Food safety and microbiological quality aspects of QDS process® and high pressure treatment of fermented fish sausages

Katharina Stollewerka, Anna Jofré a,*, Josep Comaposadab, Jacint Arnau b, Margarita Garriga a

a Food Safety Programme, IRTA, Finca Camps i Armet, 17121 Monells, Spain
b Food Technology Programme, IRTA, Finca Camps i Armet, 17121 Monells, Spain

ABSTRACT

A new fermented fish sausage product, based on monkfish, was developed by using an accelerated drying process, the QDS® process. To evaluate food safety, a challenge test was performed, in which the raw materials were inoculated with low levels of Listeria monocytogenes and Salmonella enterica (<150 CFU/g). The product was manufactured, fermented, QDS dried, and half of the samples were pressurized (600 MPa, 5 min, 13 °C). Pathogens, technological microbiota, spoilage indicator bacteria from fish (hydrogen sulphite producing bacteria, coliforms and Escherichia coli) and physicochemical parameters were monitored during manufacturing and after 6, 13, 20, and 27 days of refrigerated storage at 4 and 8 °C. Results showed that in the finished product, pathogens and spoilage indicator bacteria could not grow but decreased and E. coli was not detected during storage. Pressurization had an important reducing effect on technological microbiota, and eliminated L. monocytogenes, S. enterica, hydrogen sulphite producing bacteria and coliforms immediately after production and during refrigerated storage.

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1. Introduction

Fish is a good protein source, contains iodine, fatty acids with high nutritional value, is easy to digest (Gelman, Drabkin, & Glatman, 2000; Nordvi, Egelandsdal, Langsrud, Ofstad, & Slinde, 2007) and has therefore a wholesome and healthy image. The development of stable fish products would widen the range of health-giving fish based food products and could represent an intelligent option to upgrade low-value species and waste generated by the fish processing industry (Gelman et al., 2000).

Fermentation of fish is one of the oldest methods to prevent fish spoilage and to extend its shelf-life (Gelman et al., 2000; Ghaly, Dave, Budge, & Brooks, 2010). Nowadays, in Northern Europe various fermented fish products such as Rakorret, Tidbits and Surströmming are produced in general by spontaneous fermentation (Nordvi et al., 2007). Aryanta, Fleet, and Buckel (1991) studied the occurrence and growth of inoculated pathogens (Salmonella Typhimurium, Salmonella Sofia, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens and Vibrio parahaemolyticus) during the fermentation period of fish sausages and observed that the growth of these bacteria was significantly inhibited in the product fermented with the starter Pediococcus acidilactici. The benefits of using lactic acid bacteria (LAB) and Staphylococcus xylosus as starter cultures in fish sausages have also been reported on chemical, microbiological and technological aspects (Hu, Xia, & Ge, 2008).

Food safety of fermented fish sausages is based, as in fermented meat sausages, on the hurdle technology, where acidification, addition of NaCl, nitrite/nitrate, drying and sometimes smoking are used together for preservation (Leistner, 2007). In the present study, drying was performed by the Quick-dry-slice process® (QDS process®), an innovative technology for the accelerated production of dried sliced food products (Comaposada, Arnau, Gou, & Monfort, 2004). During the process, sausages are fermented, frozen and cut into slices, which are subsequently dried in a continuous system with convective air. The fast achievement of the finished products with this new system makes it suitable for the development of new ready-to-eat (RTE) products. Various studies confirm the applicability of the QDS system on different meat products (Comaposada et al., 2008; Ferrini, Comaposada, Arnau, & Gou, 2012; Stollewerk, Jofré, Comaposada, Ferrini, & Garriga, 2011), however, up to the present day QDS processing has not been applied on products made out of fish.

High pressure (HP) processing can be applied as an additional hurdle in the framework of the hurdle technology. It is an alternative technology which allows the production of safer dry-cured meat products without introducing important modifications in the process.
sensory characteristics (Garriga & Aymerich, 2009). HP is nowadays commercially applied on different fish products such as salted squid, fish sausages and shellfish (Cheftel, 1995; Garriga & Aymerich, 2009; Ohshima, Ushio, & Koizumi, 1993) but there is a lack on studies focused on pathogenic microorganisms inactivation.

Fish and fish products have been associated with outbreaks of food-borne diseases and the behavior and/or survival of pathogenic bacteria in fermented fish during production and throughout shelf-life has been investigated (Aryanta et al., 1991; EFSA, 2013a; Nieto & Toledo, 1989). Several studies have shown that _Listeria monocytogenes_ occurs in fish and fishery products (Kuzmanovic et al., 2011; Lambertz et al., 2012) and EFSA reported that in 2011, 6.7% of RTE single food samples of fishery products at processing did not comply with the European Commission Regulation EC N°2073/2005 microbiological criteria (EFSA, 2013b). Further, it is also worth mentioning that _L. monocytogenes_ was reported to consistently reappear at processing plants even after the application of strict sanitary and disinfection practices and could be detected at very low levels on final fish products even when strict hygienic practices were applied (Eklund et al., 1995; Garland, 1995). For _Salmonella_ spp., EFSA reported that 0.6% of the tested samples (fish and fishery products) resulted positive in 2010 (EFSA, 2012).

With the aim to evaluate the food safety of QDS dry fermented fish sausages a challenge test was performed, where the fish batter was spiked with low levels of _L. monocytogenes_ and _Salmonella enterica_. During manufacturing and the following storage period of 27 days under refrigeration at 4 and 8 °C, pathogens, spoilage indicator bacteria (hydrogen sulphite producing bacteria (SPB), co-liforms and _Escherichia coli_ and technological microbiota were monitored. The effect of an HP treatment at 600 MPa was also evaluated.

2. Materials and methods

2.1. Production, inoculation and fermentation

Fish sausages were produced from ultra-deep frozen monkfish meat (_Lophius piscatorius_) without bones and skin. Frozen blocks of 5 kg were cut with a guillotine and coarsely chopped to reduce the size of fish pieces to ca. 1–2 cm² (to simulate the pieces of fat in a “chorizo” type product). Additionally, a part of this fish was comminuted to a lower size in a cutter. Coarsely and finely chopped fish were mixed separately with the following ingredients (in g/kg): NaCl, 20; sucrose, 10; sweet red pepper, 10; dextrose, 3.5; white pepper, 2; paprika oleoresin 20.000 U.C. (Collelldeval, Banyoles, Spain), 2; garlic powder, 1.4; ascorbate, 0.5; _α_-tocopherol, 0.2; _Rosmanox_ (Sensient Technologies, Milwaukee, WI, USA), 0.15; and sodium nitrite, 0.1. Four kg of coarsely chopped and 6 kg of finely chopped fish were mixed with dextrose (3.5 g/kg) and a commercial starter culture mixture specially useful for short processing (_Lycornari VHI_–41, ClericiSacco, Cadorago, Italy) containing the fast acidifying cultures _Pediococcus pentosaceus, Lactobacillus plantarum_ (inoculated at 6 × 10⁶ and 4 × 10⁶ CFU/g, respectively) and _S. xylosus_, a gram positive catalase positive cocci (GCC+, inoculated at 1 × 10³ CFU/g). The pathogens _L. monocytogenes_ (a mixture of the strains CTC101, CTC1034 and CECT4031 at a final level of 150 CFU/g) and _S. enterica_ (serovars Typhimurium GN6, London CTC1003 and Derby CTC1022 at 30 CFU/g) coming from −80 °C stocks and diluted in 10 ml of water, were also added to the fish batter. Subsequently, the mixture was stuffed in 80 mm diameter collagen casings. Fish sausages were fermented for 32 h at 20 °C and 90% relative humidity (RH), until the pH reached 4.8. Subsequently they were frozen at −18 °C. Two independent batches were manufactured.

2.2. Slicing, QDS drying and HP treatment

Fermented sausages were sliced and placed on gratings (48 slices per grate). Subsequently, QDS drying was performed by applying convective air (velocity 3.5 m/s, at 30 °C and 35–40% RH) for ca. 45 min until slices reached a weight loss of 40%, comparable to other dry-cured meat products. Dried slices were vacuum-packed in PET/PE pouches (oxygen permeability of 50 cc/m² (24 h C) and water vapor permeability of 2.6 g/m² (24 h, 23 °C, 85% RH), Sacoliva, S.L., Castellar del Vallés, Spain) and stored at 4 °C. After 24 h half of the samples were submitted to an HP treatment of 600 MPa for 5 min at 13 °C in a Wave 6000 equipment (Hiperbaric, Burgos, Spain). The chamber volume was 120 l, the come up time was 3.83 ± 0.13 min and the pressure release was immediate (<10 s). All samples were stored under refrigeration (9 days at 4 °C and 18 days at 8 °C (AFNOR, 2004; CRL/AFSSA, 2008)).

2.3. Microbiological analysis

Microbiological counts were analyzed in duplicate during production (raw fish, fish batter, after fermentation and after drying), after pressurization and periodically during refrigerated storage (after 6, 13, 20 and 27 days) for both pressurized and non-pressurized slices. For each sampling, a quantity of 25 g was aseptically minced, diluted 1/10 in Brain Heart Infusion (BHL, BD Becton, Dickinson and Company, NJ, USA) and homogenized for 1 min in a Masticator Classic (JUL S.A., Barcelona, Spain). After doing the appropriate dilutions in 0.1% Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85% NaCl, _S. enterica_ was counted in CHROMagar™_Salmonella_ plus (Sharlab, Barcelona, Spain) by spread plating and incubation at 37 °C for 48 h; _L. monocytogenes_ was counted in Chromogenic _Listeria_ agar (Oxoid Ltd., Basingstoke, UK) by spread plating and incubation at 37 °C for 48 h; _GCC+ were counted in Mannitol salt phenol-red agar (MSA, Merck, Darmstadt, Germany) by spread plating and incubation at 30 °C for 48–72 h; LAB were counted in de Man, Rogosa and Sharpe agar (MRS, Merck) by pour plating (non-diluted homogenate) and spread plating (diluted homogenate) and incubation at 30 °C for 48–72 h in anaerobiosis; SPB were counted in Lyngby agar (Gram, Tolle, & Huss, 1987) by pour and spread plating and incubation at 25 °C for 72 h; _E. coli_ and coliforms were counted in Coli ID agar (Biomerieux®, SA, Marcy-l’Etoile, France) by pour plating and incubation at 37 °C for 48 h. When counts of _L. monocytogenes_ and _S. enterica_ were under the detection limit of 135 mm ° plates (LOD, 10 CFU/g), presence or absence of viable cells was investigated in the enriched homogenates (48 h at 37 °C) by seeding dots on selective media. For every _L. monocytogenes_ enrichment two 20-µl dots were seeded onto Chromogenic _Listeria_ agar. For _S. enterica_ 200 µl of the enriched homogenate were transferred onto 10 ml of Rappaport-Vassiliadis Enrichment Broth (Oxoid). After incubation at 41.5 °C for 48 h, 10 µl were seeded onto CHROMagar™_Salmonella_ Plus. Presumptive colonies of both _L. monocytogenes_ and _S. enterica_ were confirmed by real time PCR using primers amplifying the _hly_ and _trbC_ genes for _L. monocytogenes_ and _Salmonella_ spp., respectively (Malorny et al., 2004; Rodriguez-Lázaro, Jofré, Aymerich, Hugas, & Pla, 2004).

2.4. Physico-chemical analysis

The pH was measured with a portable Crison penetration electrode connected to a Crison pH metre PH25 (Crison Instruments S.A., Alella, Spain). Water activity (aw) values were determined using an Aqualab S3TE dew point water activity meter (Decagon Devices, Inc. Pullman, Washington, USA).

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2.5. Statistical analysis

Analysis of variance (ANOVA) and the post-hoc Tukey test at a p < 0.05 significance level were done using the Statistica 7.0 software (Statsoft, Tulsa, UK). For determining statistical differences of bacterial counts, pH and a<sub>W</sub> during manufacturing or the storage period, one-way ANOVA was performed, using either “Fish batter”, “After fermentation” or “Days of storage” as fixed factor. To allow graphical representation and statistical analysis of Salmonella spp. and L. monocytogenes counts, absence of the pathogens in 25 g of product was considered to be 0 CFU/g and the applied logarithmic scale transformation was log (N + 1), i.e. absence in 25 g of product corresponded to 0 log CFU/g.

3. Results and discussion

3.1. Physicochemical parameters

Values of pH and a<sub>W</sub> recorded along the process are shown in Table 1. The initially high pH value of fish flesh (6.76 ± 0.01) represents an important intrinsic factor with respect to safety and spoilage of the product and can be related to the low carbohydrate content found in most fish (<0.5%) and the consequent small amounts of lactic acid produced post-mortem (Gram & Huss, 1996).

During fermentation the added starter cultures converted added sugars (dextrose and sucrose) to lactic acid, responsible for the significant decrease of the pH (1.95 units, p < 0.05) and the subsequent pH profile, typical for acid dry-fermented sausages (Leistner, 1995). QDS drying did not affect the pH, which was also observed in QDS dried fermented meat sausages (Comaposada et al., 2008; Stollewerk et al., 2011), Comaposada et al. (2008) related this fact to the shorter time of the QDS drying process, which does not permit the growth of technological microbiota. Throughout the storage period of 27 days under refrigeration of non-preserved fish sausage samples, pH progressively decreased to levels around 4.4. In QDS dried meat sausages in contrast, the pH maintained during the first 15 days of storage and progressively decreased afterward (Stollewerk et al., 2011).

During QDS drying the a<sub>W</sub> was reduced to the desired level for this kind of product (0.96) in only 45 min. This time span was significantly shorter than the drying period of a traditional drying (Aryanta et al., 1991; Hu et al., 2008; Ferrini et al., 2012; Stollewerk et al., 2011). Comaposada et al. (2008) observed for both types of bacteria. Growth limits of pH 4 have not submitted to a thermal treatment as were pork fermented sausages. From a technological point of view, the application of LAB starter cultures to this type of product was proposed to give a unique lactic acid flavor and increase the texture firmness and mouthfeel due to the acid denaturation of muscle proteins (Hu et al., 2008). GCC+ have been described to be less competitive than LAB (Garriga & Aymerek, 2007) but may also possess proteolytic and lipolytic activities that hydrolyze the protein and fat components. In this way they contribute to sausage flavor and texture and play a role in color stabilization (Jerøy, Verluyten, & De Vuyst, 2006).

3.3. Spoilage indicator bacteria and pathogenic microbiota

For routine monitoring of microbial fish spoilage contamination, total viable counts and coliforms have previously been the most widely used indicator bacteria (Lorentzen, Skjerdal, & Berg, 2003). During the last decade, however, shelf-life of fish and shellfish has rather been correlated to “specific spoilage organism”-levels than to total viable counts (Gram & Dalgaard, 2002; Gram & Huss, 1996).

In the present study, hydrogen sulfite producing bacteria (Shewanella spp.), coliforms and E. coli were used as spoilage indicator bacteria. In raw fish, initial levels of SPB (4.25 log CFU/g) and coliforms (3.08 log CFU/g) were similar to counts recorded from the fish batter (Fig. 2). During fermentation, a 2-log increase was observed for both types of bacteria. Growth limits of pH 4 have not been reported for coliforms (Zangerl, 2007) and Shewanella spp. is indeed a pH sensitive bacteria but can grow at pH values > 6.0 (Gram & Huss, 1996). Apparently, pH > 6 during 24 out of 12 h of fermentation combined with the high a<sub>W</sub> levels (0.980) of the undried product permitted the growth of coliforms and SPB (Fig. 2). Nevertheless, during QDS drying, coliforms significantly decreased ca. 2.4 log and SPB ca. 1.5 log to initial levels and followed this decreasing trend until the end of the refrigerated storage. E. coli levels remained below the LOD (10 CFU/g) during the whole experiment.

To simulate a natural contamination of raw material, the fish batter was inoculated with 150 CFU/g of L. monocytogenes and ca. 30 CFU/g of S. enterica (Fig. 2). Manufacturing significantly affected S. enterica, which decreased to 3.25 CFU/g during QDS drying. Although both pathogens could not grow during storage, absence in 25 g of product was achieved after 20 days for S. enterica and after 27 days for L. monocytogenes in non-pressurized slices. Differences in L. monocytogenes and S. enterica behavior during fermentation and drying could be attributed to differences in tolerance towards stress factors, mainly acidity and a<sub>W</sub> (ICMSF, 1996).

### Table 1

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<thead>
<tr>
<th>pH</th>
<th>days of storage</th>
<th>HP treatment</th>
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<tr>
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<td>0.65</td>
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Values are mean ± SD (n = 4). For pH and a<sub>W</sub> values, significant differences are indicated by different small letters (p < 0.05).
A low aw, similarly to what happens in fermented meat products, minimizes spoilage and improves preservation of fish (Abbas, Mohamed, Jamilah, & Ebrahimian, 2008). In the case of fish fermented sausages however, the aw of the final product (0.96) was higher than the one usually observed in meat fermented sausages (<0.92). This fact reduces the capacity of the sausage environment to inhibit L. monocytogenes and S. enterica growth decreasing the food safety of the product during storage as aw growth limits of 0.92 and 0.94 have been described for L. monocytogenes and Salmonella spp., respectively (ICMSF, 1996).

Nevertheless, it is the number and intensity of all the microbial growth hurdles present in a food product that determines its safety and shelf-life. In this context, it has been described that in fermented fish products the adequate acidification in combination with organic acids (primarily lactic acid) was the most important control point for the inhibition of pathogenic microorganisms growth (Aryanta et al., 1991). In food, antimicrobial compounds can be naturally formed during processing or added as ingredients (Ray, 2004). In this regard, the elaborated dry fermented fish sausages contain lactic acid produced during LAB fermentation in addition to some ingredients with potential antimicrobial effect: NaCl, nitrite, ascorbate, red pepper, garlic and Rosmanox®/C210 (rosemary extract). The antimicrobial effect of NaCl, at least in part is due to its ability to bind to free water molecules creating an osmotic imbalance and causing cell growth inhibition (Stringer & Pin, 2005). In combination with nitrite and ascorbate, NaCl is widely used to preserve fish,
meat and poultry products (Ray, 2004). Rosemary, garlic and red pepper have also been demonstrated to affect *L. monocytogenes* and *Salmonella* spp. growth in both culture media and food products (Bajpai, Baek, & Kang, 2012; Tajkarimi, Ibrahim, & Cliver, 2010). In this context, garlic was mentioned to be one of the most used spices in fermented fish products, due to its antimicrobial effect against pathogenic bacteria associated with this type of products (Bernbom, Ng, Paludan-Müller, & Gram, 2009). Many factors such as the application form (powder, liquid or film), the food composition (varying fat content) or vacuum-packaging influence the activity of herbs and spices, which make data reported in literature difficult to compare.

Nevertheless, in sliced QDS-dried fermented fish sausages, the observed progressive reduction in pathogenic microorganisms counts during storage was due to the combination of these antimicrobials together with other intrinsic (low pH) and extrinsic (vacuum-packaging, low temperature storage) preservative factors. These conditions also inhibited growth of the spoilage indicator bacteria. It has been shown in many seafood products that numbers of specific spoilage organisms can be used to predict the remaining product shelf-life (Gram & Dalgaard, 2002). In fish, putrefaction or spoilage proceeds very rapidly once the load of specific spoilage organisms exceeds approximately 7 log CFU/g (Huss, 1994).

3.4. Effect of pressurization

The HP treatment of 600 MPa on the QDS dried fish sausages did not affect physicochemical parameters. The same observation or only slightly different pH and *a*<sub>water</sub> values after pressurization were also reported from other HP treated fermented products (Jofré, Aymerich, Grèbol, & Garriga, 2009; Marcos, Aymerich, & Garriga, 2005; Stollewerk et al., 2011). During storage, in contrast to the pH decrease (0.37 units) observed in non-pressurized fish sausage slices, pH maintained at similar levels (p > 0.05) in pressurized ones (Table 1). Constant pH values throughout the storage period in pressurized samples could be related to the strong LAB inactivation caused by HP: recorded levels were 9 log CFU/g in non-pressurized fermented fish sausage slices but <2 log CFU/g in pressurized ones (Fig. 1). Compared to LAB, pressurization also had an immediate decreasing effect, although to a lesser extent, on GCC<sup>+</sup> (p < 0.05, Fig. 1). During storage, LAB further decreased until day 6 and remained at levels under LOD while constant GCC<sup>+</sup> levels at 4 log CFU/g were recorded until day 27.

Reductions in LAB counts were also observed in ripened and non-ripened traditionally dried meat sausages treated with 400 MPa (Jofré, Aymerich, & Garriga, 2009) and 300 MPa (Marcos et al., 2005). As observed in QDS dried chorizo, the fish sausage matrix did not allow recovery of technological microbiota (Stollewerk et al., 2011).

The HP treatment also produced a significant decrease in spoilage indicator bacteria (p < 0.05, Fig. 2) and both SPB and co-liforms remained under LOD in pressurized samples throughout the whole storage period. Regarding pathogenic microorganisms inactivation, pressurization eliminated both *L. monocytogenes* and *S. enterica* immediately after application (absence in 25 g of product) and no recovery was observed during the following 27 days of refrigerated storage (Fig. 2).

The effectiveness of pressurization against *L. monocytogenes* and *Salmonella* spp. has been demonstrated in different fermented meat products at pressure levels of 400–600 MPa (Garriga et al., 2005; Jofré, Aymerich, Grèbol, et al., 2009; Stollewerk et al., 2011) and in cold-smoked dolphinfish (Montero, Gómez-Estaca, & Gómez-Guillén, 2007) at 300 MPa. At 250 MPa in contrast, Lakshmanan and Dalgard (2004) observed in chilled cold-smoked salmon that *L. monocytogenes* was not inhibited. The present results confirmed that pressurization of 600 MPa for 5 min at 13 °C, normally applied on commercial meat products (Garriga & Aymerich, 2009) was sufficient to achieve absence of both pathogens and to inhibit their recovery in slices of fermented fish sausages. These pressure levels also affected spoilage indicator bacteria, which were immediately reduced under the LOD. HP is known to effectively eliminate spoilage bacteria from foodstuff (Yuste, Capellas, Pla, Fung, & Mor-Mur, 2001). In this sense, evaluation of microbiological quality aspects revealed that 500 MPa for 2 min were enough to effectively inactivate coliforms in shellfish (<50 CFU/g; Linton, Mc Clements, & Patterson, 2003) and a 450 MPa treatment for 15 min reduced total plate counts from 5.2 × 10<sup>3</sup> CFU/g in tuna meat and 1.9 × 10<sup>4</sup> CFU/g in squid mantle flesh to <300 CFU/g (Ohshima et al., 1993).

However, food safety evaluations of HP treated food products revealed that the inactivating effect due to pressure is greatly dependent on food matrix composition and physicochemical conditions during and after the treatment (Archer, 1996). In the present study, pressurization did not affect pH and *a*<sub>water</sub> (values were kept during storage), significantly accelerated the elimination of *L. monocytogenes* and *S. enterica* and reduced spoilage indicator bacteria to levels <10 CFU/g immediately after application. Accordingly, pressurization would increase food safety and quality of sliced fermented fish sausages.

Taken together, the present challenge test demonstrates that by using the QDS process<sup>®</sup> it was possible to produce a fermented fish product, of which the food matrix, despite of the high *a*<sub>water</sub> inhibited the growth of pathogens and fish spoilage indicator bacteria. In combination with HP, it was possible to produce a safe dry-fermented QDS processed fish product that complied with the US “zero-tolerance” and the EU 2073/2005 microbiological criteria for RTE products.

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