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Food safety of salt-cured cod products.

Effects of salt-curing, rehydration and thermal treatment on survival, growth and invasiveness of *Listeria* spp.

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Tromsø, August 2010
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2 LIST OF PUBLICATIONS

The thesis is based on the following publications that are referred to in the text by their roman numerals.

Paper I

Lorentzen, G., Olsen, R. L., Bjørkevoll, I., Mikkelsen, H. & Skjerdal, O.T. (2010) Survival of *Listeria monocytogenes* in muscle of cod (*Gadus morhua* L.) during salt-curing and growth during chilled storage of rehydrated product. *Food Control*. 21, 292-297

Paper II

Lorentzen, G., Ytterstad, E., Olsen, R.L. & Skjerdal, O.T. (2010) Thermal inactivation and growth potential of *Listeria innocua* in rehydrated salt-cured cod prepared for ready-to-eat products. *Food Control.* 21, 1121-1126

Paper III

Lorentzen, G., Mennen, S., Olsen, R.L. & Skjerdal, O.T. (2010) Invasiveness of *Listeria monocytogenes* strains of Caco-2 cells in response to a period of extreme salt stress reflecting salt-curing and rehydration of cod (*Gadus morhua* L.). *Food Control* (In press.)

Additional paper

In initial experiments performed within the SEAFOODplus, I studied the survival and growth of *Listeria innocua* and *Staphylococcus xylosus* after exposure to extreme salt-stress. These results are not included in this thesis but published in a book.

Leroi, F., Jofftaud, J. J., Arboleya, J. C., Amarita, F., Cruz, Z., Izurieta, E., Lasagabaster, A., Martinez de Maranon, I., Miranda, I., Nuin, M., Olabarrieta, I., Lauzon, H. L., **Lorentzen, G**., Bjørkevoll, I., Olsen, R. L., Pilet, M. F., Prevoust, H., Dousset, X., Matamoros, S. & Skjerdal, O. T. (2008) Hurdle technology to ensure the safety of the seafood products. IN BØRRESEN, T. (Ed.) *Improving seafood products for the consumer.* Cambridge, CRC Press.

3 NORSK SAMMENDRAG

Saltfisk og klippfisk (tørket saltfisk) av torsk (*Gadus morhua* L.) er høyt verdsatte produkter på grunn av den karakteristiske smaken, teksturen og de gode lagringsegenskapene. Dette er en viktig grunn til at både saltfisk og klippfisk av torsk har blitt tradisjonelle råvarer i blant annet det portugisiske, spanske og brasilianske kjøkken. Råvarene tilberedes ved utvanning slik at saltinnholdet reduseres til ca 2% NaCl. Den utvannede fisken kan brukes i flere typer retter inkludert spiseklare produkter. På grunn av det høye saltinnholdet har det vært ansett som trygt å spise utvannede produkter av fullsaltet fisk. Formålet med arbeidet har vært å studere matvaretryggheten med hensyn på *Listeria* spp. i fiskeprodukter som har vært fullsaltet. *Listeria monocytogenes* er en matvarebåren humanpatogen bakterie som finnes i miljøet og den er blant annet påvist i fersk fisk, skalldyr, kjøtt, melk, fjørfe og i vegetabilier. *L. monocytogenes* kan gi listeriose som er en alvorlig sykdom. Utsatte grupper er spedbarn, gravide, eldre og personer med et svekket immunforsvar. I tillegg kan bakterien også forårsake sykdom hos friske personer. Sammenlignet med en rekke andre patogener, etablerer *L. monocytogenes* seg lett i en næringsmiddelbedrift. Man vier derfor denne bakterien særlig oppmerksomhet, spesielt ved produksjon av spiseklare produkter.

I artikkel I ble overlevelse av *L. monocytogenes* og *Listeria innocua* under salteprosessen og deretter en eventuell vekst under den påfølgende kjølelagringen av det utvannede produktet studert. Fersk torsk ble tilsatt enten *L. monocytogenes* eller *L. innocua* i ulike nivå før salting. Rett etter utvanning hadde antall *Listeria* spp. blitt redusert med ca 1 log enhet sammenlignet med startnivået. Ved kjølelagring av det utvannede produktet begynte *Listeria* spp. å vokse. Resultatene viste at tilstedeværelse av *Listeria* spp. i fersk torsk før salting, ga vekst i det utvannede produktet under kjølelagring.

Varmeinaktivering og vekstpotensial for *L. innocua* under kjølelagring (4 og 8 °C) ble studert i artikkel II. For å simulere spiseklare produkter ble farse av utvannet saltfisk og fersk torsk varmebehandlet ved 55 og 60 °C i opptil 10 minutter. Resultatene viste at både matriks (utvannet saltfisk eller fersk torsk) og grad av saltstress påvirket varmeinaktiveringen av *L. innocua*. Ved lagring av de varmebehandlede produktene varte lagfasen 9-10 dager uavhengig av matriks eller saltstress, mens det var variasjoner mellom lagfasenes varighet for de rå produktene.

Til tross for at *L. monocytogenes* overlever høye salt konsentrasjoner, er det ikke rapportert listerioseutbrudd tilknyttet disse produktene. Det ble derfor studert om saltstress kan påvirke sannsynligheten for at *L. monocytogenes* gir listeriose (artikkel III). Forsøkene ble utført med Caco-2 celler (humane epitelceller fra tykktarm), der invaderingsevnen (invasivitet) for saltstresset *L. monocytogenes* ble studert. Invasivitet korrelerer med bakteriens evne til å forårsake listeriose. Resultatene viste at saltstress på tilsvarende nivå som ved saltfisk produksjon reduserte invasiviteten for *L. monocytogenes*. Bakterier som ikke hadde vært utsatt for ekstremt saltstress hadde en signifikant høyere invasivitet. Resultatene antydet at *L. monocytogenes* som har vært utsatt for et høyt salt stress er mindre farlig enn når de bare har vært eksponert for et konstant lavt salt innhold.

4 ABSTRACT

Salt-cured and dried salt-cured (klipfish) cod (*Gadus morhua* L.) are highly appreciated because of their characteristic taste, texture and storage stability. The products have been and still are traditional ingredients in Portuguese, Spanish and Brazilian cuisine. Salt fish and klipfish are mainly prepared by rehydration to lower the level of salt to approximately 2% NaCl. The rehydrated cod can be used in several dishes including ready-to-eat (RTE) products. Consumption of these products has been considered safe due to the high level of salt. The overall aim of this thesis was to study the food safety of salt-cured fish products using *Listeria* spp. as an indicator. Among *Listeria* spp., the human pathogen *L. monocytogenes* is the cause of listeriosis which is a serious disease especially in infants, pregnant woman, elderly and immune-suppressed individuals. Compared to many other bacteria, *L. monocytogenes* appears to be relatively resistant to processing of food. Consequently, this pathogen is one of the primary bacteria of concern, especially in RTE products.

In paper I, the survival of *Listeria innocua* and *L. monocytogenes* in muscle of cod during salt-curing and growth during chilled storage of the rehydrated product was studied. Fresh cod was inoculated with *L. innocua* and *L. monocytogenes* at different levels before salt-curing. After salt-curing and rehydration, the levels were within 1 $\log_{10}$ CFU/g lower than prior to salt-curing in all experiments. During storage after rehydration, growth of *Listeria* spp. was observed. These experiments demonstrated that long term exposure to very high salt concentrations did not eliminate *Listeria* spp., and that *Listeria* spp. being present in the fish prior to salt-curing could recover and grow in rehydrated salt-cured cod during chilled storage.

Thermal inactivation at 55 and 60 ºC and growth potential at 4 and 8 ºC of *L. innocua* in rehydrated salt-cured cod prepared for RTE products was studied (paper II). The results demonstrated that both level of salt stress and matrix i.e. rehydrated or fresh cod muscle, did affect the thermal inactivation of *L. innocua* towards non-linearity with an upward concavity. During subsequent storage of the thermally treated samples, the lag time of the strain was 9-10 days regardless of salt stress level or matrix. In the raw products (controls), however, the lag time varied with salt stress level and matrix.

Despite survival of *L. monocytogenes* during salt-curing, no outbreaks of listeriosis have been linked to such products. Thus, the ability of *L. monocytogenes* to cause listeriosis after salt stress, measured as its ability to invade human Caco-2 cells (invasiveness), was studied (paper III). *L. monocytogenes* was cultivated in BHI to stationary phase at 4 °C and exposed to either no salt or a salt stress period comparable to that applied in the production of salt-cured and rehydrated salt-cured cod. In addition, the strains were cultivated in BHI with 2% NaCl, which is similar to the salt content in rehydrated salt-cured cod and RTE products. The results show that extreme salt-stress exposure attenuated the invasiveness of *L. monocytogenes* whereas the ability to invade Caco-2 cells was significantly higher for the non salt and 2% stressed strains. As the ability to invade the Caco-2 cells correlates with bacterial virulence, the results suggests that *L. monocytogenes* represent a lower food safety risk when exposed to salt-curing with extreme NaCl concentrations than when exposed to a constant and low level of salt.

5 INTRODUCTION

L. monocytogenes is widespread in nature and is occasionally detected in raw fish, shellfish, meat, milk, poultry and vegetables. It is often the cause of food-borne diseases and food recalls (Teratanavat & Hooker, 2004). Compared to many other non-sporing bacteria, *L. monocytogenes* has the capacity to survive many food process hurdles such as chilling, acidification and light salting. Consequently, this pathogen is one of the primary bacteria of concern, especially in the industry producing ready-to-eat (RTE) products. Among the assortment of RTE products available today, some are prepared from salt-cured fish, such as cod. Products of minced fish of fresh or rehydrated salt-cured cod are increasing in popularity and are consumed as cold cuts, snacks, or as the main ingredient in heated dishes. To our knowledge, there have been no reported cases of human listeriosis associated with salt-cured cod products so far. However, the increasing production and consumption of RTE products based on salt-cured cod and the assumption that RTE products are one reason for the increased numbers of listeriosis cases (EFSA, 2006; Lianou & Sofos, 2007), makes it relevant to carry out hazard analysis with respect to *L. monocytogenes* in products based on salt-cured fish.

Historically, *Listeria* was recognized as an animal pathogen more than 70 years ago (Murray, Webb & Swann, 1926), but it was not regarded as a significant food-borne pathogen and hence it did not receive much attention from the food industry or authorities. In 1983, the transmission of the pathogen by contaminated food was first conclusively demonstrated by epidemiologic and laboratory investigations (Schlech et al., 1983) and the role of *L. monocytogenes* as a food borne pathogen became more evident. In order to understand the sources and characteristics of the organism in food and thereby obtain knowledge on how to control the organism, a significant programme of research was initiated. A total elimination of *L. monocytogenes* from most foods will probably be unlikely, but it is possible to reduce and

control this hazard in foods by hygienic measures and thus minimize the frequency and consequences of *Listeria* infection. However, as elimination of the bacterium is not possible, consumers will be exposed to low numbers of the pathogen despite the measures taken to control the hazard according to Codex (CAC/GL 61-2007). The risk groups among consumers associated with listeriosis are neonates, elderly people, and those compromised by pregnancy or an underlying illness such as malignancy or alcoholism. Conditions that require immunosuppressive procedures are also at increased risk (Seeliger & Jones, 1986). The usual clinical manifestation is meningitis sometimes accompanied by septicaemia. The infective dose, i.e. the minimum level of *L. monocytogenes* / g or ml responsible for listeriosis varies according to risk group and virulence of the pathogen. The reported levels of the pathogen associated with outbreaks may vary from 40 to 1000000 cfu/g (Jens Kirk Andersen, DTU, Denmark, personal communication).

Salt-cured products based on cod from the North-Atlantic fisheries, are traditional and highly regarded products, especially in Southern Europe and Latin America. In 2010, the consumption was estimated to be more than 150.000 tonnes (www.ssb.no). To obtain a fully salt-cured product (salt ripened product), the industrial process of salt-curing is completed within 3 weeks at 4 ºC. The products can be stored for a long time even at abuse temperatures due to their high salt-content (15-21%) and this was the reason why salt-cured cod was so important to the food supply in the past. The salt-cured fish must be rehydrated to about 2% salt before preparation and consumption. The sensory shelf life of rehydrated salt-cured fish at chilled temperature (4 °C) varies from 4 days to approximately 3 weeks, depending on the preservation methods used (Fernández-Segovia, Guevara, Eschriche, Diaz & Serra, 2003b; Magnússon, Sveinsdóttir, Lauzon, Thorkelsdóttir & Martinsdóttir, 2006). Salt-curing implies prevention of bacterial growth. However, it has been shown that some bacteria are not eliminated during salt-curing (Barat, Gallart-Jornet, Andrés, Akse, Carlehög & Skjerdal,

2006; Vilhelmsson, Hafsteinsson & Kristjansson, 1997). Among these are the specific spoilage organism in rehydrated salt-cured cod; *Psychrobacter* spp. (Bjørkevoll, Olsen & Skjerdal, 2003). After rehydration, these surviving bacteria may recover and start to grow in the rehydrated product. Survival of *L. monocytogenes* after a pre-treatment at a low level of salt followed by an increase to 25% NaCl (Duche & Labadie, 2003) or to 20% NaCl (Adrião et al., 2008) and after exposure to a fixed level of salt (20% NaCl or higher), has been reported previously (Guillier, Pardon & Augustin, 2005; Larson, Johnson & Nelson, 1999; Shahamat, Seaman & Woodbine, 1980; Tiganitas, Zeaki, Gounadaki, Drosinos & Skandamis, 2009). However, these studies were all performed using broth, and hence, studies of *L. monocytogenes* in salt-cured and rehydrated salt-cured cod are required.

Due to the high salt-concentration, the salt-cured cod must be rehydrated for 24–48 h in chilled water to obtain a salt content of about 2% before further preparation and consumption. Rehydration has traditionally been carried out in households. Today, consumers tend to spend less time on food preparation and prefer more convenient products, RTE and ready-to-heat products (Shiu, Dawson & Marshall, 2004). Commercial rehydration and distribution often lead to a longer storage period between completed rehydration and consumption than traditional rehydration at home. It has been shown that *L. monocytogenes* grows well in rehydrated salt-cured cod when it is introduced to the rehydration water and the bacteria may reach high levels within a few days (Fernández-Segovia et al., 2003b; Skjerdal, Pedro & Serra, 2002). As mentioned previously, it is not known whether *Listeria* spp. introduced before or during the salt-curing process is able to survive and grow in the product after rehydration. As the salt-curing and rehydration steps usually occur in different geographic areas, the stakeholders may have the overview of their own part of the production chain only. Thus, knowledge of the behaviour of *Listeria* spp. in the final product, when contaminated in early processing steps is important both from a food

safety and a legal point of view. Rehydrated salt-cured cod is mostly used in dishes that are thermally treated before consumption, but the food safety risk of *L. monocytogenes* still has to be considered due to the risk of cross contamination and undercooking e.g. by microwave heating (Fernández-Segovia et al., 2000; Skjerdal et al., 2002). Furthermore, raw fully salt-cured cod is also used in some recipes, such as Esgarrat, Bacalhau Rapido and Bacalhau Cru Desfiado which are not heated prior to eating (Gallart-Jornet, Roberto & Maupoey, 2004; Modesto, 1989; Pedro, Albuquerque, Nunes & Bernardo, 2004). Regarding products of minced fish, the possibility of undercooking of RTE products is present due to difficulties in controlling the core temperature. This is particularly important in products of minced fish since contaminating microbes are likely to be more evenly distributed compared to whole pieces of fish. Overheating is undesirable since it deteriorate the sensory quality due to development of a dry texture or a burned surface. Thus, adequate thermal treatment to ensure complete inactivation of *L. monocytogenes* without loss of sensory quality is a challenge. For RTE products with a long storage time between thermal treatment and consumption, the presence of *Listeria* may represent a food safety risk since surviving bacteria grow well at refrigerated temperatures. The risk of high levels of *Listeria* in the product consumed will however become less if it has a long lag time, i.e. the time needed for recovery of heat injured cells before growth occurs. Pathogen inactivation during thermal treatment is time and temperature dependent. However, other factors such as high salt levels, extreme pH values and low water activity may induce responses in the cell that enhance the resistance to thermal inactivation (Sofos, 2002; Yen, Sofos & Schmidt, 1990). In addition, thermal resistance of pathogens may also be affected by the matrix such as meat source and fat content (Ghazala, Coxworthy & Alkanani, 1995; Veeramuthu, Price, Davis, Booren & Smith, 1998).

As mentioned before, outbreak of listeriosis linked to RTE products made of rehydrated salt-cured cod has not yet been reported. A possible reason may be that the

virulence of *L. monocytogenes* is affected, i.e. reduced by the high salt-stress during the salt-curing process. To assess the infective potential of *L. monocytogenes*, a step in the infectious process, such as invasiveness can be measured in a model system using the intestinal epithelial cell line Caco-2 cells (Larsen, Norrung, Sommer & Jakobsen, 2002). The ability to invade these cells correlates with bacterial virulence. The invasive efficiency is then expressed as the percentage of the viable count of *L. monocytogenes* released from the Caco-2 cells of the initial inoculum (Tang, Foubister, Pucciarelli & Finlay, 1993). Exposure of *L. monocytogenes* to salt, low pH, thermal treatment, alone or in combination influences the invasiveness (Conte, Petrone, Di Biase, Ammendolia, Superti & Seganti, 2000; Galdiero, D'Isanto & Aliberti, 1997; Garner, James, Callahan, Wiedmann & Boor, 2006). Thus, the environmental conditions to which the bacterium is exposed before ingestion are decisive for its infective potential when it reaches the gut, as well as the number of *Listeria* spp. present in the food product.

The overall aim of this thesis was to study food safety with respect to *Listeria* spp. in salt-cured fish products. In more detail, food safety of traditional products such as rehydrated salt-cured or dried salt-cured products (klipfish). Specifically, the following questions were addressed:

- Does *Listeria* spp. survive in the muscle of cod during salt-curing and will it grow during subsequent storage of the rehydrated product?
- How is the thermal inactivation of *L. innocua* affected after exposure to salt-stress?
- How is the invasiveness of *L. monocytogenes* in Caco-2 cells affected after salt-stress exposure?

6 GENERAL BACKGROUND

6.1 History of *Listeria* spp.

The genus *Listeria* is named after the British surgeon, Lord Joseph Lister, who pioneered the concept of antiseptic surgery in the 1860's to prevent surgical sepsis (Bell & Kyriakides, 2005). At the beginning of the twentieth century *Listeria monocytogenes* was presumably first isolated in tissue specimens of infected patients. In 1926, the species name was originally *Bacterium monocytogenes* given by Murray and colleagues to describe a new Bacillus with potent monocytosis-producing activity in rabbits and guinea pigs (Murray et al., 1926). In 1929, the first unambiguous isolations of these bacteria from humans were reported (Nyfeldt, 1929). In 1940, the organism was given its definitive name *Listeria monocytogenes* (Seeliger et al., 1986). The relationship of *Listeria* to other bacteria remained vague until the 1970's when the genus was included in the tribe *Kurthia* of the Corynebacteriaceae family in Bergey's Manual Determinative Bacteriology published in 1934. Afterwards, *Listeria* was considered a genus of uncertain affiliation and placed with *Erysipelothrix* and *Caryophanon* after the family of Lactobacillaceae in the edition of 1974. Eventually, *Listeria* was classified with *Lactobacillus*, *Erysipelothrix*, *Brochothrix*, *Renibacterium*, *Kurthia*, and *Caryophanon* in Bergey's Manual of Systematic Bacteriology (Seeliger et al., 1986).

The genus *Listeria* was assumed to be monospecific, containing only the *L. monocytogenes* species, even many years after discovery. *L. denitrificans* was discovered in 1948. Subsequently, *L. grayi*, *L. murrayi*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, was discovered in 1966, 1971, 1981, 1983 and 1985, respectively (Rocourt & Buchrieser, 2007). Later on, it was discovered that *L. denitrificans* did not belong to the genus *Listeria* and it was reclassified as *Jonesia denitrificans* (Bell et al., 2005).

L. monocytogenes is able to infect both humans and animals causing meningitis, sepsis and abortion (Yildiz et al., 2007). *L. ivanovii* is restricted to sheep and cattle, in which it

causes septicemic disease, neonatal sepsis and abortion, but no brain infection (Iwanow, 1962). The other species are considered non pathogenic, although *L. seeligeri* and *L. welshimeri* have each caused human infections (Andre, 1987; Rocourt, Hof, Schrettenbrunner, Malinverni & Bille, 1986) and *L. innocua* has been implicated in a case of ovine meningoencephalitis (Walker, Morgan, McLauchlin, Grant & Shallcross, 1994). Compared to other pathogens, *L. monocytogenes* and listeriosis is a relatively new phenomenon. In the following, only *L. monocytogenes* will be described.

6.2 Taxonomy, enumeration and identification of *L. monocytogenes*

L. monocytogenes is a non-sporing, gram positive bacterium shaped as a short rod of 0.4 – 0.5 µm in diameter and 0.5 – 2.0 µm in length. The bacterium occurs either as single cells or in short chains. When cultured at 20 – 25 °C, the cell develops filaments of 6–20 µm and a tumbling form of motility is observed.

In contaminated food samples, the number of *Listeria* is usually relatively low compared to the background flora, consequently, the numbers of *Listeria* must be enriched to enable detection (Donnelly & Nyachuba, 2007). Traditional methods according to ISO (EN ISO 11290-1) and NMKL (Method no 136, 4th ed., 2007) involve a two-stage enrichment. In more detail, a sample is homogenized and incubated in an enrichment broth with reduced selectivity, i.e. half Fraser-broth at 30 °C for 24 h. Secondly, the sample is further enriched in a broth with full selectivity, i.e. Fraser broth at 37 °C for 48 h. After the selective enrichment, the sample is spread plated on selective agar. As *L. monocytogenes* is non fastidious, it can be plated on several agarmedia, such as ALOA (Agar *Listeria* according to Ottaviani and Agosti), LMBA (*L. monocytogenes* blood agar medium), OCLA (Oxoids chromogenic *Listeria* agar medium with equal operating principle as ALOA), Oxford or Palcam agar. According to the ISO standard, ALOA, Oxford, Palcam or other chromogenic agar can be used as a solid agar medium for identification and enumeration, while using the NMKL

method, only ALOA, LMBA or OCLA are allowed. Both Oxford and Palcam medium contain various combinations of antibiotics and other selective or diagnostic chemicals which facilitate the growth and preliminary identification of *Listeria* spp. The chemicals include acriflavine, cycloheximide, cefotetan, polymyxin B, ceftazidime, fosfomycin, colistin sulphate, lithium chloride, ferric ammonium citrate and aesculin (www.oxoid.com).

After exposure to environmental stress, i.e. salt-curing or thermal treatment, it is important to also identify and enumerate the potentially injured *Listeria* as sublethal damaged bacteria may recover and grow during storage of the product and thereby regaining its pathogenicity. To include the potentially injured cells, the sample is spread plated on a non selective medium where the sublethal damaged cells usually undergo repair and become functionally normal (Miller, Brandão, Teixeira & Silva, 2006). In more detail, the sample is spread plated on a non selective agar, i.e. TSAYE (TSA added yeast extract), incubated for 5–6 h at room temperature allowing recovery, and then overlaid with a selective agar medium, i.e. Palcam or Oxford. The yeast extract in TSA facilitates cell repair as it is an important source of B-complex vitamins (Busch & Donnelly, 1992). However, enrichment of TSA is not performed routinely, since this is not adapted in standard procedures of analysis.

These traditional and culture-based methods are both time- and labour consuming. As an alternative, rapid methods enabling an earlier detection of *L. monocytogenes* might be more appropriate. Commercially available rapid methods include colorimetric DNA probe, latex bead-based lateral flow immunoassay, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunofluorescence assay (ELFA), immunomagnetic separation (IMS) and real-time polymerase chain reaction (real-time PCR) (Brem-Stecher & Johnson, 2007; Werbrouck, Botteldoorn, Uyttendaele, Herman & Van Coillie, 2007).

6.3 Salt-curing of fish and critical control point

Salt-curing has been used as a preservation method for centuries up to the present time. Products of salt-cured fish are highly appreciated partly because of storage and nutritional stability, and partly because of their characteristic taste, texture and aroma. In salt-curing, the fish is preserved mainly by lowering the water activity (a_w), but lowering the muscle pH is also a contributing factor. Methods of salt-curing include dry salting, pickle salting, injection salting, brine salting and vacuum salting. Dry salting is performed by distribution of solid salt over the fish surface, resulting in a brine due to extraction of moisture from the fish muscle (Lauritzsen, 2004). The process of dry salting and rehydration is illustrated below (Fig.1).

Figure 1. Process of dry salting and rehydration of cod. Rehydrated cod can be processed further to products like Bolinhos de Bacalhau. The level of salt during the process is indicated on the Y-axe.

In the first step of salt-curing, the fish i.e. cod, is split or filleted, and most of the backbones are removed. Afterwards, the fish is placed on stacks with salt interspersed between the layers. The stack of fish and salt is stored for up to 7–14 days allowing the salt to penetrate into the muscle. During salt-curing, liquid is gradually released from the fish muscle. After storage, used salt is removed from the fish surface and the fish are restacked with new dry salt between the layers 2–3 times during salt-curing. In cod muscle, the water content is usually reduced from approximately 82 to 54%, and the salt content increases from

approximately 1 to about 20% (Thorarinsdottir, Arason, Geirsdottir, Bogason & Kristbergsson, 2002). Other common methods are pickle salting, injection salting, brine salting and vacuum salting (Lauritzsen, 2004). Salt-curing is normally completed within 3 weeks, but the product may also be stored for a much longer time before rehydration. The rehydrated product can be used intact as an ingredient in mixed dishes and as mince in Bolinhos de Bacalhau. Raw fully salt-cured cod is however also used as an ingredient in some typical salads of Portuguese and Spanish origin, namely Esgarrat, Bacalhau Rapido and Bacalhau Cru Desfiado (Gallart-Jornet et al., 2004; Modesto, 1989). Bolinhos and the salads listed are typical examples of RTE products. RTE products are consumed without any thermal treatment in advance and an increasing number of listeriosis cases are assumed to be related to the increased consumption of RTE products (Lianou et al., 2007). The approach in controlling the food safety hazard of *L. monocytogenes* associated with RTE products involves procedures of inspection and monitoring of the food products and the production facility. Hazard analysis control point (HACCP) is a process management system, designed for use in all segments of the food industry. The HACCP concept can be used to identify and control biological, chemical, and physical hazards in food along the production chain, from raw material until consumption of the product. An HACCP plan contains critical control points (CCPs) which are steps along the production chain at which control can be applied. These are essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level. A complete and accurate identification of the CCPs is fundamental to control the food safety hazards. Furthermore, the CCPs must be carefully developed and documented. Examples of CCPs may comprise thermal treatment, chilling or acidification. Afterwards, critical limits and procedures for monitoring the CCPs are established (Kornacki & Gurtler, 2007). Implementation of HACCP is mandatory according to the EC regulation (EC 178/2002). Regarding the process of salt-curing, usually no CCPs are identified as a microbial hazard as

the risk of surviving pathogens has been assumed to be minimal. However, *L. monocytogenes* is able to survive after exposure in media with 20% NaCl (Adrião et al., 2008; Duche et al., 2003). Thus, it should be considered if *L. monocytogenes* could be evaluated as a CCP in processing of salt-cured products.

6.4. Preservation methods

The overall goal in food production is to produce safe and wholesome products. This is achieved by using a variety of processing and preservation methods. The microbial effect of preservation is to kill or inhibit growth of spoilage and pathogenic microorganisms. In addition, preservation suppresses undesirable chemical and biochemical changes and helps to maintain the products desirable physical and sensory properties (Lado & Yousef, 2007). The preservation methods available involve physical, biological or chemical treatments. Physical preservation includes heating, cooling, freezing, radiation, high-pressure processing and packaging. Biological treatments include fermentation that controls spoilage and pathogenic microorganisms and chemical treatments including addition of antimicrobial agents i.e. sodium chloride, sodium nitrite, benzoate, propionate or sorbate. The use of preservation methods imposes a stress situation that limits the growth and survival of microorganisms. The sensitivity to stress within the population of bacteria in food is assumed to follow a normal distribution. This is observed by different times of loss of viability or reduced growth. Few individual cells within the population may show extreme tolerance of stress and as such are important as the basis of colonisation of new environments. Stress is defined as any change in the genome, proteome or environment that imposes either reduced growth or survival potential (Booth, 2002). Environmental stress leads to attempts by a cell to restore a pattern of metabolism that either fits it for survival or for faster growth. In the following, use of sodium chloride, low temperature storage and thermal treatment will be further described with respect

to the inhibition or inactivation of *L. monocytogenes.* Other preservation methods will only be briefly discussed in context of the methods described.

6.4.1 Water activity and sodium chloride

Water activity is measured as the ratio of vapour pressure of the water in food, to the vapour pressure of pure water at the same temperature and is one of the most important factors controlling the rate of deterioration of food (Farkas, 1997).Microbial growth is inhibited by dehydration as the water necessary for growth is removed, i.e. if the water activity (a_w) is reduced. *L.monocytogenes* is able to survive at a_w below 0.90 that corresponds to a salt level of 15.6%. The strain tolerates extremely high levels of salt and the antilisterial activity of salt, has been thoroughly studied. It has been reported that *L. monocytogenes* survived in commercial cheese brine (23.8% NaCl, pH 4.9) stored for 259 days at 4 °C (Larson et al., 1999) and survived after exposure to 20% NaCl or higher (Guillier et al., 2005; Shahamat et al., 1980; Tiganitas et al., 2009). Furthermore, studies of survival after a pre-treatment at a low level of salt, followed by an increase to 25% NaCl (Duche et al., 2003) or to 20% NaCl (Adrião et al., 2008) have been reported. Therefore, use of high salt concentrations should not be considered as a permanent and reliable method with which to eliminate *L. monocytogenes*. After the exposure to high salt concentrations in combination with lower pH, the morphology of *L. monocytogenes* changed from short rod shapes towards filamentous and deformed shapes (Bereksi, Gavini, Bénézech & Faille, 2002; Zaika & Fanelli, 2003) with a strongly hydrophilic surface. The observed changes in cell morphology altered the adhesion properties to different surfaces (Bereksi et al., 2002). However, it has not been reported whether the changes affect the persistence of the strain in the production facilities or on the equipment. By increasing the level of salt, *L. monocytogenes* was protected against the lethal effect of thermal treatment (Juneja & Eblen, 1999). Furthermore, the thermotolerance of *L. monocytogenes* increased slightly with NaCl concentration when sodium pyrophosphate

was included in a gravy formulation. This was explained by a higher temperature of denaturation of the ribosomes, and hence, increased thermotolerance (Stephens & Jones, 1993).

Sodium chloride inhibits microbial growth primarily by loss of cellular water (Davidson, 1997). The inhibition effect is related to its ability to reduce a_w and create unfavourable conditions for microbial growth. Transport of water vapour from a food product to the surroundings depends on moisture content, the composition of food, temperature and humidity. At a constant temperature, the moisture content of food changes until it comes into equilibrium with water vapour in the surrounding air. At the equilibrium moisture content, the food product neither gains nor loses water on storage under those conditions. The a_w requirements of microorganisms varies and in the vital range of growth, decreasing the a_w increases the lag phase of growth and decreases the growth rate. In general, food-borne pathogens are inhibited by a water activity of 0.92 or less, this is equivalent to a salt concentration of 13%. Most food products, fruits, vegetables, meats and fish have a_w levels of 0.98 or higher, which is not sufficient to reduce the growth rate of undesired bacteria.

6.4.2 Low temperature storage

L. monocytogenes is a psychrotrophic bacterium with an optimum growth temperature between 30 and 37 °C, but it may grow within the range of minus 1.5 to plus 45 °C (Seeliger et al., 1986). However, the growth rate at low temperature is quite low. At 4 °C, the levelof *L. monocytogenes*, inoculated to Channel catfish, increased 2 log units during 15 days of storage (Fernandes, Flick & Thomas, 1998). The limits of growth at refrigeration temperature are strongly dependent on medium pH (Tienungoon, Ratkowsky, McMeekin & Ross, 2000). However, the overall food safety challenge with respect to *L. monocytogenes* is its ability to grow at refrigeration temperature.

Studies of the cell membrane show that the membrane phospholipids must remain in a liquid-crystalline state to maintain membrane fluidity and thereby be able to grow at low temperatures. The fatty acid composition determines whether the membrane phospholipids are in the liquid-crystalline state or not (Lado et al., 2007). In *L. monocytogenes*, the level of branched-chain fatty acids in the cell membrane is more than 95%. At 37 °C, the major fatty acids are anteiso-$C_{15:0}$ (41-52%), anteiso-$C_{17:0}$ (24-51%), and iso-$C_{15:0}$ (2-18%). While grown at 5 °C, the anteiso-$C_{15:0}$, increases to 65-85% of the total membrane fatty acids (Annous, Becker, Bayles, Labeda & Wilkinson, 1997). The reduction of long aliphatic chains ($C_{17:0}$) and the increase in asymmetric branching reduce van der Wals bonds among membrane constituents. Hence, the tight packing of membrane phospholipids at low temperature is reduced and the membrane fluidity of the pathogen is maintained (Lado et al., 2007).

Below the temperature of minus 1.5 °C, *L. monocytogenes* is able to survive, although it is not able to grow. The ability to survive during freezing is partly dependent on the temperature, freezing rate and freezing menstruum (El-Kest & Marth, 1991; El-Kest & Marth, 1992). A low freezing temperature and rapid freezing appeared to be the most favourable to bacterial survival (El-Kest et al., 1991). Freezing and storage at minus 18 °C inactivated 1 to 2 log units and injured >50% of the pathogen population. Multiple procedures of freezing and thawing are more detrimental to the survival of *Listeria* than a single cycle of freezing (El-Kest et al., 1991), due to the rupturing of the cell wall that involves leakage of cytoplasmic content. In general, freezing followed by storage causes limited inactivation of the pathogen. Thus, contamination of frozen food should be prevented.

6.4.3 Thermal treatment

Thermal treatment is one of the most reliable and commonly used methods to ensure food preservation and food safety. However, thermal treatment can alter the quality of muscle food by affecting the liquid loss (Ofstad et al., 1995) and thereby influencing texture and content of

water soluble nutrients (Gregory, 2008). Thermal treatment may also modify amino acids making them less available as nutrients (Damodaran, 2008)Survival and thermal resistance of *L. monocytogenes* under different conditions in a variety of foods have been investigated. Thermal resistance of *L. monocytogenes* is influenced by many factors such as strain variations, growth phase, growth conditions, exposure to thermal shock, acid and the composition of the heating menstruum. After the thermal treatment, the number of surviving cells detected depends on the ability to recover, recovery method, recovery medium and incubation conditions used.

Thermal resistance varies among different *L. monocytogenes* strains (Doyle, Mazzotta, Wang, Wiseman & Scott, 2001). However, due to different test conditions, it is not possible to conclude that one particular strain is the most thermal resistant. The strain Scott A has widely been used in experiments and it appears to be intermediate in thermal resistance. When *Listeria* spp. is cultivated to stationary phase, it is more resistant to thermal stress than in the exponential phase of growth (Doyle et al., 2001). When comparing *L. innocua* and *L. monocytogenes*, it has been shown that *L. innocua* seems to be more resistant to heat. Thus, *L. innocua* is considered as a suitable model for estimating thermal tolerance of *L. monocytogenes* (Char, Guerrero & Alzamora, 2009; Fairchild & Foegeding, 1993; Foegeding & Stanley, 1991).

The relationship between duration of thermal treatment and log count of survivors is commonly referred to as the survival curve. If this is linear, i.e. the inactivation follows first order kinetics, the thermal resistance parameters can be readily calculated. The time required to inactivate one log unit of the microbial population at a given temperature (i.e. D-value) is an expression of its thermal resistance. Traditionally, thermal inactivation of vegetative cells and microbial spores has been assumed to follow first-order kinetics and the calculation of the safety of commercial thermal treatment is mainly based on this assumption. However, thermal

inactivation does not always follow first order kinetics, i.e. being linear, as non-linearity of the survival curve is occasionally observed. The non-linearity is explained by each individual organism dying or being inactivated at a specific time. This results in a spectrum of thermal resistances in the population, i.e. some organisms are destroyed sooner or later than others and the differences in distribution of thermal resistances results in non linear survival curves (Peleg & Cole, 1998). Some components in food may protect *L. monocytogenes* against heat. The resistance of *L. monocytogenes* to mild heat increases with increasing pH (Juneja et al., 1999), fat content (MacDonald & Sutherland, 1993), salt concentration (Juneja et al., 1999), high concentrations of stabilizers used in ice cream (Holsinger, Smith, Smith & Palumbo, 1992), and the presence of stabilizers such as guar gum (Piyasena & McKellar, 1999). High salt concentration increases the denaturation temperature of *Listeria's* 30S ribosomal subunit, which contributes to thermal tolerance of the pathogen (Stephens et al., 1993). These variations of thermal resistance due to food composition may be associated with availability of nutrients that support the growth of *Listeria*. Starvation of the pathogen can trigger the stress-adaptive response and thereby increase the pathogen's tolerance to heat (Lou & Yousef, 1996). When *L. monocytogenes* is exposed to sublethal stress, i.e. thermal shock, acids, oxidants, starvation or high osmolarity, it may develop an adaptive response to subsequent thermal treatments (Jørgensen, Stephens & Knøchel, 1995; Lou et al., 1996; Sergelidis & Abrahim, 2009). Sublethal thermal treatment includes slow heating, holding food in warm trays, inadequate thermal treatment or hot water washing. The mode of adaptive thermal tolerance is transient (Jørgensen, Panaretou, Stephens & Knøchel, 1996) and thus, the potential of heat adaptation should be taken into account when planning the parameters of thermal processing.

6.5 The health risk aspect of *L. monocytogenes*

Listeriosis is responsible for approximately 500 fatalities annually in the USA, or about 28% of all deaths caused by known food-borne pathogens (Clark et al., 2010). If a food product contains more than 100 CFU (colony forming units) *L. monocytogenes* /g or ml, it is considered a high risk food (Rocourt & Cossart, 1997). However, 100 CFU/g does not reflect the infective dose, i.e. the number of bacteria required to cause listeriosis. This is because the precise number of the pathogen actually ingested and the exact quantity of the food product will probably remain unknown. Despite all efforts to make food safe, it is assumed that a large number of consumers are exposed to low levels of the pathogen due to the consumption of RTE products. The prevalence of *L. monocytogenes* in raw and RTE seafood and fish products, especially smoked fish can be up to 25% (Farber, 1991). The limited numbers of listeriosis outbreaks suggests that the low numbers of *L. monocytogenes* present in products is insufficient to cause severe illness in most healthy consumers. This assumption is supported by the fact that *L. monocytogenes* was isolated from 1-6% of faecal samples from healthy people (Ooi & Lorber, 2005; Rocourt et al., 1997).

There are two types of listeriosis, non-invasive and invasive listeriosis. Non-invasive listeriosis occurs when the infection remains limited to the digestive system. Invasive listeriosis involves an infection that spreads into the blood, and / or to the central nervous system, before spreading to the brain which can be potentially fatal. Non-invasive listeriosis causes mild flu like symptoms such as fever and muscle pain, as well as diarrhoea. It is difficult to estimate the number of non-invasive listeriosis cases as it is easy to mistake the condition for flu and hence, it is seldom reported. People with weakened immune systems are particularly vulnerable to invasive listeriosis. This group includes the elderly (>60yrs), pregnant women and their foetuses, babies who are less than one month old, people with a health condition that weakens the immune system, such as HIV-AIDS (human

immunodeficiency virus leading to acquired immunodeficiency syndrome), or diabetes. People having medical treatment that weakens their immune system, such as chemotherapy or radiotherapy are also vulnerable.

The prognosis for non-invasive listeriosis is good. The symptoms seem to be short lived and usually pass within three days without the need for medical treatment. Due to complications that arise from the secondary infection, the prognosis of invasive listeriosis is poor, with a fatality rate of 21% in Denmark (Antal, Høgåsen, Sandvik & Mæhlen, 2007; Gerner-Smidt et al., 2005) and 41-45% in Norway (Antal et al., 2007). Invasive listeriosis is rare, however in Europe increasing incidences of listeriosis have been observed among persons more than 60 years of age (Goulet, Hedberg, Le Monnier & de Valk, 2008) and public health officials are concerned by the increase in the number of cases since 1999 (EFSA, 2006).

6.5.1 Bacterial invasion

Invasive listeriosis begins with ingestion of contaminated food. Subsequently *L. monocytogenes* can enter the intestinal barrier. During the infection several tissues are infected, showing that the strain is able to invade a wide variety of nonphagocytic eukaryotic cells and exist as intracellular parasites (Fsihi, Steffen & Cossart, 2001; Tang et al., 1993). Tissue cultures assays of bacterial invasion have demonstrated that the strain is capable of penetrating various cell types, including hepatocytes (Dramsi, Biswas, Maguin, Braun, Mastroeni & Cossart, 1995; Gregory, Sagnimeni & Wing, 1997) and endothelial cells (Greiffenberg et al., 1998; Parida et al., 1998).

While residing in the intracellular environment the strain is protected against some antibiotics and the host's immune system. In addition, it provides the microorganism with a source of nutrients, and thus, invasion is an adaptation to exploit a niche inside the host's body. The first step in the invasion process is attachment to the host cell surface, followed by

internalization into the host cell. Afterwards, the bacteria may remain within the cell, where it may or may not replicate. Alternatively, the bacteria may escape from the cell into extracellular space where it can invade other cells.

While many strains of *L. monocytogenes* are naturally virulent and capable of causing severe illness, others are avirulent and unable to become established within the host cell. Based on serological reactions between somatic (O) / flagellar (H) antigens and their corresponding antisera, the strains of *L. monocytogenes* are divided into 12 serotypes, i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 (Liu, Lawrence, Ainsworth & Austin, 2007). Among these serotypes, about 98% of the outbreaks that occurred in the past two decades were caused by 4b, 1/2a and 1/2b (Doumith et al., 2004; Jacquet, Gouin, Jeannel, Cossart & Rocourt, 2002; Kathariou, 2002; Rocourt et al., 1997). Among the 3 serovars, serovar 4b strains have been responsible for 33 to 50% of sporadic listeriosis cases worldwide and for all major food-borne outbreaks since 1981 (Rocourt et al., 1997). Despite this, isolates recovered from food in numerous countries mostly belong to serogroup 1/2a or 1/2b (Rocourt et al., 1997). Serotypes 4a and 4c are rarely responsible for human infections, although they are both commonly isolated from animals, food or environment (Doumith et al., 2004). In experiments using mouse models, epidemic isolates often demonstrate higher infectivity and mortality than isolates from the environment (Kim, Bakko, Knowles & Borucki, 2004). By using phylogenetic analysis, the serotypes are divided into three groups, i.e. lineages. Lineage I includes 1/2b, 3b, 4b, 4d and 4e, lineage II includes serotypes 1/2a, 1/2c, 3a and 3c, and finally lineage III includes 4a, 4c and 7 (Liu et al., 2007). In general, strains of lineage I are more invasive than strains belonging to lineage II (Jensen et al., 2008).

6.5.2 Methods for measuring virulence properties

To evaluate the actual health risk associated with *L. monocytogenes* it is crucial to obtain knowledge about the virulence properties. There are several published methods for

determining the virulence properties of *Listeria* species, i.e. the presence of specific virulence genes (Jaradat, Schutze & Bhunia, 2002), by the production and expression of virulence factors (Jaradat et al., 2002; Werbrouck et al., 2009), mouse assays (Kim et al., 2004), *in-vivo* cell line bioassays (Olier, Pierre, Lemaitre, Divies, Rousset & Guzzo, 2002; Van Langendonck et al., 1998) and hydrophobicity assays (Doyle, 2000; Pedersen, Skouboe, Rossen & Rasmussen, 1998). Methods using molecular assays such as real time polymerase chain reaction (real time PCR) are used to determine the presence and expression of virulence genes (Duodu, Mehmeti, Holst-Jensen & Loncarevic, 2009; Werbrouck et al., 2007). In the following, methods involving *in-vivo* and *in-vitro* bioassays will be presented.

6.5.3 *In-vivo* and *in-vitro* bioassays

To test the virulence of *L. monocytogenes*, *in-vivo* bioassays, using animals, or *in-vitro* bioassays, using cells cultures, are both well established methods. The former method is controversial from an ethical point of view and more expensive than the latter one. The use of animals in testing virulence represents a valuable approach and mice have been preferred due to their ease of handling and their comparatively low cost. However, several other animal species such as rat, gerbil, guinea pig, rabbit and nonhuman primate have occasionally been utilized in listeriosis research (Bakardjiev, Stacy, Fisher & Portnoy, 2004; Blanot et al., 1997; Czuprynski, Faith & Steinberg, 2003; Farber, Coates, Beausoleil & Fournier, 1991; Lecuit et al., 2001; Mackaness, 1962; Schlech III, 1993; Smith et al., 2003). Guinea pigs are appropriate for oral infection as they possess a human like E-cadherin receptor that is vital for InlA-mediated internalization (Lecuit et al., 2001).

As an economical alternative to animal testing, *in-vitro* bioassays have been used in experiments to test the virulence of *L. monocytogenes*. These assays are based on the strain's capacity to adhere, enter, grow in and spread to other cells (cell-to-cell spread). *L. monocytogenes* has a large collection of specialized molecules to take on these challenges.

In detail, the strain produces internalins (particularly InlA and InlB) to assist the cell invasion, listeriolysin (LLO) to facilitate escape from primary vacuoles, actin (ActA) to aid cell-to-cell spread, phospholipase (PlcA and PlcB) to coordinate LLO and ActA for vacuole escape and spreading to neighbouring cells (Liu et al., 2007). The *in-vitro* cell bioassay is designed to simulate the barriers to *L. monocytogenes* during infection. Several cell lines are used for this purpose such as entero-cyte like Caco-2 cells, adenocarcinoma HT-29, epithelial Henle 407 and L2 (Dancz, Haraga, Portnoy & Higgins, 2002; Gründling, Gonzalez & Higgins, 2003; Kushwaha & Muriana, 2009; Midelet, Kobilinsky & Carpentier, 2006).

The *in-vitro* bioassay is performed by incubation of a known amount of *L. monocytogenes* in suspension to a confluent monolayer that are originated from a set number of seeding cells, i.e. Caco-2 cells (Liu et al., 2007). The extracellular bacteria are removed by gently washing and finally killed using gentamycin. Gentamycin is an antibiotic which does not enter mammalian cells below a specified concentration. The cells are then washed and lysed with a detergent like Triton x-100. The lysed cells with the remaining and surviving *L. monocytogenes* are serially diluted and are subsequently enumerated on a solid agar medium. By varying the testing purposes, four *in-vitro* bioassays can be performed, i.e. adhesion to the cell surface, invasion, intracellular growth, and finally the cell-to-cell spread. The latter bioassay is also described as a plaque-forming bioassay (Van Langendonck et al., 1998). By varying the lengths of additional incubation time and concentration of gentamycin it is possible to I) evaluate the capacity and efficiency to adhere and / or invade mammalian cells, II) multiply intracellularly, III) spread to neighbouring cells and finally, IV) cause cytopathogenic damage and form plaque. By using these methods, it is possible to ascertain the virulence of *L. monocytogenes*. Strains with a high virulence are more capable of adhering and entering Caco-2 cells, more efficient in escaping from the vacuoles, able to perform intracellular growth and finally to spread to neighbouring cells than the low virulence strains

(Jaradat & Bhunia, 2003; Jensen et al., 2008). High virulence strains of *L. monocytogenes* tend to produce more severe cytopathogenic damage in Caco-2 cells (Pine, Kathariou, Quinn, George, Wenger & Weaver, 1991; Van Langendonck et al., 1998) and to form more plaques with H29-cells (Roche, Velge, Bottreau, Durier, Marquet-van der Mee & Pardon, 2001), compared to the low virulence strains.

6.5.4 Invasiveness and preservatives

It is well documented that the ability of *L. monocytogenes* to invade Caco-2 cells is affected by the presence of NaCl, organic acids, pH, growth temperature and oxygen restriction as well as interacting effects. In more detail, *L. monocytogenes* grown in BHI with 3.0, 5.0 and 7.5% NaCl at pH 5.0 demonstrated a complete loss of invasiveness due to the increased level of salt, while at pH 7.0, the invasiveness was unchanged (Galdiero et al., 1997). However, increased invasiveness of *L. monocytogenes* grown in BHI with 2.2% NaCl at pH 7.0 was observed in another experiment (Garner et al., 2006). In BHI with 2% NaCl, the invasiveness of *L. monocytogenes* was higher at pH 7.4 than at pH 5.5 when cultured at 37 ºC and the same differences were observed at 7 ºC (Garner et al., 2006). When grown under oxygen restriction, *L. monocytogenes* were approximately 100 fold more invasive than similar cultures grown without oxygen restriction (Andersen, Roldgaard, Christensen & Licht, 2007).

7 MAIN RESULTS AND GENERAL DISCUSSION

Based on the aims presented in the introduction, the experiments were carried out in three parts, namely, I) study the survival of *Listeria* spp. during salt-curing and growth after storage of the rehydrated product, II) study thermal inactivation of salt-stressed *L. innocua* and the ability to recover during subsequent storage, and III) study the invasiveness of salt-stressed *L. monocytogenes* of Caco-2 cells. Each part is represented in papers I, II and III, respectively.

In paper I, six strains of *L. monocytogenes* isolated from fish and the fish processing environment and one *L. innocua* were used in studies of survival during salt-curing. In studies of thermal treatment (paper II), only *L. innocua* was used due to the risk of cross contamination to pilot scale equipment used for other purposes. Thermal inactivation of *L. innocua* was compared with *L. monocytogenes* NCTC 11994 and *L. monocytogenes* 4006 at 60 °C in 180 s in model experiments using broth. All three strains tested were found to have a very similar survival pattern, and thus, *L. innocua* was used. Regarding the invasiveness studies, seven strains of *L. monocytogenes* belonging to different serotypes and origin and one *L. innocua* (control) were used (paper III).

The survival and growth of *L. innocua* and *L. monocytogenes* in cod muscle was studied during salt-curing and subsequent chilled storage of the rehydrated product (paper I). The salt contents in fresh, salt-cured and rehydrated cod were 0.5-0.6, 17.3-19.6 and 1.2-2.9%, respectively. After salt-curing and rehydration, both strains were present in the inoculated fish samples in levels not lower than 1 log unit below the corresponding inoculation level. The growth of *L. innocua* during storage of the rehydrated fish samples at 4 and 7 °C was also analysed. When the inoculation level in the fresh cod prior to salt-curing was only 1 log CFU/g cod, the level of *L. innocua* was still below the detection level in the rehydrated cod after 10 days of storage at 4 °C, but could be detected after 10 days at 7 °C.

Parallel experiments with *L. monocytogenes* with an inoculation level of approximately log 6 before salt-curing showed the same pattern of survival during salt-curing and growth during subsequent storage as *L. innocua*. In all these experiments, the salt-curing period before rehydration was a minimum of 3 weeks in order to mimic the salt-curing process in industry.

Food Authorities have so far not considered *L. monocytogenes* as a food safety risk in the salt-curing industry and this is reasonable, as most traditional dishes of salt-cured cod are heat treated shortly after rehydration. For commercially rehydrated salt-cured cod, the chilled storage period between rehydration and preparation is often longer than when rehydrated at home. The results show that *Listeria* spp. are able to survive in cod during salt-curing. During storage of the rehydrated product, *Listeria* spp. did grow, particularly at elevated refrigerated temperatures. These results imply that the *Listeria* risk has to be reconsidered. Even though chilled rehydrated salt-cured cod products have a short sensory shelf life (Bjørkevoll et al., 2003; Fernández-Segovia, Garrigues, Carot & Escriche, 2003a; Magnússon et al., 2006), the results show that there is a possibility that low levels of *L. monocytogenes* in salt-cured cod may grow to infective levels for sensitive consumers before the rehydrated product is considered sensory unacceptable. The risk that *Listeria* infected rehydrated salt-cured cod products reach the market could be lowered by improved hygienic routines, increased frequency of analysis of the rehydrated products, and storage below 4 ºC. However, as the temperature in refrigeration cabinets used by retailers is commonly above 4 ºC, the control strategy has to focus on prevention of conditions that lead to contamination, limit growth of *Listeria* or to limit the shelf-life to a period where growth is unlikely to reach levels of infective doses. If *L. monocytogenes* is present, the overall priority is to eliminate the pathogen prior to consumption.

Rehydrated salt-cured cod is commonly used in several dishes including RTE products. Regarding products using minced fish, the possibility of undercooking is present due to difficulties in controlling the core temperature. Thus, the aim of paper II was to study thermal inactivation of *L. innocua* as a function of salt stress exposure and matrix, i.e. rehydrated salt-cured cod or fresh cod. As some products of thermally treated minced fish are consumed cold several days after preparation, the survival and growth potential during storage of *L. innocua* after thermal treatment was studied. In total, three experiments were performed, covering both salt stressed and non salt stressed *L. innocua* and both matrixes, namely, rehydrated salt-cured cod with *L. innocua* added 1) before salt-curing (salt stressed), 2) during rehydration (non salt stressed) and finally 3) in fresh cod. All samples were minced and heated at 55 °C up to 10 min, and the survival curve was calculated and fitted using the Weibull model (Peleg et al., 1998; van Boekel, 2002). A non-linear trend with tailing was observed in rehydrated cod with salt stressed *L. innocua*. This observation was supported by the parameters obtained with the Weibull model. However, in rehydrated cod and in fresh cod both with the non salt stressed strain, the survival curves were almost linear. The different shapes of the survival curves could probably be explained by the previous salt stress exposure of the strain prior to thermal treatment and different matrices. In more detail, in the rehydrated cod with the salt stressed strain, the strain was exposed to a severe salt-stress of approximately 18% NaCl for 21 days followed by reduction to 2–3% NaCl for 48 h. It has been shown that physical stress of bacteria prior to thermal treatment leads to formation of subcultures with different thermal tolerance resulting in concavity of the survival curves (Peleg et al., 1998; van Boekel, 2002), therefore the observed non-linearity was not surprising. In the case of rehydrated cod, the strain was added during the rehydration process and thus exposed to salt stress of a certain level. In this product, the salt stress was far less compared to the former, and a more linear survival curve, as observed, could therefore be

expected. In fresh cod, linear survival curves could also be expected as a low exposure to salt-stress occurred. Even though the survival curves for fresh and rehydrated cod both with non salt stressed *L. innocua* were linear, the slope of the curves was different. More precisely, the time needed to obtain a 3 log reduction of non salt stressed *L. innocua* in rehydrated cod and fresh cod were about 6–8 and 4 min at 55 °C, respectively. The time required to obtain a 3 log reduction of salt stressed *L. innocua* in rehydrated cod was in the range of 6–8 min, i.e. similar to that observed for the corresponding non salt stressed strain in the rehydrated product. The observations indicated that the matrix; rehydrated or fresh cod muscle, may affect the thermal inactivation of the strain. However, as the salt levels in rehydrated cod and fresh cod, both with non salt stressed *L. innocua*, were approximately 2–3 and 0.5%, respectively, the observed differences in thermal inactivation could also be due to a different level of salt.

In total, the results presented in paper II demonstrate that both salt stress and state of the matrix, i.e. rehydrated or fresh cod muscle did affect the thermal inactivation of *L. innocua* towards a lower probability of dying for the remaining cells. Assuming linear trends only by using first order kinetics, the calculated thermal death time had been shorter than was actually required to obtain a defined reduction of *Listeria*.

To study the ability to recover and grow after the thermal treatment, the samples were stored at 4 and 8 °C up to 10 days. In addition, samples without thermal treatment, controls, were stored under the same conditions. The ability to recover and grow was determined by the length of the lag time. The lag time of the strain in raw products (controls) varied according to salt stress and state of the matrix, i.e. rehydrated salt-cured cod or fresh cod. In the thermally treated samples, however, the lag time of the strain was similar regardless of salt stress level or matrix. These results indicate that the history of salt stress exposure of *Listeria* prior to thermal treatment does not need to be considered in determination of shelf life in products of

thermally treated fish mince. In fact, detailed knowledge about the salt stress history before thermal treatment is of less importance for the growth potential of *L. innocua* in thermally treated products than in raw products.

If *L. monocytogenes* is detected in a product, it is considered as a food safety risk if it is consumed, i.e. involving a risk of developing listeriosis. As a third and final part of the thesis, the invasiveness of *L. monocytogenes* exposed to extreme salt stress was studied using a Caco-2 cell bioassay (paper III). Invasiveness is the ability of the strain to enter the cell barrier and it correlates with bacterial virulence. The invasiveness of *L. monocytogenes* cultivated to stationary phase was tested after exposure to 21% NaCl for 96 h at 4 °C, followed by 2% NaCl for 48 h at 4 °C and compared with the corresponding non salt stressed strains. To study any effect on the growth phase of *L. monocytogenes*, the invasiveness was tested both at early and late stationary phase. The selected salt stress scenario is comparable to that applied in the production of salt-cured and rehydrated salt-cured cod. For comparison, invasiveness studies of *L. monocytogenes* cultivated with BHI with 2% NaCl, reflecting the salt levels of some RTE products were included. The viable count of released *L. monocytogenes* from Caco-2 cells was then determined as the invasion efficiency, expressed as the percentage of the initial bacterial inoculum (Tang et al., 1993).

In general, the invasion efficiencies of *Listeria* spp. of Caco-2 cells were below 1% in all experiments. This is in the same range or slightly lower than that found in comparable studies (Garner et al., 2006) including studies where some of the same strains were used (Duodu, Holst-Jensen, Skjerdal, Cappelier, Pilet & Loncarevic, 2010).

The invasion efficiencies of *Listeria* spp. cultivated in BHI to early stationary phase ranged from 0.0032 (*L. monocytogenes* 4006) to 0.13% (*L. monocytogenes* 3998) of the initial inoculum. The invasion efficiency of *L. monocytogenes* 1001 isolated from light salt-cured salmon and 3998, a clinical isolate, were both significant higher ($p<0.05$) than the other

strains. Although these are different serotypes, namely 1/2b and 4b, they both belong to lineage I (Liu et al., 2007). The remaining strains with lower invasion efficiency belonged to both lineages I and II. No statistically significant differences among the lineages were observed because lineage I strains covered the invasive spectrum of lineage II. Strains of lineage II have been reported to be less invasive than strains belonging to lineage I (Jensen, Larsen, Ingmer, Vogel & Gram, 2007) and strains of lineage II are isolated from foods significantly more frequently than from human listeriosis cases (Gray et al., 2004). As in our study, no significant differences of invasion efficiency between strains isolated from humans and those isolated from food were observed (Larsen et al., 2002).

To study potential effects of growth phase effects on the invasion efficiency of *L. monocytogenes*, the non salt stressed strains were cultivated for an additional 48 h. The invasion efficiency ranged from 0.007 (*L. monocytogenes* 1994) to 0.092% (*L. monocytogenes* 1001) and no significant grouping among the strains was observed. Compared to early stationary phase, the invasiveness of *L. monocytogenes* 1001 and 3998 declined while that of *L. monocytogenes* 3442 increased. Growth phase effects on *Listeria* spp. Caco-2 invasion capacity were less pronounced for the remaining strains.

Strains cultivated to stationary phase and stationary phase + 48 h were exposed to a period of extreme salt stress followed by invasiveness studies. The invasion efficiencies of the strains cultivated to stationary phase were significantly attenuated ($p<0.05$) after exposure to 21% NaCl for 96 h at 4 °C, followed by 2% NaCl for 48 h at 4 °C when compared to non salt stressed strains. The invasion efficiency ranged from 0.00069 (*L. monocytogenes* 11994) to 0.017% (*L. monocytogenes* 3998). The invasion efficiency after exposure to an extreme salt stress for strains cultivated at stationary phase + 48 h was still low, indicating that a decline in invasion efficiency occurs irrespective of early or late stationary phase. The strains segregated into two groups ($p<0.05$) where *L. monocytogenes* 1001 and 2208 displayed the highest

invasiveness, ranging from 0.006 to 0.018%. The other group comprised of *L. monocytogenes* 2209, 3998, 11994 and 3442. The groups did not reflect serotype or lineage.

A distinct segregation of strains according to serotypes or lineages was not observed after extreme salt stress exposure. Knowledge about serotype and lineages is however important in evaluating the ability of a strain to invade Caco-2 cells since more than 98% of human listeriosis cases are caused by just three serotypes, 1/2a, 1/2b and 4b (Doumith et al., 2004; Jacquet et al., 2002; Kathariou, 2002; Rocourt et al., 1997).

Observations of attenuated invasiveness due to salt may appear contradictory to other studies, where addition of 2.2 or 5% NaCl to the *L. monocytogenes* cultures resulted in increased or unchanged invasion efficiency, respectively, compared to non salt stressed strains (Garner et al., 2006; Jensen et al., 2008). The difference may possibly be due to the extreme salt stress exposure of *Listeria* spp. before dilution to 2% NaCl. A second possibility is that the strains used in the present study may have premature stop codons in their inlA genes and therefore respond with a lower invasion efficiency of Caco-2 cells (Ward et al., 2010). It was hypothesized that the salt stress scenario with 21% NaCl for 96 h before dilution to 2% NaCl for 48 h resulted in attenuated invasiveness. Thus, invasion efficiency of *Listeria* spp. cultivated in BHI with 2% NaCl to early stationary phase was tested.

The Caco-2 invasion efficiencies of *Listeria* spp. cultivated in BHI with 2% NaCl were studied. Among the strains of *L. monocytogenes* the invasiveness ranged from 0.0012 (*L. monocytogenes* 4006) to 0.42% (*L. monocytogenes* 1001) of the initial inoculums. No significant segregation due to serotype or lineage was observed. When comparing the invasiveness of *Listeria* spp. cultivated in BHI with 2% NaCl, and BHI only, a slight increase of invasiveness was observed for the strains cultivated in BHI with 2% NaCl. These results are in agreement with other studies carried out with different strains of *L. monocytogenes*

(Garner et al., 2006). Therefore, it seems likely that the attenuated invasiveness observed after extreme salt stress is not due to the selection of strains, but to the extreme salt stress exposure.

The results indicate that *L. monocytogenes* exposed to an extreme salt stress display a lower invasion efficiency than using no salt or 2% NaCl. This implies that knowledge of the salt stress period applied to a food product, i.e. the process of salt-curing and rehydration, is required when performing an adequate risk analysis with respect to *L. monocytogenes*. As the ability to invade Caco-2 cells correlates with the virulence of *L. monocytogenes* (Jaradat et al., 2003; Roche et al., 2001), it is suggested that salt-cured products, like salt-cured cod, are less likely to cause listeriosis if the *L. monocytogenes* has been introduced to the food prior to salt-curing. This may have contributed to the low level of outbreaks of listeriosis that have been traced to salt-cured fish or rehydrated products of salt-cured fish. However, before including these aspects in risk analysis, the results obtained in this work with *Listeria* spp. cultivated in BHI require verification in studies involving fish. Furthermore, studies to reveal whether the attenuated invasiveness due to salt stress is a permanent or a transient mode are also required. Despite the necessity of further studies, our findings support the hypothesis that environmental conditions influence the infectious dose of *L. monocytogenes* and that extreme salt stress as a procedure in the processing of food is an important parameter in this context.

8 CONCLUSIONS

In this thesis it has been demonstrated that long term exposure to very high salt concentrations does not eliminate *Listeria* spp., and that the presence of *Listeria* in fish prior to salt-curing can lead to recovery and growth in rehydrated salt-cured cod during chilled storage.

In subsequent experiments with thermal treatment of rehydrated and fresh cod, it was demonstrated that both salt stress and state of the matrix i.e. rehydrated or fresh cod muscle did affect the thermal inactivation of *L. innocua* towards non-linearity with an upward concavity. During the subsequent storage of the products at 4 and 8 ºC, the lag time of the strain in raw products (controls) varied according to salt stress and state of the matrix. In the thermally treated samples, however, the lag time of the strain was similar regardless of salt stress level or matrix. The results indicate that a detailed knowledge of the history of salt stress before thermal treatment is of less importance for the growth potential of *L. innocua* in thermal treated ready-to-eat (RTE) products.

In the study of invasiveness, it has been demonstrated that the ability of salt-stressed *L. monocytogenes* to invade Caco-2 cells is affected by the concentration of NaCl. Specifically, the ability of *L. monocytogenes* at stationary phase to enter Caco-2 cells was significant lowered ($p>0.05$) after being exposed to an extreme salt stress, compared to the corresponding non salt stressed strains. It is suggested that the extreme salt-stress exposure is responsible for attenuated invasiveness as *Listeria* spp. grown in BHI with 2% NaCl, i.e. the same salt concentration as in salt stressed culture after rehydration, did not reveal attenuated invasiveness.

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