Food Control 21 (2010) 292–297

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Survival of *Listeria innocua* and *Listeria monocytogenes* in muscle of cod (*Gadus morhua* L.) during salt-curing and growth during chilled storage of rehydrated product

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ARTICLE INFO

Article history:
Received 16 January 2009
Received in revised form 5 June 2009
Accepted 12 June 2009

Keywords:
Salt-cured cod
Listeria spp.
Ready-to-eat-product

ABSTRACT

The aim of this work was to study survival of *Listeria innocua* and *Listeria monocytogenes* in muscle of cod during salt-curing and growth during chilled storage of the rehydrated product. 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 the first 5 days of storage after rehydration, growth of *L. innocua* was observed in 1 out of 5 experiments at 4 °C, but a 10–100-fold increase were observed in all experiments from day 5 to day 10. The growth started earlier and was more rapid when samples were stored at 7 °C. Growth of *L. monocytogenes* at 4 °C appeared to start earlier than for *L. innocua,* but a 10–100-fold increase was observed also for this bacterium. The lag phases in rehydrated products were longer than in experiments with cod muscle juice. The differences could be explained by a different level of salt stress. This work demonstrates that long term exposure to very high salt concentrations does not eliminate *Listeria* spp., and that Listeria being present in the fish prior to salt-curing can recover and grow in rehydrated salt-cured cod during chilled storage.

1. Introduction

Salt-cured products based on cod from the North-Atlantic fisheries, are traditional and highly regarded products, especially in Southern Europe and Latin America. The annual consumption can be estimated to more than 150,000 tonnes (Norwegian Export Council, 2009). To obtain a fully salt-cured product (salt ripened product), the process of salt-curing in the industry is completed within 3 weeks at 4 °C. The products can be stored for a long time even at abuse temperatures due to its high salt content (15–21%). This storage stability is the reason why salt-cured cod has been important for the food supply in earlier days. After the introduction of freezing and chilling facilities, dishes made from salt-cured cod products are still highly appreciated because of the characteristic taste, texture and aroma (Borgstrom, 1968; Gallart-Jornet, Roberto, & Maupoey, 2004; Lauritzsen et al., 2004). Salt-curing of food is an effective hurdle for microbial growth. However, in the case of salt-cured cod it has been shown that some bacteria are not eliminated during salt-curing (Barat et al., 2006; Vilhelmsson, Hafsteinsson, & Kristjansson, 1997) and among these are the specific spoilage organism in rehydrated salt-cured cod; *Psychrobacter* spp. (Bjørkevoll, Olsen, & Skjerdal, 2003). Studies regarding survival of food borne pathogens like *Listeria* spp. in salt-cured cod have to our knowledge not been reported, but long term survival of *Listeria monocytogenes* has been shown in tryptose soy broth containing 25% NaCl at 4 and 22 °C (Shahamat, Seaman, & Woodbine, 1980).

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 2–3% before preparation. The rehydration has traditionally been carried out in the households. Today, consumers tend to spend less time on food preparation and prefer more convenient products like ready-to-eat and ready-to-heat-products instead (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, Guevara, Eschriche, Diaz, & Serra, 2003; Skjerdal, Pedro, & Serra, 2002). However, it is not known if *Listeria* spp. introduced before or during the salt-curing process, are able to survive and grow in the

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doi:10.1016/j.foodcont.2009.06.012

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 process 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 heat 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 rehydrated 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 et al., 2004; Modesto, 1989; Pedro, Albuquerque, Nunes, & Bernardo, 2004). To our knowledge, it has not been reported cases of human listeriosis associated with salt-cured cod products so far, but several have been related to other ready-to-eat-products of fish (EFSA, 2006). New guidelines from the EU emphasise the importance and duty to carry out risk assessments of ready-to-eat-products based on survival and growth of *L. monocytogenes* in durability and challenge studies (Beufort et al., 2008).

The purpose of this work has been to study if *Listeria* spp. are able to survive in muscle of cod during salt-curing and grow during subsequent storage of the rehydrated product at 4 and 7 °C to simulate chilled and abuse temperatures, respectively. The studies were carried out as challenge tests with fresh cod fillet inoculated with a reference strain of *Listeria innocua* and 6 strains of *L. monocytogenes* isolated from fish producing facilities in Norway. In initial experiments, sterile cod muscle juice supplemented with salt was used as a model system to obtain data of growth rate and survival in a salt-containing media. In this work, the salt tolerance and growth rates among the Listeria strains were compared.

2. Material and methods

2.1. Bacterial strains

The bacteria used in the study were *L. innocua* CCUG 15531 T (Culture Collection, University of Gothenburg, Sweden) which is identical with ATCC 33090 and DSM 20649, and 6 isolates of *L. monocytogenes* obtained from low temperature sources (<10 °C) in Norway (Table 1). The bacteria were maintained in brain heart infusion (BHI) broth (Difco, Maryland, USA) supplemented with 50% glycerol (v/v) (Merck, Darmstadt, Germany) and kept at −80 °C. To prepare the inoculum for each trial, the frozen bacterial culture was thawed for 15 min at room temperature and transferred to baffled shake flasks containing 20 mL of BHI broth and incubated at 20 °C for 24 h with shaking at 5 rpm until a late exponentially growth phase was obtained. Before each experiment, the cultures were diluted to the levels required.

Table 1
Isolates of *L. monocytogenes* used in this study were obtained from 6 low temperature sources (<10 °C) in the Norwegian fish industry.

No.	Source	Serotype	Collected in
984	Sea water from well boat during transportation of salmon to the slaughterhouse	1	1991
1001	Light salt-cured salmon	1	1991
1182	Waste water from a drainage in a salmon processing plant	4	1993
1200	Seawater taken in October nearby a Salmon slaughter house	1	1993
3442	Salmon stored in box	1	1996
4006	Frozen block of cod	4	1997

2.2. Media and analysis

Enumeration of *Listeria* spp. were performed using plate count agar (PCA) (CM 0463, Oxoid, Basingstoke, UK), and PALCAM selective agar (CM 0877, Oxoid) in the studies with monoculture (cod muscle juice) and inoculated cod, respectively. In studies with cod, the samples were prepared by homogenising 25 g of muscle with 225 mL water with 3% NaCl (w/v) in a stomacher bag with filter (Seward Medical, London, UK) for 2 min in a Lab Blender 400 Stomacher (Seward Medical) at normal speed. The PALCAM agar plates were incubated in an anaerobic jar with microaerophile vacuum conditions of 0.4 bar as recommended by the producer (Oxoid). The jars were incubated at 30 °C for 48 h (Oxoid, 2009).

The salt content was measured in fresh, salt-cured and rehydrated fish by the principle of Volhard's method (AOAC, 1990) by using a Dicromat II-6 Salt Analyser (PCL Control Instrumentation Ltd., Leicester, UK). In total, 4 parallels of each sample category were analysed.

2.3. Survival and growth of L. innocua and L. monocytogenes

2.3.1. Model experiments with cod muscle juice

Cod muscle juice (Dalgaard, 1995) supplemented with 1%, 3%, 15% or 21% NaCl, was used to obtain salt concentrations equivalent to those in fresh, rehydrated, salt-cured and dried salt-cured cod (klipfish), respectively. The growth of *L. innocua* CCUG T 15531, *L. monocytogenes* isolate no 3442 and 4006 at 4 and 7 °C were studied in cod muscle juice supplemented with 1% or 3% NaCl (w/v) at an initial inoculum level of 2.46, 2.67 and 1.20 $\log_{10}$ CFU/mL, respectively. The levels of the strains were analysed every 24 h for 6 days (Table 2). The survival of *Listeria* spp. in 15% and 20% NaCl were studied in three sets of experiments at two initial inoculation levels, 7.6 and 2.1 $\log_{10}$ CFU/mL (Table 3). All experiments were repeated twice.

2.3.2. Challenge experiments with salt-cured, rehydrated and chilled stored cod

Newly caught cod (*Gadus morhua* L.) were filleted and skinned post rigor (4–5 days post mortem). To study the survival of *Listeria* spp. during the salt-curing process, 20 cubic fish muscle pieces, approximately 25 g each, were shaken in a beaker (2000 mL) containing 100 or 1000 mL of saline water (NaCl, 0.9% w/v) with *L.*

Table 2
Kinetic parameters describing the growth of *L. innocua* CCUG 15531 T (L.i. 15531 T), *L. monocytogenes* (L.m.) isolate no 3442 and 4006 at an initial inoculation level of 2.46, 2.67 and 1.20 $\log_{10}$ CFU/mL, respectively. The cod muscle juice was supplemented with 1% and 3% NaCl and incubated for 6 days at 4 and 7 °C. Specific growth rate: μ_{max} (h^{-1}).

Strain/isolate	μ_{max} (h^{-1})[a]				Time to 100-fold increase (days)			
	4 °C		7 °C		4 °C		7 °C	
	1% NaCl	3% NaCl	1% NaCl	3% NaCl	1% NaCl	3% NaCl	1% NaCl	3% NaCl
L.i. 15531 T	0.008 ± 0.001	0.006 ± 0.001	0.012 ± 0.002	0.011 ± 0.001	3.1	3.5	2.2	2.3
L.m. 3442	0.006 ± 0.001	0.006 ± 0.002	0.013 ± 0.003	0.013 ± 0.002	2.8	2.8	0.8	0.9
L.m. 4006	0.008 ± 0.001	0.005 ± 0.001	0.010 ± 0.001	0.013 ± 0.001	4.0	3.4	0.8	0.8

[a] Significant differences ($p < 0.01$) between incubation temperatures, but not salt levels.