Physiochemical and microbiological quality of lightly processed salmon (*Salmo salar L.*) stored under modified atmosphere.

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Abstract

Low-temperature cooking such as sous-vide has become a favored method for processing seafood. In order for this method to be applicable for retail products, combinations with other processing steps are needed to keep the products safe and durable while maintaining high quality. The present experiments were designed to investigate the influence of low-temperature treatment (40, 50, or 60 °C) in combination with various packaging technologies (modified atmosphere (MA) or soluble gas stabilization (SGS)) on both the microbial growth as well as the physiochemical quality. Salmon loins were either kept natural or inoculated with *Listeria innocua* prior to drying (16-18h) in either 100% CO_2 (SGS) or atmospheric air (MA packaging). All samples were sous-vide treated, repackaged in MA and stored at 4 °C for 24 days. The results showed shelf life to be significantly improved with the implementation of SGS, prolonging the of lag-phase and slowing the growth rate of both naturally occurring and inoculated bacteria. Variations in packaging technology did not significantly influence any of the tested quality parameters including drip loss, surface color, and texture. Consumers increasing demand for lightly processed seafood products makes *Listeria spp*. an increased problem, however the present experiment has shown that it is possible to lower processing temperatures to as little as 40 or 50 °C and still obtain inhibition of *Listeria*, but with improved chemical quality compared to traditional processing.

Keywords

Atlantic salmon, lightly processed, *Listeria spp*, microbial quality, modified atmosphere packaging, physiochemical quality, soluble gas stabilization, sous-vide.

1. Introduction

Easy-to-prepare and ready-to-eat meals are increasingly perceived as an optimal solution in a modern lifestyle. This has led to a tremendous increase in the demand for tasty, nutritious, high quality, and yet convenient food products.

Seafood is the second largest export sector in Norway, after oil and gas, and salmon make up the largest individual product group within this sector. This has led to Norway being the world's largest producer of farmed salmon (Asche, Roll, & Tveteras, 2009). Currently, Norwegian salmon is mostly sold in whole fresh or frozen form (Straume, 2017) however, contemporary trends for consumption of lightly processed seafood (Speranza, Corbo, Conte, Sinigaglia, & Del Nobile, 2009) have increased the market for valueadded salmon products. This emphazising the need for devolping and testing of processing methods which allow production of tasty, safe, and durable salmon products with minimal heat treatment.

The consumer demands make sous-vide cooking a favored light processing option. By heating in sealed pouches at a lower temperature, sous-vide cooking offers multiple benefits compared to traditional cooking of seafood (Baldwin, 2012). The benefits include reduced heat damage to proteins and lipids and diminishing the loss of liquid, nutrients, and aromatic compounds while improving the perceived texture (Singh et al., 2016). Two factors are influential in relation to the shelf life extension obtain by sous-vide cooking; the intensity of the applied heat and the control of subsequent storage temperatures (García-Linares, Gonzalez-Fandos, García-Fernández, & García-Arias, 2004). Increased temperature or time during heat treatment has the potential to prolong the shelf life further, but at the same time leads to a significant decrease in organoleptic quality. Low storage temperature is necessary to ensure the microbial safety of sous-vide cooked products (García-Linares et al., 2004), yet many food products are subject to temperature abuse during transport, selling, or storage. This emphasizes the need for further preservation steps in a form of hurdle technology (Baldwin, 2012). Multiple technologies are being used for this purpose, and modified atmosphere (MA) packaging has become a well-established method (Bouletis, Arvanitoyannis, & Hadjichristodoulou, 2017; Lambert, Smith, & Dodds, 1991).

Depending on species and temperature MA packaging has been found to extend shelf life of seafood products by several days compared to air storage (Powell & Tamplin, 2012; Sivertsvik, Rosnes, &

Kleiberg, 2003; Speranza et al., 2009; Torrieri, Cavella, Villani, & Masi, 2006; Tsironi & Taukis, 2010; Özogul, Polat, & Özogul, 2004). The amount of dissolved CO_2 in the foods is proportional to the inhibitory effect of MA packaging (Devlieghere, Debevere, & Van Impe, 1998a, 1998b). Thus constricting the optimal use of MA packaging by the need for a high gas to product ratio to avoid packaging deformation due to CO_2 dissolvement when high CO_2 levels are introduced (Rotabakk, Birkeland, Jeksrud, & Sivertsvik, 2006). Dissolvement of CO_2 prior to retail packaging, a method known as soluble gas stabilization (SGS) (Sivertsvik, 2000) has the ability to overcome this drawback. Regardless of the choice of modified atmosphere applied, the altering of the gas composition in the packages also alters the microbial community (Yesudhason, Lalitha, Gopal, & Ravishankar, 2014). The identified dominant spoilage strains for MA packaged seafood includes lactic acid bacteria (LAB) (Gram & Huss, 1996), *Brochotrix thermosphacta (Macé et al., 2012; Sivertsvik, 2003)* as well as *Photobacterium phosphoreum* (Dalgaard, Mejlholm, Christiansen, & Huss, 1997). Both *B. thermosphacta* and *P. phosphorerum* have been shown to be limited by either heat (Gram & Huss, 1996) or by CO_2 levels equivalent to those obtained by SGS-treatment (Abel, Rotabakk, & Lerfall, 2019). The processing inhibition of aerobic spoilage microflora has the potential to rendering the food unsafe for consumptions before it appears spoiled (Sivertsvik, Jeksrud, & Rosnes, 2002), thus making the control of pathogens such as *Listeria monocytogenes* and *Clostridium spp.* an even more important task.

Multiple studies have been performed on the effect of either heat-treatment or packaging technology on seafood shelf life or product quality; however, research regarding combinations of such technologies on *both* shelf life and product quality are limited. Hence, the aim of this study is to gain knowledge of quality deterioration and microbial development in lightly processed salmon, by studying the effect of combined low heat treatment, MA packaging, and SGS technology on the microbial load as well as perceived product quality parameters.

2. Materials and methods

A three-factor challenge- storage study was conducted, the factors being degree of heat treatment (core temperature of 40, 50, or 60 °C), packaging technology (MA packaging or SGS followed by MA packaging), and microbial flora (natural or inoculated with *Listeria innocua*) (Table 1). The microbial and physiochemical development was evaluated coninuesly for a period of 24 days (at day 0, 6, 10, 13, 17 or 24). The experiments were executed in two rounds, separated based on choice of packaging technology.

2.2 Raw material

Pre-rigor filleted farmed Atlantic salmon (*Salmo salar L.*) (fillet weight of 1-1.4kg) were obtained from Salmar AS (Frøya, Norway). Fillets were obtained on the day of slaughtering and stored in a fridge (4.2±2.4 °C) on ice for 3 days to ensure post rigor state before processing. Backfins, belly flaps, and tails were trimmed in order to obtain a product with equal height (approx. 3cm). Fillets were portioned into equal size of 79.8±2.3g (Figure 1).

2.3 Bacterial strains

A pure *L. innocua* culture (-80 °C) (ATCC 33090) were obtained from the culture collection at University of Gothenburg (CCGU). The cultures were thawed and recovered on brain heart infusion (BHI) agar (Oxoid CM1136, Oxoid Ltd., Basingstoke, UK) at 37 °C for 24 hours. Single colonies were inoculated into separate vails of BHI broth (CM1032, Oxoid Ltd., Basingstoke, UK) for enrichment and incubated at 8 °C for 5 days. The procedure resulted in cold-adapted cultures in an early stationary growth phase. Samples were diluted to OD_{600} of approximately 0.1 (0.104-0.110) in order to obtain a cell concentration of approximately $1x10^5$ colony forming units (CFU) x ml^{-1} ($2.7x10^5$ CFU x ml^{-1}).

2.4 Inoculation

100 µL inoculum were dispersed on the surface of half the samples (estimated 10^4 CFU x $surface^{-1}$) and all the samples were air-dried for 10 min. The rest of the samples were kept natural, without any inoculation. All the samples were packed in batches (n=13) on trays (C2325-1C, Færch Plast, Holstebro, Denmark) in vacuum pouches (425x650 mm PA/PE sous-vide pouch, Maske AS, Trondheim, Norway, filling degree approx. 17%). The pouches were filled with either atmospheric air (in case of MA packaged samples) or pure CO_2 in excess (in case of SGS samples) using a chamber machine (Webomatic SuperMax s3000, Webomatic, Bochum, Germany). Samples were stored at 3.7±0.5 °C for 16-18 h to dry completely and to ensure CO_2 saturation of the SGS-samples. Four replicates were prepared and analysed of each sample at each sampling point, a total of 28 for each treatment group.

2.5 Heat treatment and packaging

Samples were repacked in vacuum pouches (135x180 mm PA/PE sous-vide pouch, Maske AS, Trondheim, Norway) using a chamber machine (Webomatic SuperMax s3000, Webomatic, Bochum, Germany). A sous vide water bath (Diamond M, Fusionchef by Julaba, Germany) was used for all heat treatments. Temperatures were 45 °C (44.6±0.4 °C), 55 °C (54.5±0.2 °C), or 65 °C (64.6±0.1 °C). Treatment times were 15, 18, or 21 min, respectively. Treatment times were chosen based on pre-experiments conducted to establish time needed to obtain a core temperature 5 °C lower than the water bath temperature (core temperature of 40, 50, or 60 °C, respectively). All sample pouches were cooled in ice water and fish samples repackaed in 300 ml semi-rigid crystalline polyethylene terephthalate (CPET) trays (C2125-1B, Færch Plast, Holstebro, Denmark) using a semi-automatic tray sealing packaging machine (TL250, Webomatic, Bochum, Germany). All trays were equipped with an absorbent. During packaging, the air was evacuated (final vacuum pressure of 25 mbar) and flushed with the pre-set MA gas mixture prior to application of a cover film comprised 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 were mixed to 60%

CO_2 balanced with N_2 (both MA and SGS) using a gas mixer (MAP Mix 9000, Dansensor, Ringsted, Denmark). All handling were done aseptically. Oxygen transmission rate (OTR) was 66-78 cm^3 x 25 µm x m^{-2} x 24 h^1 x bar^1 at 23 ˚C for the tray, 2.5 cm^3 x 40 µm x m^{-2} x 24 h^1 x atm^1 at 23 ˚C for the cover film, and 50 cm^3/m^2 × 24 h^1 × bar^1 at 23 °C for the vacuum pouches. Packaging resulted in a sample filling degree of approximately 1:3.

After packaging, the trays were stored at 2.4±1.0 ˚C for up to 24 days.

2.6 Chemical analysis

2.6.1 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) as described by Abel, Rotabakk, Rustad, and Lerfall (2018). The gas compositions were measured at storage day 0, 6, 10, 13, 17, and 24.

2.6.2 Water-, lipid-, and protein content

Water content was determined gravimetrically by drying the samples for 24 hours at 105˚C (ISO.6496, 1983). Lipids were extracted and the total amount calculated gravimetrically as described by Bligh and Dyer (1959). Protein content was calculated based on the total Kjeldahl nitrogen method, using an automated Kjeldahl digester (KjeldDigester K-449, Büchi, Flawil, Switzerland) and titration-system (KjelMaster K-375, Büchi, Flawil, Switzerland) equipped with an autosampler (KjelSampler K-376, Büchi, Flawil, Switzerland). Only the raw material underwent composition analysis.

2.6.3 Drip loss and water holding capacity (WHC)

Drip loss was calculated by the difference in weight of the tray plus absorbent between day 0 and days 6, 10, 13, 17, and 24. WHC was measured as described by Skipnes, Østby, and Hendrickx (2007) using metal carriers (Part No. 4750, Hettich Lab Technology, Germany) and centrifuged (Rotina 420 R,

159 Hettich centrifuge) for 15 min at 4 °C, using a free swing rotor at RCF = 530×g. The WHC was measured
160 in triplicates of each group on day 6 and 24 of storage to obtain start and end values.

161 ### 2.6.4 Surface color

162 Sample surface color (CIE Lab) was assessed by a digital photo imaging color-measuring system
163 (DigiEye full system, VeriVide Ltd., Leicester, UK). Analysis were carried out in a standardized lightbox
164 (6400 K) using a digital camera (Nikon D7000, 35 mm lens, Nikon Corp. Japan). The pictures were
165 analyzed with DigiPix software ver 2.8.0.2 (VeriVide Ltd., Leicester, UK). Changes in perceived color
166 were calculated as ΔE in accordance with the formula $E = \sqrt{{S_L}^2 + {S_C}^2 + {S_H}^2}$ where $S_L = \Delta L/2$, $S_c =$
167 $\Delta C/(1 + 0.048 * C_1)$, $S_H = \Delta H_{ab}/(1 + 0.014 * C_1)$ and $\Delta H_{ab} = \sqrt{\Delta a^2 + \Delta b^2 - \Delta C^2}$ as described by
168 CIE Int. Commission on Illumination (1994). ΔE values higher than 4 are normally visible to the human
169 eye (Lerfall, 2011).

170 ### 2.6.5 Texture

171 Instrumental textural analyses were performed using a Texture Analyzer TA-XT2 (SMS Ltd., Surrey,
172 England) fitted with a 30 kg load cell and a Warner Bratzler probe (SMS Ltd., Surrey, England). The
173 force-time graph was obtained by the Texture Exponent software for Windows (version 6.1.7.0, SMS
174 Ltd., Surrey, England), which was used for the data analyses. The analyses were performed in four
175 times replicates for each group immediately after processing and cooling. The analysis was done at a
176 speed of 1 mm x s^{-1}, and measurements were performed until 100% penetration was achieved.
177 Portioned raw and treated samples (Figure 1) were placed with the probe adjacent to the mid line, to
178 ensure measures were a result of shearing rather than flaking of the muscle fibers.

179 ### 2.6.6 Degradation products of adenosine triphosphate (ATP)

180 Degradation products of ATP was analysed on a Phenomenex synergi 4u hydro-RP80 A (150×4.6mm,
181 4µm) HPLC column after a method by Sellevold, Jynge, and Aarstad (1986), using an Agilent 1290
182 chromatograph (Agilent technologies, Paolo Alto, CA, USA) (isocratic, flow 1.0 mL/min) connected to

183 an Agilent 1260 diode array UV-VIS detector, as described by Lerfall, Jakobsen, and Bjørge Thomassen
184 (2018). Standard curves of ATP (Sigma, ≥99%, CAS:34369-07-8), ADP (Sigma, ≥95%, CAS:20398-34-9),
185 AMP (Sigma, ≥99%, CAS:149022-20-8), IMP (Sigma, ≥98%, CAS:352195-40-5), HxR (Sigma, ≥99%,
186 CAS:58-63-9) and Hx (Sigma, ≥99.0% CAS:68-94-0) in deionized water were used for identification of
187 quantification.

188 ## 2.7 Microbial analysis

189 Microbial analyses were prepared using 10 g of fish sampled aseptically from the inoculated surface.
190 The fish sample was homogenized in 90ml sterile 0.85% NaCl (w/v) and 0.1% peptone (w/v) water for
191 60 sec. Decimal dilution series were prepared in similar solution in accordance with NMKL-standard 91
192 (NMKL, 2010).

193 Natural bacterial flora (NBF) samples were analyzed for total aerobic plate count, *Clostridium* spores,
194 *Listeria* spp., and LAB. Inoculated samples were analyzed for total aerobic plate count and *Listeria* spp.
195 Negative control samples were tested for total aerobic plate count, *Clostridium* spores, *Listeria* spp.,
196 and LAB.

197 Total aerobic plate count was analyzed as pour plates with a top layer of Lyngby iron (LI)-agar
198 (CM0964, Oxoid Ltd., Basingstoke, UK) prepared as described by the manufacturer, and incubated
199 aerobically at 22 °C (21.6±0.4 °C) for 3 days, in accordance with NMKL-184 (NMKL, 2006). Presence of
200 sulfite-reducing *Clostridium* spores was analyzed in accordance with NMKL-56 (NMKL, 2008) on Shahidi
201 Ferguson Perfringens (SFP) agar base (DIFCO28110, Thermo Fisher Scientific, Waltham, MA, USA)
202 prepared as described by the manufacturer, but without the addition of egg yolk. Dilution of the
203 sample material was heated at 80 °C for 10 minutes prior to plating in order to inhibit any vegetative
204 cells. Samples were incubated anaerobically at 15 °C (15.1±0.6 °C) for 5 days. Presence of *Listeria* spp.
205 were tested on Brilliance™ listeria agar (BLA) containing Brilliance™ listeria selective supplement
206 (Oxoid CM1080 and Oxoid SR0227, Oxoid Ltd., Basingstoke, UK) prepared as described by the
207 manufacturer, and incubated aerobically at 37 °C (37.1±0.2 °C) for 24±2 h. Presence of LAB was tested

as described in NMKL-140 (NMKL, 2007) on de Man, Rogosa and Sharpe (MRS)-agar (Oxoid CM0361, Oxoid Ltd., Basingstoke, UK) with 10 mg/l amphotericin B, and inoculated at 25 °C (25.8±0.2 °C) for 3 days.

Sampling was performed of the raw material, inoculated samples, and on all stored samples after 0, 6, 10, 13, 17, and 24 days storage in accordance with experimental design (Table 1).

2.8 Statistics

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

Data were analysed in 4 time replicate and is presented as mean ± standard deviation (SD) unless otherwise stated.

3. Results and discussion

A GLM showed all parameters (core temperature, packaging technology, and storage time) as well as all the interaction effects to be of significant influence on the amount of microbial growth ($p<0.001$) for both natural- and inoculated samples. No correlation was found between packaging technology and color, WHC, drip loss, or formation of ATP-degradation products. Processing temperature influenced all of the tested parameters ($p<0.001$)

3.1 Quality analyses

A concern when it comes to the implementation of SGS is the influence on the quality of the product. The appearance of food products is of major importance to consumers, both with regards to acceptability and preference. When it comes to salmon, the color is generally perceived as one of the most important quality parameters (Anderson, 2000). In the present experiments, the only results that

yielded ΔE values above the noticeable limit was comparisons between temperature treatments, showing increased lightness (L*) and decreased redness (a*) and yellowness (b*) with increasing temperatures (Figure 2), as reported by Bhattacharya, Choudhury, and Studebaker (1994). This result can be explained by the increased protein denaturation and coagulation of sarcoplasmic proteins on the surface caused by the increased temperatures. Broadly speaking, fish muscle proteins are separated into three groups, with the more important proteins being myosin, actin and the sarcoplasmic proteins. Multiple studies have demonstrated the temperature stability of these proteins, and it is generally agreed that heat denaturation of myosin in salmon occurs in a range of 43-50 °C, actin around 76-78 °C and the sarcoplasmic proteins, which a more diverse group, in a broad range from 57-67 °C (Ovissipour, Rasco, Tang, & Sablani, 2017). In the present study, the heat treatments were carried out at 45, 55, and 65 °C, respectively. This would indicate that only limited protein denaturation would have taken place at 45 °C, whereas myosin would be completely denatured at 55 °C and at least some of the sarcoplasmic proteins in the 65 °C samples. Temperatures never reach levels of actin denaturation.

The results of the color analysis also show that neither choice of packaging technology nor storage time gave rise to any perceivable changes in color (average a*=14.5±3.4, b*=9.4±2.2, L*=67.0±2.4, ΔE=2.2±1.3). These findings are in agreement with those by Rotabakk, Birkeland, Lekang, and Sivertsvik (2008) on halibut, or by Mendes and Gonçalves (2008) in sea bream and sea brass. As for the color analysis, WHC was found not to be significantly influenced by treatment temperature, packaging technology, or duration of storage ($p=0.054-0.926$). Average WHC was measured to be 70.9%. The lack of differences in WHC is in agreement with the fact that no significant differences ($p<0.001$) were observed in drip loss as a result of either temperature or packaging technology (Table 2). In contrast, a close relationship has previously been established between protein denaturation and WHC (Kong, Tang, Rasco, Crapo, & Smiley, 2007) and hence drip loss. WHC is highly dependent on the properties of myosin, thus expecting WHC to decrease once myosin denaturation temperatures have been reached (Ofstad, Kidman, Myklebust, & Hermansson, 1993). The reason for the discrepancy is not

understood. Moreover, it has been reported that an increase in dissolved CO_2 will alter WHC and increase drip loss (Davis, 1998; Randell et al., 1999) as reported for halibut (Rotabakk et al., 2008) and shrimps (Sivertsvik & Birkeland, 2006). The effect was then ascribed to volume reduction caused by the uptake of CO_2 by the product, an effect which is counteracted by the use of SGS prior to MA packaging (Rotabakk et al., 2008). An alternative explanation could be that the presence of CO_2 during the heat treatment increased the cook loss thereby limiting the drip loss later in the storage; however, cook loss was not measured in the present study. The significant differences between processing temperature in results for all the tested quality parameters show the potential to improve quality by lowering of the processing temperature. At the same time, the lack of significant differences between packaging technology treatments facilitates the lowering of this temperature without affecting the quality by itself.

Just as perceived color, tenderness of foods is another important parameters regarding consumer satisfaction (Bhattacharya, Choudhury, & Studebaker, 1993). Particularly the shear force has been cited as an influential factor for consumer opinions (Jonsson, Sigurgisladottir, Hafsteinsson, & Kristbergsson, 2001; Sigurgisladottir et al., 1999). Protein denaturation is known to play an important role in the toughening of the texture of muscle products (Hatae, Yoshimatsu, & Matsumoto, 1990). The influence of both treatment temperature and packaging technology were analyzed, but the results were inconclusive as variation in raw material were found to be bigger than variations between treatments (data not shown).

Fish deterioration is monitored in many different ways, common amongst these are ATP-degradation products, which is considered a good indicator of fish freshness (Shumilina et al., 2016). Post-mortem degradation of ATP in fish muscle occurs due to a combination of endogenous and bacterial enzymes and goes through the intermediate products ADP, AMP, IMP, HxR, and Hx. Most important degradation products include Hx and IMP, which has been associated with the development of unpleasant and enhancing flavors in stored fish, respectively (Mørkøre et al., 2010). Regardless of treatments, all

283 samples showed a significant drop in IMP levels between storage day 0 and 6. Furthermore, at day 0
284 no difference was found between temperature treatments within packaging-groups, however at the
285 end of the storage period, samples treated to 40 °C were significantly lower than those at treated to
286 50, and 60 °C ($p=0.003$-0.042) (Figure 3). This can be explained by the enzymatic nature of the
287 degradation of IMP to HxR. Higher treatment temperatures yield a higher degree of enzyme
288 denaturation, causing the degradation of IMP to slow (Surette, Gill, & LeBlanc, 1988). The temperature
289 dependence is further highlighted by the fact that the levels of HxR were significantly higher in samples
290 heated to 40, followed by 50 and lastly 60 °C. For the duration of the entire storage period, only
291 samples heated to 60 °C with SGS showed any development in HxR levels (Figure 3). The developmental
292 trend indicates that the conversion rate from IMP to HxR equals that of HxR to Hx. Concerning Hx, no
293 significant initial differences were observed between temperatures within packaging groups. During
294 the storage period levels developed into samples heated to 40 °C having significantly higher levels than
295 those at 50 °C, which in turn was significantly higher than those heated to a core temperature of 60 °C
296 ($p<0.013$). As for the formation of HxR, this development is explained by lower temperatures causing
297 lesser enzyme denaturation. Further, the formation of Hx is partly caused by bacterial action (Surette
298 et al., 1988), and as seen from the bacterial counts, bacterial levels were significantly higher with
299 lowering of the core temperature (Table 2), thus further explaining the increased formation of Hx at
300 lower core temperature. Analysis of ATP degradation products in the raw salmon used in the two round
301 of experiments (MA or SGS) showed only HxR levels to be significantly different ($p=0.037$) (data not
302 shown). This could explain the differences observed between packaging groups in HxR- as well as in Hx
303 levels, however, it does not explain why significant differences were observed between packaging
304 technologies for IMP. Due to the differences in initial levels of ATP degradation products observed
305 between the two batches of raw material, it is not possible to distinguish potential effect of variation
306 in packaging technology from the batch variations, rendering comparisons between packaging
307 technologies infeasible.

3.2 Microbial community and processing

The raw material for the two rounds of experiments was obtained separately in order to obtain equal length from slaughtering to processing and analyzing, and thereby equal rigor-state. Unfortunately, the raw material characteristics were significantly different for the two batches, both regarding protein content ($p=0.003$), lipid content ($p=0.002$), and water content ($p=0.002$). It has previously been shown (Abel et al., 2018) that the important parameter for absorption of CO_2 and consequently the bacteriostatic effect of packaging, is the total content of water and liquid lipid. When combing water- and lipid content from each of the two batches of salmon, no significant differences were seen between them ($p>0.642$) rendering the differences in each compound not important. All other inputs were identical between the two rounds (water bath temperatures $p>0.701$, packaging gas CO_2 concentration $p=0.551$, and storage temperature $p>0.921$) and thus the two round is assunmed equal.

Regarding microbial growth, a clear pattern evolved during the study when examining both the natural flora and the *L. innocua*-inoculated samples. Regardless of packaging technology, no growth was observed on any samples heated to a core temperature of 60 °C, the same was the case for SGS treated samples heated to core temperature of 50 °C. Temperatures were chosen based on pre-trials, which had shown growth after heat treatment at 40, 50, and 60 °C (data not shown). The pre-experiments were carried out without the use of modified atmosphere, which might explain the inconsistencies. Furthermore, regardless of packaging technology higher temperature treatment always lead to a lower level of recovered bacteria, as expected (Figure 4 and Figure 5). Headspace gas composition analysis showed that SGS treated samples had significantly higher O_2 levels ($p<0.001$) after equilibrium has been reached (day 6 and onwards). It has previously been shown the presence of CO_2 during the heating step facilitates a higher heat inactivation of the bacteria (Abel et al., 2019; Loss & Hotchkiss, 2002), however, this was not observed in the present study (Figure 4 and 5). In the case of *L. innocua*-inoculated samples, heat treatment to a core temperature of 40 °C led to an insignificant reduction in CFU ($p>0.072$) unlike that seen for the 50 °C samples ($p<0.001$), regardless of presence of CO_2. This led to no differences in bacterial count at day 0 between samples packed using MA or SGS when heated

334 to 40 °C core temperature. On the other hand, samples heated to a 50 °C core temperature showed
335 significantly higher initial CFU levels ($p<0.001$) when treated with CO_2 prior to heating (SGS samples).
336 This can be explained by a visible bloating that arose during the heat treatment. The solubility of CO_2
337 is highly temperature dependent (Sivertsvik, Jeksrud, Vågane, & Rosnes, 2004), thus heating at
338 temperatures as high as 55-65 °C for prolonged periods will decrease the solubility of CO_2, causing it
339 to desorb from the salmon and into the headspace of the vacuum pouch. This forms a layer of gas
340 surrounding the sample, protecting the samples from the heat of the water bath, thus redering the
341 heat load less than anticipated. Despite the higher initial count from the SGS-treated samples, the
342 bacterial counts are equal already at day 6 and surpassed by the MA packaged samples from day 10
343 and onwards ($p=0.001$-0.035). This effect is ascribed to the fact that samples treated in 55 °C water
344 and packed using SGS showed no significant bacterial growth throughout the entire 24 days of storage
345 (Figure 4 and Table 3). The experiments were performed in two rounds, and no significant differences
346 were observed between bacterial counts in either inoculum or from samples right after inoculations.
347 This means that the outgrowing on the MA packaged samples can be ascribed to the processing and
348 not variations between samples. It has been suggested by multiple studies that increased CO_2 level will
349 increase the inhibitory effect of *Listeria spp.*, e.g. by reducing the growth rate (Augustin & Carlier, 2000;
350 Devlieghere et al., 2001; Farber, Cai, & Ross, 1996; Provincial et al., 2013), as seen in the present study
351 (Table 3). However, to the best of our knowledge, this is the first time it has been proven that growth
352 of *L. innocua* can be completely inactivated for as long as 24 days of storage, under the given
353 conditions. Industry practice calls for a heat-treatment at no less than 70 °C for 2 min (concerning
354 *Listeria spp.*; Advisory Committee on the Microbiological Safety of Food (2009)) in order to ensure a
355 safe product, often with unwanted quality deterioration as a result. The present results show that
356 inhibitatory effect on *Listeria spp.* can be reached at much lower processing temperatures, at least for
357 a refrigerated storage period of up to 24 days. SGS treatment of samples does not only benefit the
358 high-temperature samples. Even when treated to 40 °C, introduction of SGS results in a prolonging of
359 the lag phase of *L. innocua* (Table 3) from seeing significant count increase already between day 0 and

6, to a lag phase of more than 6 days. This leads to no significant difference at the end of storage between 50 °C treated samples packed using MA packaging and 40 °C treated samples packed using SGS (Figure 4). This highlights that SGS compared to MA packaging makes up for at least a 10 °C difference in core temperatures, when it comes to inhibition of *Listeria*, with potential organoleptic quality improvements as a result. The experiments were only performed under ideal storage conditions e.g. low storage temperature, elimination of cross contamination etc., thus the effect might be different in case of temperature abuse or deviating conditions. However, the comparison between MA packaging and SGS is believed to be true as tests are performed under the same condition.

Similar positive results were seen for the natural samples. Unlike *L. innocua* inoculated samples, the bloating of the natural samples during heat treatment was not enough to influence the bacterial inhibition by heat (figure 5). A log reduction in CFU of 0.82, 1.17, 1.14 or 3.14 (MA-40°C, MA-50°C, SGS-40°C or SGS-50°C, respectively) was obtained ($p<0.003$ for all groups). The difference in heat inactivation can be ascribed to the lower temperature tolerance of natural flora that mainly consists of LAB, as can be seen by the lack of significant differences between bacterial count for natural samples grown on either non-selective or selective LAB growth media (equivalent to Figure 5, with bacterial counts at day 24 for 40 °C core temperature samples: LI 5.8-5.98±0.35 log CFU x g^{-1} while MRS 5.33-5.59±0.20 log CFU x g^{-1}). This further explains why significant bacterial growth was only observed for samples heated to 40 °C, with the exception of growth on samples heated to 50 °C packed in MA. Comparing bacterial growth on samples heated to 40 °C packed using either MA or SGS showed no significant difference in bacterial count immediately after heat treatment. This was in spite of the raw material used for the SGS-treated samples showing a significantly higher bacterial count prior to heat treatment ($p<0.001$) (Figure 5). At the end of the storage period, no significant differences were observed between bacterial counts from samples packed in MA and SGS ($p>0.545$), however, at days 6 till 17 MA packaged samples had significantly higher bacterial counts compared to SGS ($p<0.029$) (Figure 5). Manufacturers are not only interested in obtaining the longest possible period below the recommended maximum level of 10^6 CFU x g^{-1} (Health Protection Agency, 2009), but they are equally,

386 if not more, interested in prolonging the period with what is perceived as "good quality", which is what
387 SGS-treatment facilitates under the conditions of the present study (Figure 4 and Figure 5). Spoilage
388 of MA packaged seafood has often been associated with the growth of LAB which, due to its
389 proteolytic abilities, can cause serious deterioration of the quality of the products, including increased
390 drip loss and loosening of the texture (Gram & Huss, 1996). Reducing the growth of LAB therefor has
391 the potential to significantly increase the quality of the product. The use of SGS packaging significantly
392 lowered the maximum growth rate by half and increased the lag phase of the natural flora compared
393 to that observed in MA packed samples (Table 3). The results indicate a potential inhibatory effect on
394 the natural flora of salmon, as introduction of SGS in the current study compensates for a 10 °C
395 reduction in processing temperature, as seen for the listeria inoculated samples. Further certainty
396 regarding the effect on the natural flora, can only be achieved through further challenge testing,
397 considering both strains and conditions.

398 The non-pathogenic *L. innocua* has a high phenotypic similarity to *L. monocytogenes* and is often
399 used as surrogate for *L. monocytogenes*. Both species share ecological niches (Hudecova, Buchtova,
400 & Steinhauserova, 2010), show no differences in growth patterns (McLaughlin, Casey, Cotter, Gahan,
401 & Hill, 2011), and no differences in response to the use of MA packaging (Hugas, Pagés, Garriga, &
402 Monfort, 1998). On the other hand, *L. innocua* have been found to be more heat resistant than *L.*
403 *monocytogenes* under certain *conditions* (Lorentzen, Ytterstad, Olsen, & Skjerdal, 2010). This makes
404 *L. innocua* a suitable, yet more conservative, surrogate for studying effect of processing on *L.*
405 *monocytogenes* under the conditions in present experiment (Hu & Gurtler, 017).

406 # 4 Conclusion

407 In conclusion, SGS has long been expected to have beneficial properties with regard to prolonging shelf
408 life, however, the impact on chemical quality has been questioned. The present study underlines the
409 microbiological benefits of CO_2 in SGS by prolonging the lag phase of both *Listeria innocua* and the
410 naturally occurring flora, slowing the growth rate, and even completely hindering the growth of *L.*

innocua for 24 days of storage as compared to MA packaging. Furthermore, no negative effect of SGS was observed for any of the chemical parameters tested, including WHC, drip loss, surface color, and texture. Consumers show an increased demand for lightly processed convenient seafood products. The present experiment has shown that it is possible to lower processing temperatures to a little as 40 or 50 °C and still obtain an inhibitory effect on *Listeria spp*., one of the biggest risks regarding food safety, while improving chemical quality compared to traditional processing.

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6 Author Contribution

The study was designed in cooperation between N. Abel, B.T. Rotabakk, and J. Lerfall. Data was collected by N. Abel, V. B. Ahlsen, and J. Lerfall. Results were interpreted by N. Abel, J. Lerfall, B.T. Rotabakk, and T. Rustad. The manuscript was drafted by N. Abel and revised by B.T. Rotabakk, T. Rustad, V. B. Ahlsen and J. Lerfall.

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612

613

Table 1: Experimental design and response variables.
MA=modified atmosphere, SGS=soluble gas stabilization

Design variables	**Levels**
Core temperature	40, 50, or 60 °C
Packaging method	MA packaging or SGS
Microbial community	Natural or inoculated with *Listeria innocua*
Storage time	0, 6, 10, 13, 17, or 24 days
Response Variables	**Analyses**
Quality (only on natural samples)	Color, composition, drip loss, headspace gas composition, metabolites of ATP, texture, water-holding capacity.
Microbiological	Aerobic plate count, H_2S-reducing clostridium, Lactic acid bacteria, *Listeria spp*.
Tested samples	Raw, processed, stored n=4 for each group, at each sampling point

622 *Table 2: Main effect of treatment temperature and packaging technology on drip loss, WHC, headspace CO_2 concentration,*
623 *and bacterial count of natural and inoculated samples. Key results are elaborated in Figure 4 and Figure 5*

		Drip loss [%] df=175	**WHC [%]** df=54	**Head space CO_2 [%]** df=257	**Log CFU x g^{-1} Inoculated** df=141	**Log CFU x g^{-1} Natural** df=139
Core temp	40	7.4±2.2[a]	70.4±5.4[a]	50.6±7.4[a]	3.85±0.77[a]	3.97±1.4[a]
	50	8.9±1.3[a]	71.2±4.1[a]	49.8±6.3[a]	2.18±1.05[b]	0.90±1.2[b]
	60	7.4±1.4[a]	71.0±5.1[a]	49.7±6.7[a]	N.D.	N.D.
		p=0.065	p=0.868	p=0.635	p<0.01	p<0.01
Packaging technology	SGS	8.0±1.9[a]	72.0±5.6[a]	54.5±5.8[a]	2.26±2.01[a]	1.95±2.05[b]
	MA	7.8±1.9[a]	69.7±3.7[a]	45.4±4.2[b]	1.69±1.42[a]	1.17±1.87[a]
		p=0.329	p=0.087	p<0.01	p=0.053	p=0.020

df = degrees of freedom

N.D.=not detected.

Superscript letters (a-c) indicates significantly mean value differences (p<0.05) according to one-way ANOVA

624

625

626 *Table 3: Growth kinetic parameters (maximum specific growth rate (μ_{max}, day^{-1}) and lag phase (day) for samples subjected to*
627 *different heat treatments (40 or 50 °C) and packaging technology (MA and SGS). Samples treated at 60 °C are not included*
628 *due to lack of detectable growth. The parameters are estimated from the primary model of* **Baranyi and Roberts (1994)** *using*
629 *log-transformed bacterial counts.*

Treatment	*μ_{max} [day^{-1}]*	*Lag phase [day]*	*R^2*	*SE(fit)*
Natural flora				
40°C – MA	0.48±0.08	2.8±1.4	0.929	0.515
50°C – MA	0.2±0.05	3.1±2.0	0.827	0.827
40°C – SGS	0.22±0.03	4.0±0.2	0.934	0.336
50°C – SGS	N/A[a]	N/A[a]	N/A[a]	N/A[a]
Inoculated flora				
40°C – MA	0.70±0.06	-	0.951	0.421
50°C – MA	0.38±0.15	1.59±2.4	0.839	0.563
40°C – SGS	0.06±0.03	6.48±0.1	0.643	0.233
50°C – SGS	0.10±0.88	24.0±5.1	-0.0745	0.426

630 R^2, coefficient of determination, SE (fit), standard error of fit to the model, [a] not estimated due to no detectable growth during storage.

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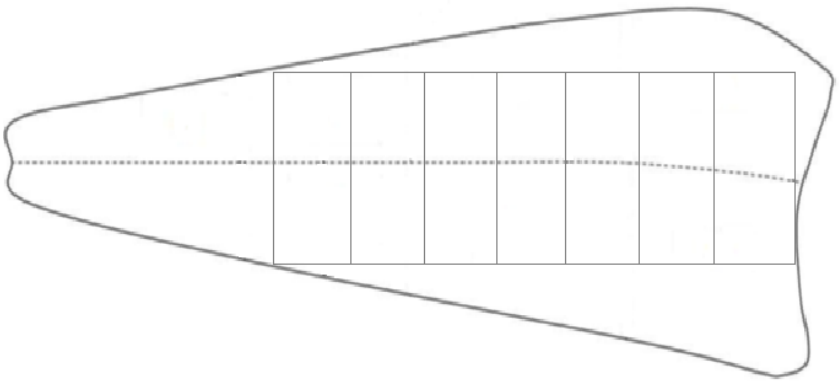
632



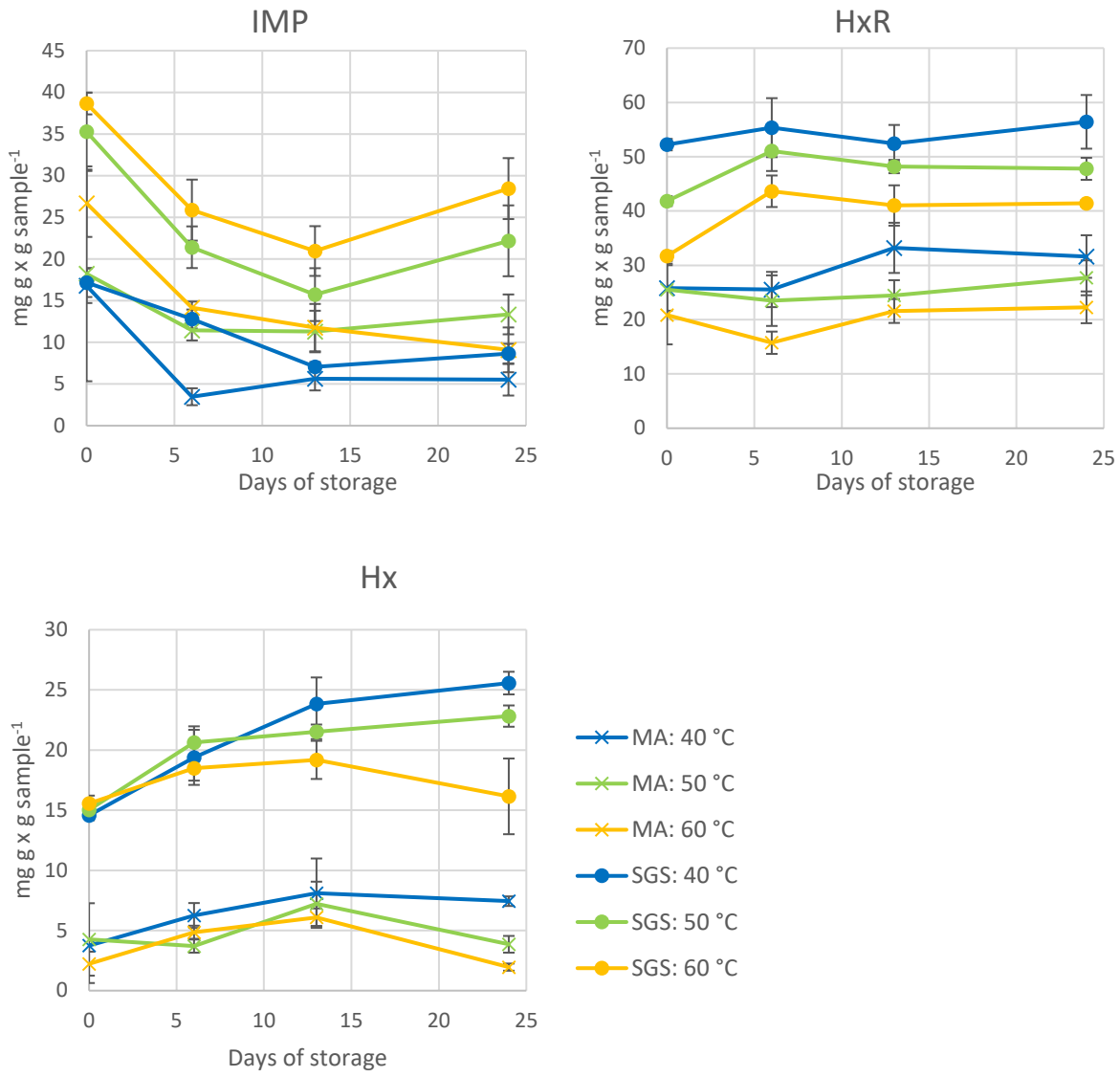
633

634 *Figure 1: Schematic illustration showing the sampling of salmon portion after removal of backfin, belly flap, and tail.*

635

Figure 2: Image of salmon samples immediate after heat treatment in temperatures of 45 (left), 55 (middle), or 65 °C (right). All samples are with no CO_2 exposure prior to heat treatment (MA-samples).

641



642

643 *Figure 3: Development of ATP-degradation products inosine monophosphate (IMP) (upper left), inosine (HxR) (upper right)*

644 *and hypoxanthine (Hx) (lower left) during 24 days of storage, separated based on treatment temperatures and packaging*

645 *technology applied. X = MA packaged samples, O = SGS treated samples, blue = 40 °C core temperature samples, green = 50*

646 *°C core temperature samples, yellow = 60 °C core temperature samples. Error bars indicates mean ±1 standard deviation.*

647

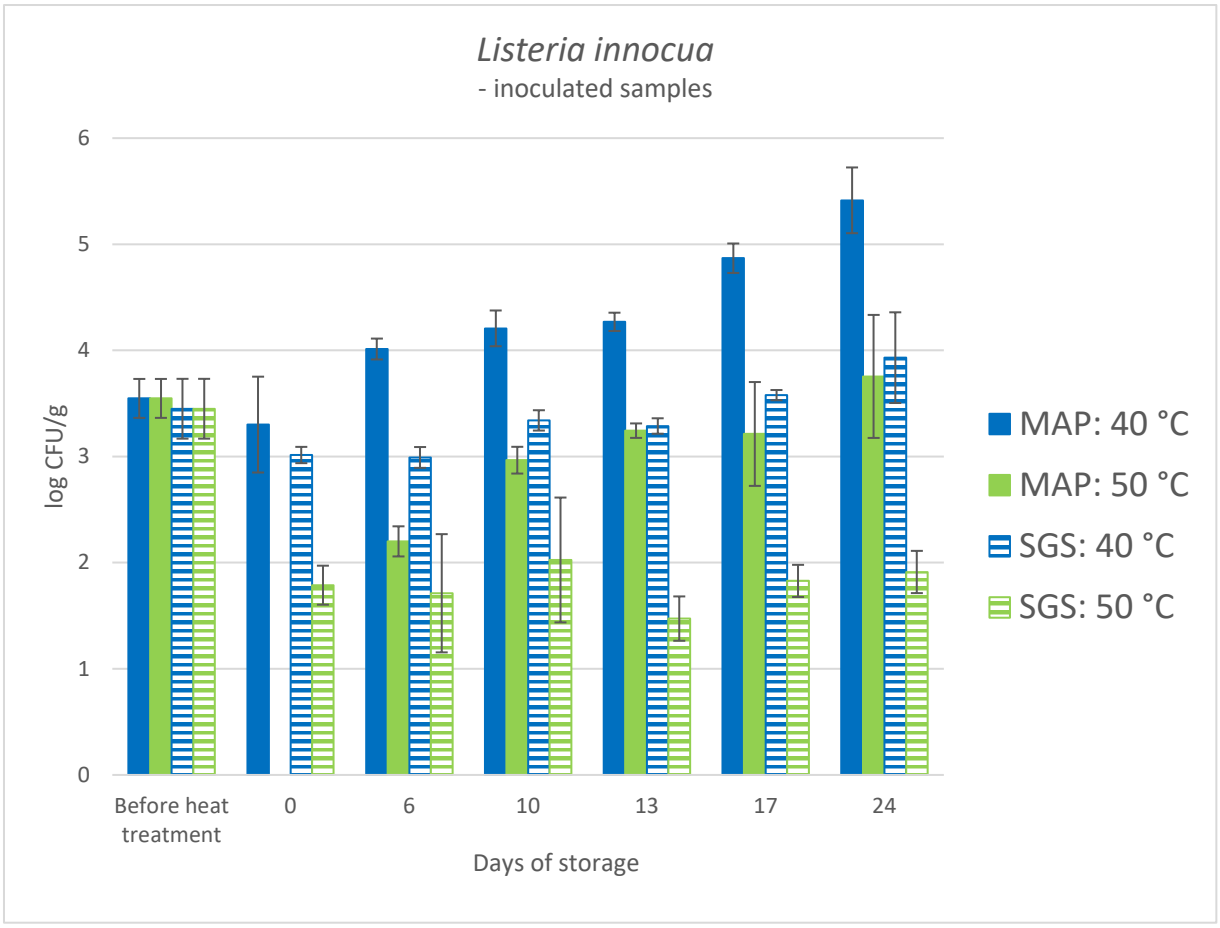


Figure 4: Growth of Listeria innocua on inoculated samples, separated based on heat treatment temperature and packaging technology applied. 60 °C samples, regardless of packaging technology, showed no growth at any point, and thus have been left out. Solid = MA packaged samples, striped = SGS treated samples, blue = 40 °C core temperature samples, green = 50 °C core temperature samples. Error bars indicates mean ±1 standard deviation.

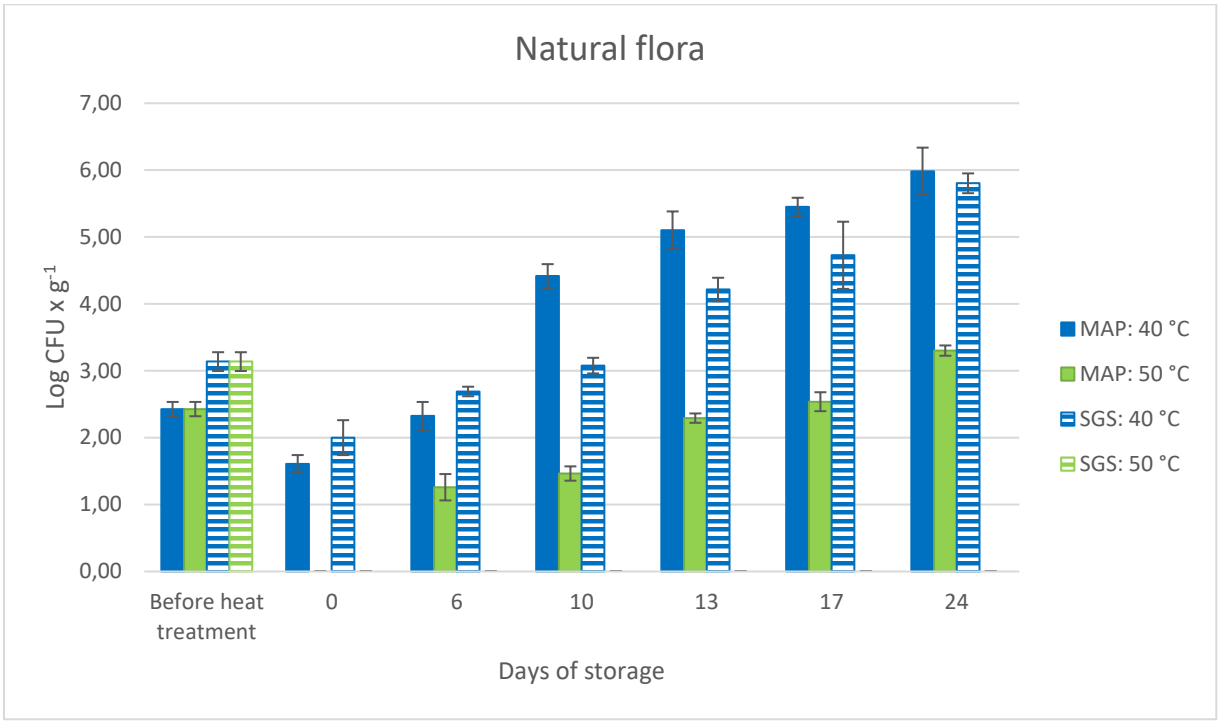


Figure 5: Growth of microbial flora on natural samples, separated based on heat treatment temperature and packaging technology applied. 60 °C samples, regardless of packaging technology, showed no growth at any point, and thus have been left out. Solid = MA packaged samples, striped = SGS treated samples, blue = 40 °C core temperature samples, green = 50 °C core temperature samples. Error bars indicates ±1 standard deviation.