Final Report from OECD Co-operative Research Programme, Research Fellowship 2012

Cover page

- **Name:** Lisbeth Truelstrup Hansen
- **The subject title:** Microbial ecology and pathogen inhibition in seafood preserved with lactic acid bacteria
- **Theme:** The Food Chain
- **Host institution**: ONIRIS: Nantes-Atlantic National College of Veterinary Medicine, Food Science and Engineering
- **Host supervisors**: Drs. Hervé Prévost and Marie-France Pilet
- **Dates of fellowship**: March 1-June 28, 2012
- **Your consent to your report being posted on the Co-operative Research Programme's website, or alternatively, a short paragraph about your fellowship which could be used anonymously:** I consent to posting the report on the website.

1. Relevance

- Relevance to the Co-operative Research Programme objectives

International trade of food requires the availability of commodities that are free from plant, veterinary and human pests and/or health hazards. This project aimed to address the need for innovative molecular based methodologies to determine the adequacy of novel food preserving hurdle technologies applied to minimally processed ready-to-eat food products, which are some of the most value-added yet vulnerable products seen from a risk assessment point of view.

- Relevance to the "Food Chain" Theme

The achievement of a pathogen free food chain is the ambition of the agri-food industry, national governments and consumers alike. The proposed research addresses the two OECD Co-operative Research Program "Food Chain" research theme areas, which focus on development of new technologies for the treatment and prevention of outbreaks of "old" pathogens, i.e., *Listeria monocytogenes*, in the vulnerable but popular ready-to-eat (RTE) foods. The importance of this work is highlighted by the continued problems experienced by the food industry as exemplified by the Maple Leaf outbreak in Canada in 2008 causing 20 deaths (Weatherill et al. 2009) and severe financially losses (~C$50 million) to the company, an estimated 1455 annual listeriosis cases leading to hospitalization (and a 19% death rate) in the USA (Scallan et al. 2011) and the fact recent data showed the number of listeriosis cases to be on the rise in the European Union (EFSA, 2009). In spite of efforts from both government and industry, a large outbreak caused by contaminated Cantaloupe melons originating in Colorado, USA (CDC, 2011) shocked consumers. In Canada, reports of recalls due to the presence of *L. monocytogenes* on products ranging from fresh-cut mushrooms, lettuce and RTE sausages are common place (CFIA, 2012). Due to the documented persistence of *L. monocytogenes* in the food processing environment a multiple barrier/hurdle approach will be needed along the food chain.

This collaborative project evaluated if a multi-tiered approach using both culture and culture-independent methods can be used to elucidate the microbial ecology and provide essential information about the efficacy of multiple barrier/hurdle approaches including ones based on biopreservation with lactic acid bacteria (LAB), to control this microbial hazard in the final packaged RTE food product. The achieved results will be useful for both the industrial development of effective applications of novel hurdle methods, protective LAB cultures and value-added biopreserved food products, and for the public policy makers needing to draft new science-based regulations on the use of these preservation methods.

- Relevance to Agricultural and Food Policy

Our continued issues with human bacterial pathogens in the food chain emphasize the need for science-based policies and regulations. Using *L. monocytogenes* as an example of a problematic microorganism, we have yet to fully understand its ecology, i.e., how does it enter the food chain? Once established in the food processing environment, the bacterium is known to present a high risk of cross-contamination. This has led regulators and policy makers to require that the industry apply a risk-based environmental and food product sampling approach. In addition to keeping the production environment *Listeria*-free it is recommended that additional hurdles are incorporated into all RTE products to ensure that growth of *L. monocytogenes* is prevented during the shelf-life of the product. The industry is responsible for documenting that the hurdle approach they use is suitable. The method developed in this project will aid industry as well as governmental representatives in the assessment of the microbial ecology and provide essential information about the efficacy of multiple barrier/hurdle approaches including ones based on biopreservation with LAB, to control this microbial hazard in the final packaged RTE food product.

2. Objectives of the fellowship

Introduction.

Keeping RTE food products free from contaminating pathogens presents a major challenge for food processors worldwide. It is commonly recognized that to achieve a pathogen free food chain it is necessary to

apply a multiple barrier or hurdle approach to build a "farm-to-fork" food safety continuum. This fellowship project evaluated the suitability of a molecular microbial ecology analysis method to assess the microbial ecology of RTE value-added seafood products. In many of these products, it is desirable to apply a multi-hurdle treatment that both secures shelf-life extension and inhibition of important foodborne pathogens, in particular *L. monocytogenes* which is a psychrotrophic pathogenic bacteria known to frequently occur in a wide range of RTE food products and persist in the food processing environment, as documented for example by its resistance to desiccation (Truelstrup Hansen and Vogel, 2011).

Previous work conducted in Nantes and elsewhere has shown that some LAB strains of aquatic food origin can successfully be used to limit the growth of *Listeria* without any adverse effects on the sensorial quality on seafood products including cooked shrimps and cold-smoked salmon (Nilsson et al., 1999; Brillet et al. 2004; Brillet et al. 2005; Vescovo et al. 2006; Tome et al., 2008; Matamoros et al. 2009; Fall et al. 2010).

From an industrial and regulatory point of view it is imperative, that it can be documented that the biopreservation treatment effectively inhibits and controls both the endogenous spoilage microflora and the target pathogens consistently. As traditional culture methods are estimated to detect only about 10-20% of the actual microflora, molecular microbial community analysis based on the *16S rRNA* genes are increasingly being used to provide a more comprehensive analysis of the structure and function of microorganisms in food products (Justé et al. 2008; Roh et al. 2010). However, use of the *16S rRNA* suffers from the limitation that many bacterial species harbour more than one copy of the gene and variations in these *16S rRNA* sequences are known to cause multiple bands or signals from the same bacterial species (Case et al. 2007; Macé et al. 2011). The use of protein encoding genes, which occur in a single genomic copy, have therefore been proposed and RpoB, a sub-unit of the DNA-dependent RNA polymerase holo-enzyme, has surfaced as a suitable candidate (Dahllöf et al. 2000; Adékambi et al. 2008).

Microbial ecosystems in fish products has only once before been subjected to an *rpoB* based analysis, however, the researchers had to use a two-step amplification approach before carrying out the resolving temporal temperature gradient electrophoresis (TTGE) (Giacomazzi et al. 2004). A simplified protocol would be desirable and allow for the assessment of whether *rpoB* based community analyses would improve current techniques.

Objectives.

The overall objective of the work carried out during the research fellowship was to develop and apply an *rpoB*-PCR TTGE analysis to elucidate the microbial ecology evolving in value-added seafood products. Cooked shrimp and modified atmosphere packaged salmon containing the natural endogenous microflora was analyzed using the *rpoB*-PCR TTGE as well as using culture methods and *16S rRNA*-PCR TTGE.

The specific objectives were as follows:

1. Can diversity in the *rpoB* gene be used to yield insight into the dominant prokaryotic community found in fish products such as salmon and shrimp?

2. Does the presence of only one gene copy per organism of the protein encoding *rpoB* gene result in a better description of the microbial community as compared to the more commonly used *16S rRNA* genes, which occurs in multiple copies in many bacteria?

3. What is the detection limit for a specific bacterial group when using *rpoB*-PCR TTGE?

3. Major achievements (up to three)

(1) New alignments of the *rpoB*-gene from *Photobacterium phosphoreum*, *Shewanella putrefaciens*, *Carnobacterium maltaromaticum*, *Morganella psychrotolerans* (new sequences) as well as sequences obtained for public databases for *L. monocytogenes*, other important food borne pathogens and endogenous fish spoilage bacteria were carried out to determine the suitability of published primers and/or need to design new primers for *rpoB*-PCR TTGE analysis of seafood products. More than 400 sequences were aligned.

(2) Using the primer set published by Perumbakkam and Craig (2011), a new PCR-TTGE method was developed and used to analyze a library of 33 culture collection strains representative of the bacteria mentioned above. All bacteria were successfully amplified and separated by TTGE with 32 out of 33 bacteria running as one distinct band in the polyacrylamide gel.

(3) The new *rpoB*-PCR TTGE method was applied successfully to samples of seafood including cooked shrimp and modified atmosphere packaged salmon products. The microflora of these product samples were at

the same time extensively characterized by culture methods, *16S rRNA*-PCR TTGE and for one set of samples a novel Q-PCR method specific for *P. phosphoreum* was also used. Standard software (Bionumerics) was used to analyse the *rpoB*-PCR TTGE gel patterns, which generally showed agreement with the results obtained by the other methods. The advantage of the *rpoB*-based TTGE method lies in the fact, that each bacterium (32/33) gave rise to just one band in the polyacrylamide gel thus facilitating the gel analysis. However, the detection limit for specific bacteria was found to be 10^5 CFU/g for the new method as opposed to 10^4 CFU/g for the *16S rRNA*-PCR TTGE method, which is perhaps to due multiple copies of *16S rRNA* being found in each cell contrary to the single copy of *rpoB*.

References.

Adékampi, T., M. Drancourt and D. Raoult. 2008. The *rpoB* gene as a tool for clinical microbiologists. Trends Microbiol. 17, 37-45.

Brillet., A., M.F. Pilet, H. Prévost, M. Cardinal and F. Leroi. 2005. Effect of inoculation of *Carnobacterium divergens* V41, a biopreservative strain against *Listeria monocytogenes* risk, on the microbiological, chemical and sensory quality of cold-smoked salmon. Int. J. Food Microbiol. 104, 309-324.

CDC. 2012. Cantaloupe outbreak. http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html (Accessed August 28, 2012).

CFIA. 2012. Food Recalls. http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recalls-and-allergy-alerts/eng/1299076382077/1299076493846 (Accessed August 28, 2012)

Dahllöf, I., H. Baillie and S. Kjelleberg. 2000. *rpoB*-based community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. Appl. Environ. Microbiol. 66, 3376-3380.

EFSA. 2009. The community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. The EFSA J. 223, 1-141.

Fall, P.A., F. Leroi, F. Chevalier, C. Guerin and M.F. Pilet. 2010. Protective effect of a non-bacteriocinogenic *Lactococcus piscium* CNCM I-4031 strain against *Listeria monocytogenes* in sterilized tropical cooked peeled shrimp. J. Aquat. Food Prod. Technol. 19, 84-92.

Giacomazzi, S., F. Leroi, C. L'Henaff and J.J. Joffraud. 2004. rpoB-PCR amplified gene and temporal temperature gradient gel electrophoresis: a rapid tool to analyse bacterial strains representative of cold-smoked salmon microflora. J. Appl. Microbiol. 38, 130-134.

Jaffrès, E., D. Sohier, F. Leroi, M.F. Pilet, H. Prévost, J.J. Joffraud and X. Dousset. 2009. Study of the bacterial ecosystem in tropical cooked and peeled shrimps using a polyphasic approach. Int. J. Food Microbiol. 131, 20-29.

Justé, A., B.P.H.J. Thomma and B. Lievens. 2008. Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. Food Microbiol. 25, 745-761.

Macé, S., J. Cornet, F. Chevalier, M. Cardinal, M.F. Pilet, X. Dousset and J.J. Joffraud. 2012. Characterisation of the spoilage microbiota in raw salmon (Salmo salar) steaks stored under vacuum or modified atmosphere packaging combining conventional methods and PCR-TTGE. Food Microbiol. 30, 164-172.

Matamoros, S., F. Leroi, M. Cardinal, F. Gigout, F.K. Chadii, J. Cornet, H. Prévost and M.F. Pilet. 2009. Psychrotrophic lactic acid bacteria used to improve the safety and quality of vacuum-packaged cooked and peeled tropical shrimp and cold-smoked salmon. J. Food Prot. 72, 365-374.

Nilsson, L., L. Gram and H.H. Huss. 1999. Growth control of *Listeria monocytogenes* on cold-smoked salmon using competitive lactic acid bacteria flora. J. Food Prot. 62, 336-342.

Perumbakkam, S. and A.M. Craig. 2011. Design and in vitro evaluation of a new rpoB-DGGE primers for ruminants. FEMS Microbiol. Ecol. 76, 156-169.

Roh, S.W., K-H. Kim, Y-D. Nam, H-W. Chang, E-J. Park and J-W. Bae. 2010, Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. ISME J. 4, 1-16,

Scallan, E., R.M. Hoekstra, F.J. Angulo, R.V. Tauxe, M-A. Widdowson, S.L. Roy, J.L. Jones, and P.M. Griffin 2011. Foodborne illness acquired in the United States – major pathogens. Emerg. Infect. Dis. 17, 7-15.

Tome, E., P.A. Gibbs and P.C. Teixeira. 2008. Growth control of *Listeria innocua* 2030c on vacuum-packaged cold-smoked salmon by lactic acid bacteria. Int. J. Food Microbiol. 121, 285-294.

Truelstrup Hansen, L. and B. Fonnesbech Vogel. 2011. Desiccation of adhering and biofilm *Listeria monocytogenes* on stainless steel: Survival and transfer to salmon products. Int. J. Food Microbiol. 146, 88-93.

Vescovo. M., G. Scolari and C. Zacconi. 2006. Inhibition of *Listeria innocua* growth by antimicrobial-producing lactic acid cultures in vacuum-packed cold-smoked salmon. Food Microbiol. 23, 689-693.

Weatherill, S. et al., 2009. Report of the independent investigator into the 2008 Listeriosis outbreak. Health Canada, Ottawa, Canada. (http://www.listeriosis-listeriose.investigation-enquete.gc.ca/lirs_rpt_e.pdf).

a. Follow-up

- Is a publication envisaged? Will this be in a journal or a publication? When will it appear?
  We are planning on publishing our results in a journal such as Journal of Microbiological Methods or Letters in Applied Microbiology. The manuscript will be finalised later this year.
- Is your fellowship likely to be the start of collaboration between your home institution and your host?
  Indeed, we are hoping to continue our collaboration through joint projects and if possible through having our graduate students perform parts of their experimental work in the labs in France and Canada thus benefitting from the different skill sets that we bring forth.
- Is your research likely to result in protected intellectual property, novel products or processes?
  The *rpoB*-PCR TTGE method that we have developed can be used to complement or replace currently used *16S rRNA* based methods depending on the objective of the study. The use of molecular based community characterization methods will be essential as RTE products preserved using novel hurdle methods including those based on biopreservation with LAB cultures seek approval and acceptance in industry as well as by the regulators.

b. Satisfaction

- Did your fellowship conform to your expectations?
  Yes, it did very much so. Discussions and meetings had been held with the host both by e-mail contact but also through a face-to-face meeting in November 2012, where the last details were planned. The host was well prepared and provided me with all the facilities that were required to successfully carry out the work.
- Will the OECD Co-operative Research Programme fellowship increase directly or indirectly your career opportunities? Please specify.
  The OECD Research Fellowship enabled me to spend 17 weeks at a French university in a vibrant research environment with extensive expertise in molecular community analysis. I enjoyed the opportunity to focus on a research field (bioinformatics and molecular microbial ecology) that I had wanted to explore for a long time but found difficult to secure the time required in my busy day-job as an educator, supervisor and administrator. The new skills will promote not only my career but more importantly that of the graduate students under my supervision as we implement these new skills into our research in Canada. Also, it is envisioned that the continued joint collaboration with ONIRIS will enable French students to spend time in an English-speaking university environment while some of my students will get the opportunity to spend time in a French-speaking environment at a top ranking French university.
- Did you encounter any practical problems?
  The universities in Nantes have created a lovely residence for visiting scientists (Maison des chercheurs etranger, MCE) which makes the arrival and lodging in Nantes very easy. The only reoccurring problem I had was with the internet access, which both at ONIRIS and MCE was loaded with restrictions due to various internet safety protocols. This meant, for example, that I was not able to use Skype to communicate with my graduate students in Halifax. However, other than that I encountered no problems.
- Please suggest any improvements in the Fellowship Programme.
  The Fellowship Program ran very smoothly. My contact with the secretariat was very pleasant and payments prompt.

c. Advertising the Co-operative Research Programme

- How did you learn about the Co-operative Research Programme?
  My university did not advertise the programme – perhaps this is due to Dalhousie not having a big agricultural focus. A friend of mine from another university made me aware of the program.
- What would you suggest to make it more “visible”?

The program could be advertised by contacting the offices of research as the individual universities. Most offices send out regular e-mails to advertise funding opportunities. My colleagues both here in Canada and internationally are now aware of the program – the good old word-of-mouth method.

- Are there any issues you would like to record?

  None. I sincerely thank both my wonderful hosts and the OECD Co-operative Research Program for making my 17-week stay in Nantes, France possible and a very pleasant and successful experience.