilvægum skemmdarbakteríum í fiski. Sýnt var fram á að magnbundin greining á *Pseudomonas* tegundum eru mögulegar á innan við 5 klukkustundum með góðri samsvörun við ræktanir sem annars taka 2-3 daga.

Í öðrum hlutanum er komið inn á þrif í fiskvinnslum og virkni hefðbundinna þrifaferla til fjarlægingar á örveruþekjum með tilliti til þriggja breyta; hitastig skolvatns (7 eða 25°C), styrkleiki sápu (2 eða 4%) og gerð yfirborðs (stál eða plast). Örveruþekjan var framkölluð með því að nota óskilgreinda bakteríuflóru úr þorskhakki og henni komið á þar til gerð yfirborð.

Í þriðja hlutanum er fengist við spurningar um framvindu bakteríusamfélaga við geymslu á fiski þar sem dæmi eru tekin af þremur fiskitegundum. Þorskur og ýsa eru dæmi um beinfiska á meðan skata er flokkast til brjóskfiska. Ýmsir beinfiskar eru mikilvægir nytjastofnar og hafa því hlotið meiri athygli þegar kemur að rannsóknum á örverufræði þeirra og skemmdarferlum. Í þessum hluta er sýnt fram á og staðfest að *Photobacterium phosphoreum* er sú bakteríutegund sem oftar en ekki nær yfirhöndinni við geymslu á þorski og ýsu við mismunandi aðstæður. Með notkun ræktunaraðferða og sameindalíffræðilegra greininga er framvindu örverusamfélaga við kæsingu á skötu lýst og sýnt fram á viðveru áður ólýstra bakteríutegunda í umtalsverðu magni í þessu sérstæða umhverfi.

List of original papers

This thesis is based on the following papers:

I. **Eyjólfur Reynisson**, Mathilde H. Josefsen, Mikael Krause, Jeffrey Hoorfar. 2006. Evaluation of probe chemistries and platforms to improve the detection limit of real-time PCR. *Journal of Microbiological Methods* 66: 206– 216.

II. **Eyjólfur Reynisson**, Hélène Liette Lauzon, Hannes Magnússon, Guðmundur Óli Hreggviðsson, Viggó Þór Marteinsson. 2008. Rapid quantitative monitoring method for the fish spoilage bacteria *Pseudomonas. Journal of Environmental Monitoring.* 10 (11): 1357-1362

III. **Eyjólfur Reynisson**, Birna Guðbjornsdottir, Viggo Þór Marteinsson and Guðmundur Óli Hreggviðsson. 2009. Decontamination Efficiency of Fish Bacterial Flora from Processing Surfaces. *Food Technology and Biotechnology*. 47 (1) 75-82.

IV. **Eyjólfur Reynisson**, Hélène L. Lauzon, Hannes Magnússon, Rósa Jónsdóttir, Guðrún Ólafsdottir, Viggó Marteinsson, Guðmundur Óli Hreggvidsson. Bacterial Composition and Succession during storage of North-Atlantic Cod (*Gadus morhua*) at superchilled Temperatures. *BMC journal of Microbiology*. In press.

V. **Eyjólfur Reynisson**, Hélène Liette Lauzon, Lárus Thorvaldsson, Björn Margeirsson Árni Rafn Rúnarsson, Viggó Þór Marteinsson, Emilía Martinsdóttir. Effects of cooling technologies on developing bacterial populations and spoilage indicators during storage of whole, gutted haddock (*Melanogrammus aeglefinus*). Submitted to *European food research and technology*.

VI. **Eyjólfur Reynisson**, Rósa Jónsdóttir, Viggó Marteinsson, Jeffrey Hoorfar, Guðmundur Óli Hreggvidsson. Microbial diversity of uncultivated bacteria and isolation of new species during curing process of skate (*Diptirus batis*). Manuscript in preparation.

Preface

This thesis is composed of an introduction followed by discussions on the present topic and finally the six papers of which this thesis is built upon. The introduction puts the work into context with an overview of the historical background of fish microbiology which is of concern and with relations to the classical microbiology and the more recent molecular microbiology. The current research issues of microbial diversity in the world are presented and how the understanding of the diversity has deepened with the emergence of new technologies. This enhanced understanding has furthermore raised questions on how microbial species are defined which is also discussed. Marine and fish microbiology is reviewed with the perspective of undesirable bacteria in fish and their relation to quality and safety of seafood. Improvements in methodologies in recent years has enabled us to better understand the microbial world and some of these methods are described and put into context with what was done in the present study.

During the work of this thesis I had the opportunity of working in two separate fields of microbiology; the conventional, cultivation food microbiology and more recent molecular microbiology dealing with ecological questions and the development of new detection methodologies. In spite of obvious overlap between these areas the practical differences were surprisingly large in my opinion. In the thesis I try to bridge the fields both in practical work and discussion.

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Papers I-IV

1. Abbrevations

BHQ	Black hole quencher
CarA	Carbamoyl phosphate synthase, subunit A
CFU	Colony forming units
CGH	Core genome hypothesis
Ct	Cycle threshold
Cy3	Cyanine 3 (fluorescent dye)
Cy5	Cyanine 5 (fluorescent dye)
DDQ	Deep dark quencher
DGGE	Denaturing gel gradient electrophoresis
DNA	Deoxyribonucleic acid
EU	European union
FACS	Fluorescent assisted cell sorting
FAM	6-carboxy-fluorescein (fluorescent dye)
FISH	Fluorescent in situ hybridisation
GAST	Global alignment for sequence taxonomy
GOS	Global ocean sampling expedition
LNA	Locked nucleic acid
MA	Modified atmosphere
MAP	Modified atmosphere packaging
MLSA	Multi locus sequence analysis
MLST	Multi locus sequence typing
mRNA	Messenger RNA
OTU	Operating taxonomic units
PCR	Polymerase chain reaction
PEP	Polyethylene plastic
RNA	Ribonucleic acid
SS	Stainless steel
SSO	Specific spoilage organism
SYBR	Double stranded DNA fluorescent dye
TAMRA	A common fluorogenic quencher
TM7	A specific bacterial phylum without a cultivable representative
TMA	Tri-methyl-amine
TMAO	Tri-methyl-amine-oxide
TOPO	Topoisomerase
t-RFLP	Terminal restriction fragment length polymorphism
V3	Variable domain no. 3 in the 16S rRNA gene
V6	Variable domain no. 6 in the 16S rRNA gene