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Effect of heat treatment and packaging technology on the microbial load of lightly processed seafood.

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Abstract

Increasing demands for lightly processed seafood stresses the need for development of non-intensive processing methods that ensures a safe product. The limitation to the shelf life of seafood is often ascribed to microbial activity. An experiment was design to investigate the influence of heat-treatments in combination with packaging technologies (vacuum (VAC), modified atmosphere (MA) packaging, or soluble gas stabilization (SGS)) on the microbial survival of inoculated species. Fish patties were inoculated with either *Brochothrix thermosphacta* or *Listeria innocua* before heat-treatment, packaging, and storage at 2°C for 16 days. Results showed increased heat-treatment lowered the bacterial load throughout the experiment. The choice of packaging technology had a bigger effect on the results, where VAC-samples had a significantly higher bacterial load than MA- and lastly SGS-packaged samples, regardless of heat-treatment (*L. innocua*: 8.7±0.1, 8.3±0.1, 8.2±0.1logCFU/g, *B. thermosphacta*: 9.9±0.1, 9.2±0.1, 8.6±0.1 logCFU/g, respectively, at end of storage). Furthermore, use of CO_2 significantly increased the bacterial inhibition by heat (0.5-0.6logCFU/g) and extended the lag phase of *B. thermosphacta*, as well as decreasing the growth rate of both inoculum species. It is concluded that use of SGS has the opportunity to fulfill the consumers' demand of fresh, lightly processed seafood with a reasonable shelf life.

Keywords

Brochothrix thermosphacta, heat treatment, *Listeria spp.*, modified atmosphere, soluble gas stabilization, vacuum.

1. Introduction

Recent developments with increased time pressure from both work and past time activities has led to an increase in the demand for convenient and tasty ready-to-eat food options. Furthermore, contemporary trends for consuming fresh or lightly processed seafood, stresses the need to develop processing methods that allow a fulfillment of these demands, while still offering a reasonable shelf life (Speranza, Corbo, Conte, Sinigaglia, & Del Nobile, 2009). The shelf life of fresh seafood is limited

owing to multiple factors. However, the main restriction in shelf life of fish and seafood are often ascribed to the spoilage potential of microorganisms (Gram & Huss, 1996). Multiple technologies are being used to overcome these hindrances, and modified atmosphere (MA) packaging in combination with refrigeration has become one well-established method (Lambert, Smith, & Dodds, 1991). The effect of MA packaging is often ascribed to the bacteriostatic effect of CO_2 (Genigeorgis, 1985) as CO_2 increases the lag phase and generation time of most aerobic microorganisms (Gill, 1996).

The bacteria responsible for the spoilage of fish varies with species, harvesting environment, and preservation technologies (Milne & Powell, 2014). Multiple studies have looked into the bacteria responsible for spoilage in various fish species, and even though interspecies variations exist, results are in consensus. The identified dominant spoilage strains for MA packaged seafood include lactic acid bacteria (LAB) (Gram et al., 1996), *Brochothrix thermosphacta (Macé et al., 2012; Sivertsvik, 2003)* as well as *Photobacterium phosphoreum* (Dalgaard, Mejlholm, Christiansen, & Huss, 1997). *P. phosphoreum* is of special interest in relations to MA packaged seafood, where it has been established as one of the major spoilage organism (Dalgaard et al., 1997; Sivertsvik, Rosnes, & Kleiberg, 2003). However, *P. phosphoreum* is highly heat sensitive, and thus it is seldom a problem in heat-processed ready-to-eat products, even when only low heating temperatures are used (Gram et al., 1996). *B. thermosphacta,* on the other hand, has been shown in foods heated as high as 76 °C for extended periods and it has been found to be able to grow at temperatures as low as 0 °C (Aaslyng, Vestergaard, & Koch, 2014). *B. thermosphacta* plays a major role in the spoilage of MA packaged fish (López-Gálvez, De La Hoz, Blanco, Ordóñez, & López-Gálvez, 1998; Ordóñez, López-Gálvez, Fernández, Hierro, & De La Hoz, 2000) as well as cooked and MA packaged shelf fish (Fall, Leroi, Cardinal, Chevalier, & Pilet, 2010). Thus *B. thermosphacta* is often considered as a specific spoilage organism (Mamlouk et al., 2012). *Listeria monocytogenes* can cause serious foodborne diseases and is often a reason for food recalls (Teratanavat & Hooker, 2004). This is due to *Listeria* ability to overcome many food processing hurdles and its ability to grow at refrigeration temperatures and/or in reduced oxygen atmosphere. Furthermore, *Listeria* is commonly found in seafood as well as in

processing plants (Vázquez-Sánchez, Galvão, & Oetterer, 2017). This makes the control of Listeria a challenge which has to be solved in relation to ready-to-eat seafood (Marshall, 2008).

Multiple studies have demonstrated that MA packaging can increase the shelf life of many foods as compared to air- or vacuum packaging, this includs various fish species (Bouletis, Arvanitoyannis, & Hadjichristodoulou, 2017). It has been shown that growth inhibition of bacteria in MA packaged foods is significantly conditioned by the concentration of dissolved CO_2 in the product (Devlieghere, Debevere, & Van Impe, 1998a, 1998b). However, MA packaging has some drawbacks, including the risk of imploding of the packages, known as packaging collapse, limiting the optimization of its use (Floros & Matsos, 2005). An alternative method to MA packaging, which overcomes these problems, is to dissolve the CO_2 into the product before retail packaging. This method of packaging is called soluble gas stabilization (SGS) (Sivertsvik, 2000).

Various processing- and packaging technologies for fish and seafood have been well studied in relations to microbial content and food safety. However, research regarding combinations of such technologies are limited, especially for lightly processed ready-to-eat fish products. Thus, the aim of this study is to expand the knowledge of microbial survival in lightly processed seafood, by studying the effect of combined low heat processing and gas packaging on the microbial load.

2. Materials and Methods

A storage experiment was conducted in order to establish the effect of heat treatment and packaging technology on microbial growth. The experiment was performed using fish mince patties inoculated with either *B. thermosphacta* or *L. innocua.* The experimental factor were heat treatment (decimal reduction of 3.5 and 4.5), packaging technology (SGS, MA packaging or VAC), and storage time (0, 4, 7, 11, and 16 days) (Figure 2). Experiments were performed in three rounds, separated based on packaging technology applied (n=145 for each round).

2.1. Production of the fish mince patties

Frozen mince of silver smelt (*Argentina silus, 20 kg)* was purchased from Norwegian Seafood Company (Ålesund, Norway). The fish mince had a water content of 69.8±0.5% and lipid content of 1.2±0.1%. The fish mince was thawed at 4 °C for 24 h prior to being mixed in a bowl chopper (Blixer 6, Robot Coupe, France) at 20 000 rpm. Salt (0.5%) was added prior to the addition of 6.5% of potato starch and 38% of skimmed milk (0.1% lipid). A total mixing time of 150 sec was applied. The mince was stuffed in plastic casings (Ø=60 mm, L=30-40 cm, approx. 1 kg), closed with metal clips and heat-treated in a steam cabinet (SelfCookingCenter®, Rational, UK) at 100 ˚C for 1 h. After the heat treatment, the mince product was cooled in the fridge at 4.2±0.8 ˚C for 2-4 h, prior to being frozen at -22.8±1.0 °C until use.

2.2. Bacterial strains and inoculum preparation

Frozen cultures (-80 °C) of *B. thermosphacta* (ATCC 11509) and *L. innocua* (ATCC 33090), were obtained from the culture collection at University of Gothenburg (CCGU). The cultures were grown on brain heart infusion (BHI) agar (Oxoid CM1136, Oxoid Ltd., Basingstoke, UK) for two days at 22 °C and 37 °C, respectively. Single colonies were transferred to BHI broth (CM1032, Oxoid Ltd., Basingstoke, UK) for enrichment, and incubated at 8 °C for 2 days for *B. thermosphacta* and for 5 days for *L. innocua*, resulting in cold-adapted cultures in early stationary growth phase. Samples were diluted to OD_{600} of approximately 0.5 (0.504-0.507) in order to obtain a cell concentration of approximately 10^9 colony forming units (CFU) x ml^{-1} ($2.5x10^9$ CFU x ml^{-1} for *L. innocua* and $3.0x10^9$ CFU x ml^{-1} for *B. thermosphacta*).

2.3. Sample preparation and inoculation

The mince product was thawed for 48 h at 4 °C prior to being sliced in portions of 82.5 g (82.7±1.8 g) equivalent to slices of approximately 2.7 cm, and placed in 63 mm aluminum weigh dishes. The samples were drip inoculated, meaning that 100 µL inoculum were dispersed on to the surface in droplets (Figure 1). Samples were inoculated with single cultures. Samples were split in three, 1/3 were inoculated with B. thermosphacta (n=145), 1/3 were inoculated with L. innocua (n=145), and

the remaining were kept natural as control samples (n=145). The samples air-dried in a fume hood for 10 min prior to packaging. The samples were packed in batches (n=9-11) on trays (C2325-1C, Færch Plast, Holstebro, Denmark) in vacuum pouches (425x650 mm PA/PE sous vide pouch, Maske AS, Trondheim, Norge) filled with either atmospheric air (MA packaging and VAC samples) or pure CO_2 (SGS samples) in excess. Samples were stored at 4 °C (3.7±0.5 °C) for 16-18 h to dry completely. For all heat treatment and packaging combinations, control samples were prepared without inoculation. All samples were made as five replicates.

2.4. Heat treatment and packaging

After drying, the samples were repacked in vacuum pouches (135x180 mm PA/PE sous vide pouch, Maske AS, Trondheim, Norge). Heat treatment of the samples were carried out using a sous vide water bath (Diamond M, Fusionchef by Julaba, Germany). Temperatures were 47 °C (46.4±0.2 °C) and 52 °C (51.5±0.2 °C), or 60 °C (59.6±0.1 °C) and 64 °C (63.37±0.15 °C), for *B. thermosphacta* and *L. innocua*, respectively. Treatment temperature-time combinations were chosen in order to obtain 3.5 or 4.5 log reduction, D-value ($D_{3.5}$ and $D_{4.5}$) for both bacterial species. D-values were calculated based on theoretical values for surface heat treatment (Batt, Patel, & Robinson, 1999; Embarek & Huss, 1993). Heat treatment temperatures and times are presented in Table 1.

After heat treatment, all sample pouches were immediately cooled in ice water to prevent any further heating. Once cold, the samples were repacked in 230 ml semi-rigid crystalline polyethylene terephthalate (CPET) trays (C2125-1A, Færch Plast, Holstebro, Denmark) using a semi-automatic tray sealing packaging machine (TL250, Webomatic, Bochum, Germany). The trays obtained a degree of filling of approximately ⅓. The air was evacuated (final vacuum pressure of 25 mbar) and subsequently flushed with the gas mixture prior to adhering the top film of a 40 µm combination of polyethylene (PE), ethylene vinyl alcohol (EVOH), polyamide (PA), and polyethylene terephthalate (PET) (Topaz B-440 AF, Plastopil, Almere, The Netherlands). Food grade CO_2 and N_2 was mixed using a gas mixer (MAP Mix 9000, Dansensor, Ringsted, Denmark) to obtain packaging gas mixture of 60% CO_2-40% N_2 for all MA- and SGS packaged samples. All sampling and repackaging were done

aseptically. Oxygen transmission rate (OTR) was 66-78 cm^3 x 25 µm/m^2 x 24 h^1 x bar^1 at 23 ˚C for the tray and 2.5 cm^3 x 40 µm/m^2 x 24 h^1 x atm^1 at 23 ˚C for the cover film. VAC samples were not repacked.

After packaging, the trays were stored at 2 ˚C (2.6±0.5 ˚C) for 16 days. An overview of the experimental design is shown in Figure 2.

2.5. Headspace gas analysis

The headspace gas composition (O_2 and CO_2) was measured using an oxygen and carbon dioxide analyzer (Checkmate 9900 analyzer, PBI-Dansensor, Ringsted, Denmark). 20ml of the headspace gas was collected with a syringe after intrusion of the top film. Before probing the samples, a rubber septum (Nordic Supply, Skodje, Norway) was placed onto the top foil in order to avoid rupture and to avoid introduction of false atmosphere. In addition, the gas compositions was measured in empty trays immediately after packaging and in sample trays after 4, 8, 12, and 16 days storage.

2.6. Water content, lipid content, and pH

The water content of the fish patties (n=8) was determined gravimetrically by drying the samples for 24 h at 105 ˚C (International Organization for Standardization, 1999). Lipids were extracted and total amount calculated gravimetrically from the mince product by homogenizing the sample in a chloroform/methanol/water mixture follow by lipid-phase separation by centrifugation, as described by Bligh and Dyer (1959). Eight samples were taken and each sample was divided into two; one for analysis of water content and one for analysis of lipid content, respectively. pH was measured in all samples on sampling days. Analysis were done using a pH-probe (____).

2.7. Microbial analysis

10 g of fish patty were sampled aseptically from the inoculated surface of the samples (Figure 1) and homogenized in 90ml sterile water with 0.85% NaCl (w/v) and 0.1% peptone (w/v) for 60 sec. Appropriate decimal dilution series were performed in sterile 0.85% NaCl (w/v) and 0.1% peptone

191 (w/v) in water. *B. thermosphacta* inoculated samples were plated on streptomycin-thallous acetate
192 (STA) agar containing STA selective supplement (Oxoid CM0881 and Oxoid SR0162, Oxoid Ltd.,
193 Basingstoke, UK) prepared as described by the manufacturer, and incubated aerobically at 22 °C
194 (22.0±0.3 °C) for 48±2 hours. *L. innocua* inoculated samples were plated on Brilliance™ listeria agar
195 (BLA) containing Brilliance™ listeria selective supplement (Oxoid CM1080 and Oxoid SR0227, Oxoid
196 Ltd., Basingstoke, UK) prepared as described by the manufacturer, and incubated aerobically at 37 °C
197 (37.1±0.2 °C) for 24±2 h. Control samples were tested for presences of *Brochothrix* species, *Listeria*
198 species, and total viable organisms. Analysis of control samples were performed as described above
199 on both STA and BLA media, as well as plate count agar (PCA) (Oxoid CM0325, Oxoid Ltd.,
200 Basingstoke, UK), which was incubated at 30 °C (30.1±0.2 °C) for 4 days. All sampling was done in
201 accordance with NMKL-standards (NMKL, 2010) and manufactures recommendations. Initial analysis
202 also included quality control of all inoculated samples on PCA, however, these analyses were
203 excluded due to finding background microbiota from the raw material, making quality control of the
204 handling and sampling impossible.

205 Sampling was performed of the raw material, inoculated samples, and on all samples after 0, 4, 8, 12,
206 and 16 days storage.

207 2.7.1. Identification of background microbiota

208 The fish patty showed growth of unknown organisms when sampled on PCA. Single colonies from
209 various PCA plates were re-cultivated twice to obtain pure colonies; first time using BHI agar and
210 secondly on tryptone soya agar (TSA) (Oxoid CM0131, Oxoid Ltd., Basingstoke, UK), both rounds were
211 incubated at 30 °C for 24 h. Colonies were identified using the Omnilog ID system (Biolog, Harvard,
212 CA, US) in accordance with manufacture manual protocol B. In short; colonies were picked with a
213 sterile cotton-tip swaps and diluted in inoculating fluid (0.40% sodium chloride, 0.03% Pluronic F-68,
214 and 0.02% Gellan Gum). 100 µL were loaded into each well of a Gen III Microplate (Biolog, Harcard,
215 CA, US). The microplates were incubated at 30 °C for 30 h. Results were compared with the Omnilog
216 Biolog Database (biology, Harvard, CA, US).

Identification analyses found all of the unknown growth to be *Bacillus spp.* The fish mince patties in the present study were boiled prior to use, however, *Bacillus* spores has been shown to survive extended periods of high temperatures and being able to regain vegetative status afterward (Soni, Oey, Silcock, & Bremer, 2016), explaining why they could be found in the fish patties. The Bacillus showed no increase in numbers throughout the storage period but kept constant at a level of 3.0±0.5 log CFU x g^{-1}. The presence *of Bacillus*, however relatively low, meant that results for *L. innocua* or *B. thermosphacta* growth on PCA media was excluded from the rest of the study. As the *Bacillus* showed no growth during the storage period, it is believed not to constitute any competition to the inoculated bacteria, thus not influencing the results obtained from the selective media (BLA and STA).

2.8. Statistics

Statistical analyses, including outlier test, analysis of variance (ANOVA), and general linear modeling (GLM) were performed using Minitab 17.0 (Minitab, Coventry, UK). Outlier testing was performed using Grubbs outlier test at level $p<0.05$. GLM was performed using Tukey's HSD test at level $p<0.05$. To meet the requirements of equal variance and normal distribution, all statistical analyses of microbial growth were done on log-transformed data.

Data are given as mean±standard deviation (SD) unless otherwise stated

3. Results and discussion

GLM analyses showed all parameters (D-value, packaging technology and storage time) as well as all of the interaction effects to be of significant influence on the amount of bacterial growth ($p<0.001$). Despite showing interaction effects, all parameters were tested in combinations using ANOVA.

The experiment was performed in three separate runs, separated based on packaging technologies used. Neither bacterial count of the inoculum nor of the inoculated sample controls showed any significant differences between the runs ($p>0.05$), thus all data has been treated as one experiment.

3.1. Microbial load and packaging technology

A clear pattern evolved for the duration of the study when examining growth of *B. thermosphacta* under different conditions. Regardless of D-value, *B. thermosphacta* showed significantly lowest growth when stored using SGS, followed by MA packaging and lastly VAC (Figure 3), these findings are in agreement with the finding of Birkeland and Rotabakk (2014). In the case of *L. innocua,* the pattern is not as clear. Unlike that seen for *B. thermosphacta*, results between packaging technologies are not as distinct. The level of growth after 16 days of storage is $D_{4.5}$ SGS^{A} < $D_{4.5}$ MA^{B} < $D_{3.5}$ SGS^{B} < $D_{3.5}$ MA^{C} < $D_{4.5}$ $VAC^{C,D}$ < $D_{3.5}$ VAC^{D} [1](Figure 4). As mentioned, it is known that shelf life increases with increasing CO_2 concentrations (Hotchkiss & Langston, 1995). It has further been shown that higher degree of CO_2 saturation can be achieved when using SGS compared to MA packaging (Sivertsvik & Birkeland, 2006). This is in agreement with the findings for headspace gas composition in the present study, which showed significantly higher equilibrium CO_2 concentration in the headspace indicating a higher CO_2 concentration in the fish patties of the SGS- compared to MA packaged samples (Figure 5). This explains why the growth of *B. thermosphacta* and *L. innocua* is lower for SGS than for MA packaging (and VAC) when comparing within same D-values. This correlated well with the fact that use of SGS previously has shown promising results on product susceptible to infection with *B. thermosphacta* and *L. innocua*, including Atlantic salmon (Sivertsvik et al., 2003), cooked peeled shrimps (Sivertsvik et al., 2006), and chicken breast fillets (Al-Nehlawi, Saldo, Vega, & Guri, 2013; Rotabakk, Birkeland, Jeksrud, & Sivertsvik, 2006). On the other hand, Rotabakk, Birkeland, Lekang, and Sivertsvik (2008) found *B. thermosphacta* not to be influenced by the introduction of SGS. This agrees with previous studies, which have showed *B. thermosphacta,* amongst other, to benefit from the elevated CO_2 levels used in MA packaging or SGS. This effect is ascribed to the CO_2 tolerance of *B. thermosphacta* making it able to outcompete the otherwise dominant strictly aerobic microbiota (Koutsoumanis, Taoukis, Drosinos, & Nychas, 2000). In the

[1] Superscript refers to significant variation ($p<0.05$) by one-way ANOVA and Tukey's pairwise comparison test.

present study, the fish patties were sterile, except for presence of *Bacillus spp.* background microbiota and the introduced inoculum species; all being facultative anaerobes. This means that the use of CO_2 will not change the competition and thus not favor one species over the others. The potential growth-limiting effect of vacuum storage is ascribed to the removal of oxygen (Cutter, 2002), however, as facultative anaerobes, *B. thermosphacta* and *L. innocua* can survive and grow under such conditions. The shelf life prolonging effect of CO_2 is further highlighted by the fact that when comparing within same D-value a log difference for *B. thermosphacta* density of 0.7 and 1.3 were observed when comparing VAC to MA- and VAC to SGS, respectively. The log differences in the final density of *L. innocua* were 0.3 and 0.4 for VAC to MA- and VAC to SGS comparisons, respectively ($p<0.01$, details not shown). This shows that the final density is directly related to the amount of CO_2 dissolved in the product.

In more details, results show that *B. thermosphacta* inoculated samples packaged using SGS had no significant difference in Log CFU regardless of D-value and storage time (Figure 3). As mentioned, the heat treatments chosen were based on obtaining a D-value of approximately 3.5 and 4.5 respectively; however, these results show that when using SGS packaging, the packaging technologies is of bigger influence than the use of $D_{4.5}$ or $D_{3.5}$. Differences between D-values showed significant influence for all MA packaged- and VAC samples at the end of the storage period. This demonstrates that in the current study the choice of packaging technology is of greater influence to the final bacterial density that the difference in heat treatment with regards to *B. thermosphacta*. With consumer demands of fresh and lightly processed seafood, high levels of thermal treatment is often unwanted. Use of MA packaging for fish products is the common industrial practice (Birkeland et al., 2014), however, the results of the present study show that at least a difference of one D-value reduction in heat treatment is possible if using SGS rather than MA packaging, thus helping to fulfill the demands of the consumers.

289 Regardless of D-values, *B. thermosphacta* SGS samples showed the first significant increase in log
290 CFU from day 0 to day 8, whereas the MA and VAC samples show growth already between day 0 and
291 4. This indicates that use of high levels of CO_2 is able to extend the lag phase for this bacterium. No
292 such increase in lag phase were observed form *L. innocua* inoculated samples. To the best of our
293 knowledge, no previous studies have looked at growth patterns for *L. innocua* and/or *B.*
294 *thermosphacta* under both MA packaging and SGS, however studies have found SGS, compared to
295 MA packaging, to be able to extend the lag phase in total aerobic- and psychrotrophic plate counts
296 (Sivertsvik et al., 2006), thus agreeing with the present findings. Despite not showing a significant
297 extension of the lag phase for *L. innocua*, the overall results show a significantly lower growth rate
298 compared to those of the MA- and VAC packaged samples, which is in agreement with the findings of
299 Provincial et al. (2013). This has the potential to increase the shelf life of the food products. It has
300 previously been suggested that if the chosen treatment is not sufficient to kill the bacteria, *Listeria*
301 can have the ability to recover during storage, and thus start to grow later in the storage period
302 (Rode, Hovda, & Rotabakk, 2015). However, Rode et al. (2015) further suggested that in the presence
303 of CO_2, this recovery would be postponed even further. This could explain the lowering of the growth
304 rate seen in the present study.

305 The use of Brilliance™ Listeria media has previously been found to limit growth of *Listeria* to non-
306 injured cells, thus excluding most or all heat-injured cells (Beck Hansen & Knøchel, 2001). This could
307 indicate that the levels of inhibition is overestimated in the present study. On the contrary, the use of
308 Brilliance™ Listeria media is recognized by the Food and Drug Administration (Jinneman & Chen,
309 2017) and is an often used method in analysis of listeria in lightly processed seafood (Mejlholm,
310 Bøknæs, & Dalgaard, 2015; Mengden, Röhner, Sudhaus, & Klein, 2015; Young, Anang, & Tiwari,
311 2014), amongst others. The results in the present study are therefore believed to be representative
312 to the true values. Furthermore, at least the data allow a comparison between the different
313 treatments in the present study.

CO_2 not only has an antimicrobial effect during the storage period. Loss and Hotchkiss (2002) demonstrated that the addition of CO_2 to milk prior to pasteurization significantly reduced the number of surviving microorganisms compared to samples pasteurized without the addition of CO_2. In the present study, only the SGS samples had been treated with CO_2 prior to the heat treatment (Figure 2). The findings of Loss et al. (2002) are in agreement with the samples inoculated with *B. thermosphacta*, which show significantly higher inactivation in SGS-samples compared to MA- and VAC-samples (data not shown). The effect of pre-heating addition of CO_2 has been ascribed to the lowering of pH which in turns increases the thermal sensitivity of microorganisms (James, Loessner, & Golden, 2005). In the present study, analysis showed pH to be significantly lower in SGS samples (pH=6.09±0.05) compared to samples not treated with CO_2 (pH=6.51±0.03), thus potentially explaining the increased efficiency of the heating step. On the other hand, no such pattern is seen with regards to *L. innocua* (Figure 4). The reason for this is not understood.

As mentioned earlier *L. monocytogenes* is often considered to be the main concern regarding food safety in ready-to-eat seafood. The non-pathogenic *L. innocua* has a high phenotypic similarity to *L. monocytogenes* and is often used as a substitute organism when studying *L. monocytogenes*. Some strains of *L. innocua* have been found to be more heat resistant than *L. monocytogenes (Lorentzen, Ytterstad, Olsen, & Skjerdal, 2010)*. However, both species share ecological niches (Hudecova, Buchtova, & Steinhauserova, 2010), show no differences in growth patterns (McLaughlin, Casey, Cotter, Gahan, & Hill, 2011), or no differences in response to the use of MA packaging (Hugas, Pagés, Garriga, & Monfort, 1998), thus making *L. innocua* a suitable, yet more conservative, surrogate for studying effect of processing on *L. monocytogenes*.

4. Conclusion

To sum up, the present study has demonstrated that the use of high CO_2 levels in SGS significantly increase the bacterial inhibition by heat, extended the lag phase, and reduced the final microbial density by up to 95% for *B. thermosphacta*, as compared to samples packed with either regular MA packaging or in vacuum, regardless of heating protocol used. In the case of *L. innocua*, a significant

decrease in growth rate, as well as a reduction in final CFU of up to 60% was obtained. In conclusion, this shows that the choice of packaging technology applied is of bigger influence than the differences in the heating protocols applied in the present study.

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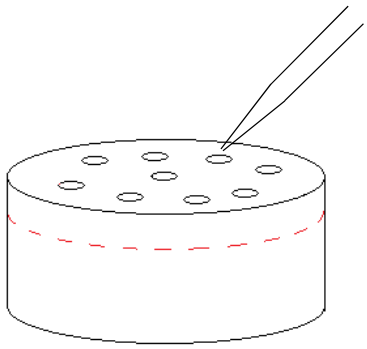


Figure 1: Schematics of fish patty slice with 100 µL inoculum dispersed on the surface by pipetting. Dashed line indicates approximate level of surface sampling for microbial analysis.

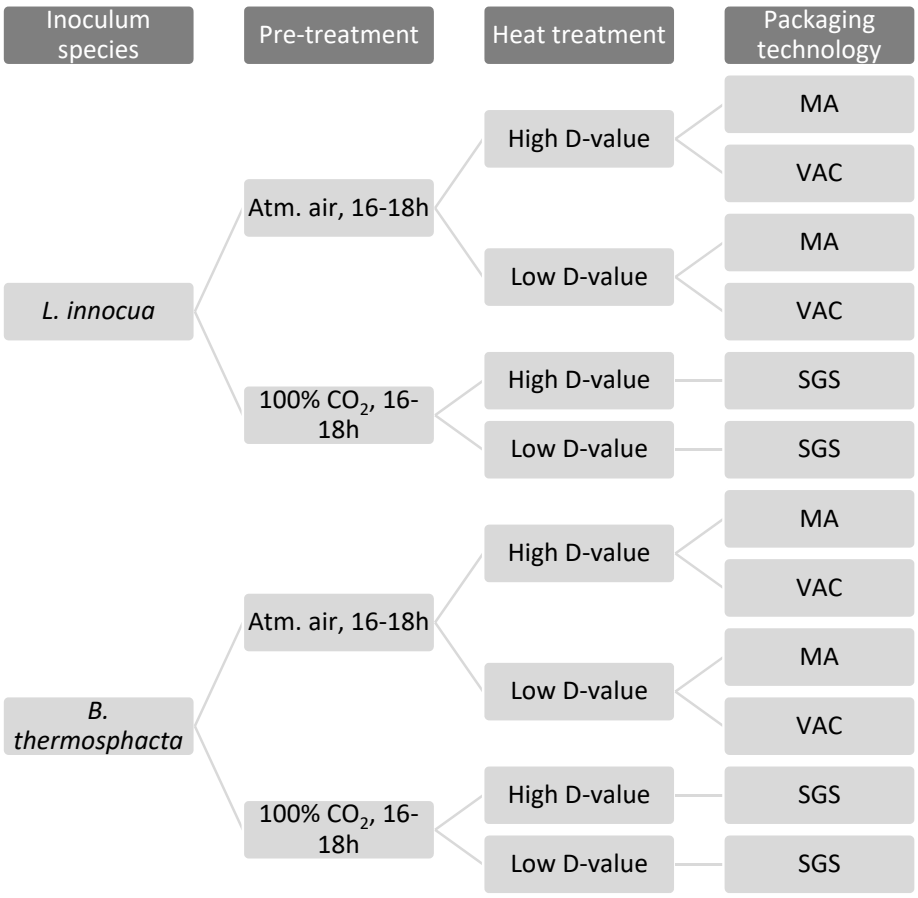
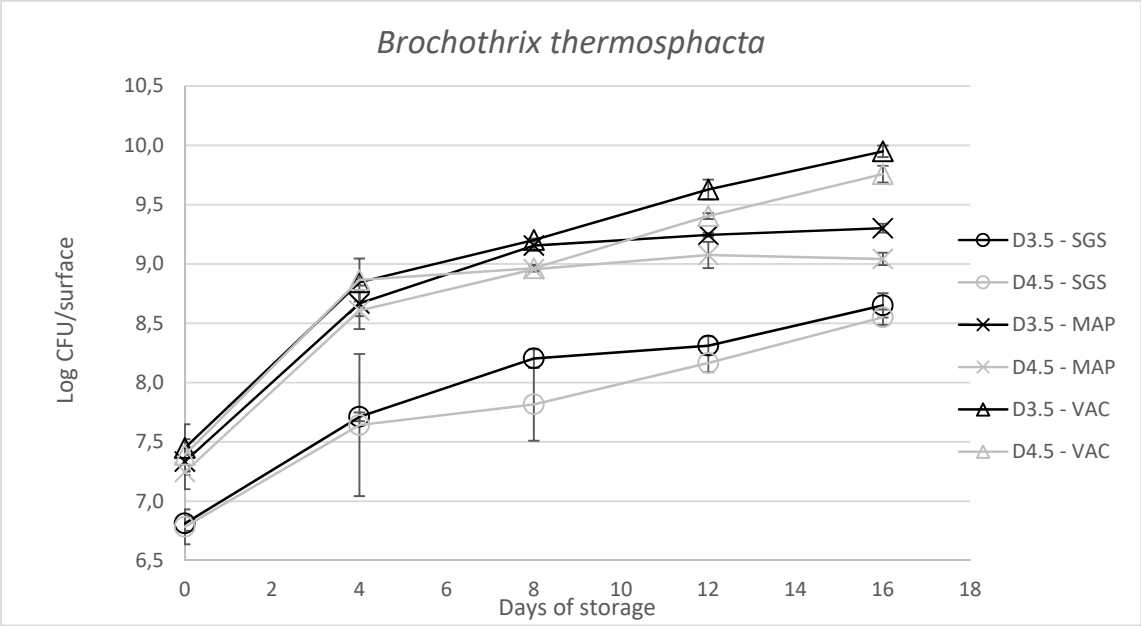


Figure 2: Summary of the experimental design, including sample characteristics separation based on every step of processing. "High D-value" = samples heated to an equivalent of D=4.5 (L. inocua 64°C for 300sec, B. thermosphacta 52°C for 60 sec), "Low D-value" = samples heated to an equivalent of D=3.5 (L. inocua 60°C for 900sec, B. thermosphacta 47°C for 210 sec). "MAP" = Samples packaged in modified atmosphere, "VAC" = vacuum packed samples, "SGS" = Samples packaged using soluble gas stabilization.



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509 *Figure 3: Growth of Brochothrix thermosphacta during 16 days of storage, separated based on heat treatment temperature-*
510 *time combinations and packaging technology. Line color: black = samples heated to an equivalent of D=3.5, grey = samples*
511 *heated to an equivalent of 4.5. Line markings: Δ = vacuum packaged samples, X = Modified atmosphere packaged samples,*
512 *O = Soluble gas stabilization treated samples.*

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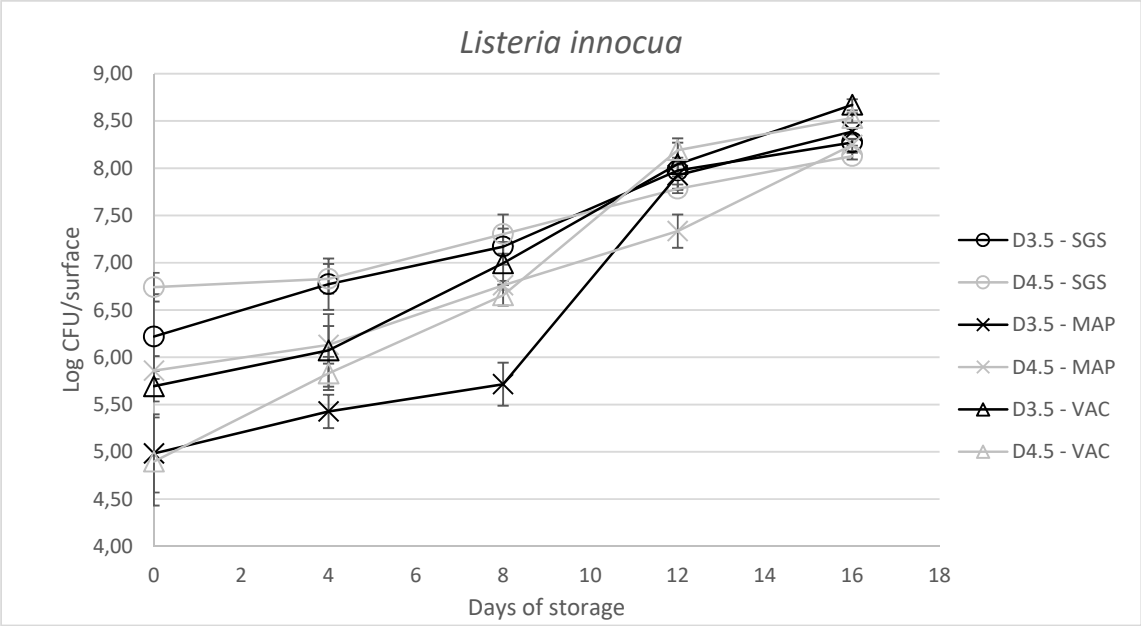

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515 *Figure 4: Growth of Listeria innocua during 16 days of storage, separated based on heat treatment temperature-time*
516 *combinations and packaging technology. Line color: black = samples heated to an equivalent of D=3.5, grey = samples*
517 *heated to an equivalent of 4.5. Line markings: Δ = vacuum packaged samples, X = Modified atmosphere packaged samples,*
518 *O = Soluble gas stabilization treated samples.*

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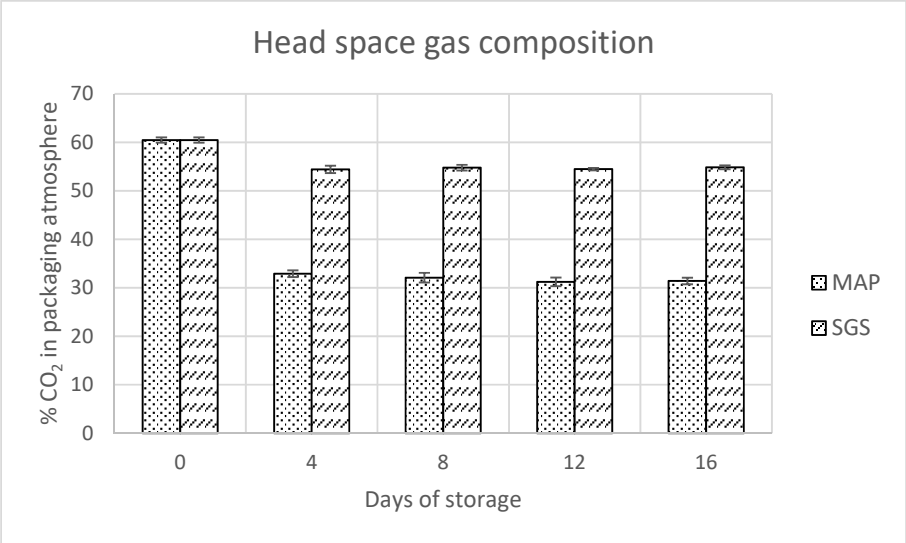

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522 *Figure 5: Headspace CO_2 concentration of MAP- and SGS-samples during the 16 days storage period, presented as mean ±*
523 *standard deviations. Dotted column = Modified atmosphere packaged samples, hatched column = soluble gas stabilization*
524 *treated samples.*

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