AGAR FILMS CONTAINING GREEN TEA EXTRACT AND PROBIOTIC BACTERIA FOR EXTENDING FISH SHELF-LIFE

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ABSTRACT

A bioactive film composed of agar, incorporating green tea extract and probiotic strains (*Lactobacillus paracasei* L26 and *Bifidobacterium lactis* B94) was applied on hake fillets in order to evaluate the effect of the films during 15 days of storage. Hake was previously inoculated with *Shewanella putrefaciens* and *Photobacterium phosphoreum* (10^3-10^4 CFU/g) to simulate a spoilage process. The green tea and/or probiotic film provoked a reduction, particularly of H_2S-producing bacteria counts and total viable bacteria throughout the storage period. The probiotic strains added to the film could pass to the fish producing an increment of lactic acid bacterial counts, even in the presence of green tea extract. The effect of the films also caused a decrease in the indexes of fish quality (total volatile basic nitrogen (TVB-N), trimethylamine nitrogen (TMA-N) and pH). The total viable counts, H_2S-producing microorganisms and TVB-N were maintained below the limits of acceptability during 15 days for the fillet covered with the green tea + probiotic film, compared to the rest of the samples. Films with green tea and probiotic were able to extend shelf-life of hake at least for a week and increase the beneficial lactic acid bacteria in fish.

Key words: active edible packaging, film, green tea, probiotic bacteria, fish shelf life.

1. Introduction

Fish is highly perishable during refrigerated storage mainly due to rapid microbial growth of microorganisms naturally present in fish or from contamination, which can occasionally result in either economic or health-related problems (Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010). In this connection, *Shewanella putrefaciens* and *Photobacterium phosphoreum* are some of the specific spoilage microorganisms that cause, among others, unpleasant off-odors in fish like the production of trimethylamine from trimethylamine oxide (López-Caballero, Álvarez Torres, Sánchez-Fernández & Moral, 2002; López-Caballero, Sánchez-Fernández & Moral, 2001). For this reason, some strategies have been recently developed with the objective to inhibit spoilage and pathogenic microorganisms.

One of these strategies is the application of active edible films which have proved to enhance shelf-life or even safety of fish (Gómez-Estaca, López de Lacey, Gómez-Guillén, López-Caballero, & Montero, 2009; Gómez-Estaca, et al., 2010). In addition, edible films can avoid moisture loss, gas exchange, oxidation and photo-degradation. Furthermore, safety, nutritional and even sensory properties of edible films can be improved by the addition of several active ingredients into the polymer matrix such as essential oils, organic acids, chitosan, etc. (Gómez-Estaca, et al., 2009; Gómez-Estaca, et al., 2010; Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2009).

Along with these ingredients, green tea possesses healthy beneficial effects, antimicrobial and antioxidant properties, primarily due to its own composition rich in polyphenols and other compounds like minerals and amino acids (Graham, 1992; López de Lacey, 2012). These activities make tea extracts suitable for its use as an active ingredient in edible films. Lately green tea extracts added to edible films (formulated with *Gelidium corneum*-gelatin blend or soy protein isolate) have been reported to improve the physical properties (tensile strength and water vapour permeability) and antimicrobial properties (against *Escherichia coli* O157:H7 and *Listeria monocytogenes*) of these films (Hong, Lim, & Song, 2009).

Recently, some studies have reported that the incorporation of probiotic bacteria in films contribute to the stability and security of food: for example, alginate-gellan incorporated with *Bifidobacterium lactis* Bb-12 for coating of fresh fruits (apple and papaya) (Tapia et al., 2007), and sodium-caseinate films incorporated with *Lactobacillus sakei* to control *L. monocytogenes* in culture medium and in fresh beef (Gialamas et al., 2010). However, no references of the joint incorporation of probiotic bacteria and phenolic compounds in edible films have been reported.

The aim of this work was to investigate the effect of applying the films incorporating green tea extract and probiotic strains to fish during chilled storage. Changes on the natural microflora, paying special attention to *S. putrefaciens* and *P. phosphoreum*, and to the biochemical changes in muscle, were studied in order to evaluate the role of the films during the spoilage of fish.

2. Materials and methods

2.1. Extraction of green tea

Chinese green tea (*Camellia sinensis L.), Wu Lu Mountain* variety*,* was used for preparing the extract and was purchased from a local specialized tea store. Dry green tea was ground into fine powder using a Osterizer blender (model nº 4153-50, Oster®, Sunbean Products Inc., Boca Raton, FL, USA). To prepare the extract, the powder (35 g) was mixed with distilled water (350 mL) at 80 °C for 30 minutes with continuous stirring and was centrifuged at 13200 g for 10 minutes at 5 °C. The supernatant was filtered twice through filter papers (Whatman Nº 1, GE Healthcare UK Ltd., Buckinghamshire, UK). Finally, the filtered extract was stored at - 20 °C before the preparation of the film and analysis.

2.2. Bacterial strains and culture conditions

Two spoilage bacteria, obtained from the Spanish Type Culture Collection (CECT), were used for the inoculation of the fish: *Photobacterium phosphoreum* CECT 4192

and *Shewanella putrefaciens* CECT 5346T. The strains were stored at -80 ºC in Brain Heart Infusion Broth (Oxoid, Basingstoke, UK) with 25% glycerol (Panreac, Moncada i Reixac, Barcelona, Spain) until use. The day before the assay, the strains were grown in BHI broth (Oxoid) supplemented with 1% NaCl, and were incubated at 30 ºC for *S. putrefaciens* and at 15 ºC for *P. phosphoreum* for 24 h.

Two commercial probiotic bacteria purchased in DSM (DSM Food Specialties Ltd., Sydney, Australia) were used for the preparation of the bioactive films: *Lactobacillus paracasei* spp. *paracasei* LAFTI-L26 and *Bifidobacterium animalis* spp. *lactis* LAFTI-B94. The selection of the two strains was based on its antimicrobial properties against *E. coli*, *L. monocytogenes* and *Salmonella typhimurium* (Mahoney & Henriksson, 1999; Pidcock, Heard, & Henriksson, 2002) and its resistance to green tea extract studied (López de Lacey 2012). These strains were acquired lyophilized, and were kept at -20 °C. Appropriate quantity of each lyophilized bacteria was grown in MRS broth (Oxoid) and incubated at 30 ºC for *L. paracasei* and MRS broth + cysteine (0.05%) (Panreac) under anaerobic conditions for *B. lactis*, for 48 h. Each probiotic culture was properly diluted in 0.9 % NaCl solution with the aim to obtain a target inoculum of 10^8-10^9 CFU/mL.

2.3. Formulation of films

Agar film forming solutions were prepared by dissolving 1.5 g of agar (Gold Agar, Hispanagar, Burgos, Spain), 1 g of glycerol, 2 g of glucose in 100 mL of water (agar film) or a 50/50 v/v mixture of distilled water and green tea extract (agar-green tea film). Glycerol was employed as a plasticizer. The mixtures were stirred to obtain a good blend, and the films made by casting 40 ml on 144 cm^2-square plates, drying afterwards at 40 ºC in a forced-air oven for 16-18h to yield a uniform thickness in all cases (200 µm). The films were conditioned in desiccators for 2 days at 22 ºC at 63% relative humidity (Gómez-Estaca et al., 2010).

For preparing the bioactive film, 100 μL of each probiotic bacteria solution (*L. paracasei* LAFTI-L26 and *B. animalis* spp. *lactis* LAFTI-B94*)* were spread on each squared film (12×12 cm) with a sterile loop. The inoculum was applied on agar and agar-green tea film. Four different types of films were then obtained: (i) agar film, (ii) agar probiotic film, (iii) agar green tea film and (iv) agar probiotic green tea film.

2.4. Fish preparation, inoculation and storage

Fillets of defrosted hake (*Merluccius capensis*), purchased at a local market, were cut in portions (c.a. 100 g each). Each portion was inoculated with 5 mL of each spoilage bacteria suspensions (appropriately diluted in 0.9 % NaCl solution) with the objective to have an initial concentration of 10^{3}-10^{-4} CFU/g (Montero, Gómez-Estaca & Gómez-Guillén, 2007). Finally, each piece of fish was covered with two squared films (12 × 12 cm), and then vacuum-packed in bags (Cryovac BB-1, Grace, Barcelona, Spain), one covered fillet per bag. Batches were stored at 4 °C during 15 days. Five lots were prepared, all of them from hake inoculated with the microorganisms already mentioned: hake (H), hake with film (HF), hake with film and probiotic bacteria (HFP), hake with green tea film (HFT) and hake with green tea film and probiotic bacteria (HFTP).

2.5. Microbiological analysis

The microbial counts on the sample were performed immediately after inoculation and after 2, 7, 10 and 15 days of storage. The microbiological analyses were as follows: a total amount of 10 g of fish (after removal of the film), from at least 3 different packages, was collected and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 90 mL of buffered 0.1% peptone water (Oxoid, Basingstoke, UK) in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain.) After 1 min in a Stomacher blender (model Colworth Stomacher 400), appropriate dilutions were prepared for the following microorganisms determinations: (i) total bacteria counts (TBC) on spread plates of Iron Agar 1% NaCl incubated at 15 ºC for 72 h; (ii) H_2S-

producing bacteria, as black colonies and presumptive *S. putrefaciens,* on spread plates of Iron Agar 1% NaCl incubated at 15 ºC for 72 h; (iii) luminescents bacteria on spread plates of Iron Agar 1% NaCl incubated at 15 ºC for 5 days as presumptive *P. phosphoreum;* (iv) total viable bacteria on pour plates of PCA incubated at 30 ºC for 72 h; (v) *Pseudomonas* spp. on spread plates of Pseudomonas Agar Base (Oxoid) with added CFC (Cetrimide, Fucidine, Cephalosporine) supplement (Oxoid) incubated at 25 °C for 48 h; (vi) lactic acid bacteria on overlay plates of MRS Agar (Oxoid) incubated at 30 ºC for 72 h; (vii) Enterobacteriaceae on double-layered plates of Violet Red Bile Glucose agar (VRBG, Oxoid) incubated at 30 ºC for 24 h. All microbial counts are expressed as the log of the colony-forming units per gram (log CFU/g) of sample (detection limits were 2 log CFU/g and 1 log CFU/g for spread plates and pour plates techniques, respectively). All analyses were performed in triplicate.

2.6. pH

Approximately 5 g of muscle were homogenized with a double quantity (g/mL) of distilled water. After 5 min at 25 ºC, pH was determined with a pHm93 pH meter and a combined pH electrode (Radiometer, Copenhagen, Denmark). The experiments were repeated at least in triplicate.

2.7. Total volatile basic nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N)

Total volatile basic nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N) determinations were performed by the methods described by Antonacopoulos and Vyncke (1989) and Malle and Tao (1987), respectively. Briefly, 10 g of the ground sample were weighed in a suitable container and homogenized with 100 mL 6% perchloric acid solution for 2 min. After filtering, the extract was alkalinized with 20% sodium hydroxide solution for TVB-N determination or with 20% sodium hydroxide solution and formaldehyde for the TMA-N determination. Then, the extracts were

submitted to steam distillation. The volatile base components were absorbed by an acid receiver (boric acid 3 ‰) and determined by titration (HCl 0.1 N).

2.8. Instrumental colour analysis

The colour parameters lightness (L*), redness (a*), and yellowness (b*) were measured using a Konica Minolta CM-3500d colorimeter (Aquateknica S.A., Valencia, Spain). Iluminant D65 and 10º observer angle were used. Measurements were taken at a number of locations in different fish portions and each point is the mean of at least 16 measurements (Núñez-Flores, Castro, López-Caballero, Montero & Gómez-Guillén, 2013).

2.9. Statistical analysis

The results are expressed as mean ± standard deviation (sd). Statistical tests were performed using the SPSS® computer program, version 19.0 (SPSS Statistical Software, Inc., Chicago, IL, USA). One-way and two-way analysis of variance (ANOVA) was carried out. Differences between pairs of means were assessed on the basis of confidence intervals using the Tukey-b test. The level of significance was $p \leq 0.05$.

3. Results and discussion

3.1. Microbial counts

Microbial counts of hake fillet covered with different films are shown in Figure 1. Fillets were previously inoculated with two spoilage bacteria *S. putrefaciens* and *P. phosphoreum* in order to simulate the fish spoilage and then to assess the effect of the films during storage. In spite of the inoculation of fish with *P. phosphoreum*, the luminescent bacteria could not be detected during the first days of storage (Fig. 1d), probably because bacteria were recovering.

Counts in control hake (H) increased through storage except for lactic acid bacteria; for this group, counts gradually decreased during storage, which could be due to the low

storage temperature (Fig 1f). At the end of the period, H_2S-producing bacteria became the dominant group in hake (H) since their counts constitute the majority percentage of the total flora at 15 ºC. The prevalence of *S. putrefaciens* was also observed (Baixas-Nogueras, Bover-Cid, Veciana-Nogués and Vidal-Carou, 2003) in Mediterranean hake during storage. Hake covered with agar film (HF) registered similar values to the control lot practically for all microorganisms studied.

The application of films on hake fillet during the storage, especially those that contain green tea, reduced ($p \leq 0.05$) the microorganisms studied, mainly reflected in total viable bacteria at 15 ºC, representative of spoilage flora in fish (Núñez-Flores, Castro, López-Caballero, Montero & Gómez-Guillén, 2013; López-Caballero, Pérez-Mateos, Gómez-Guillén & Montero, 2005) and H_2S-producer organisms. In the present experiment, the films contain glycerol in their formulation. The potential decrease in water activity of the film surface due to the presence of glycerol (and then in the fish surface) is not considered essential in reducing microorganisms, because the microbial reduction is much lower or even insignificant on films that do not contain tea (with or without bacteria), (Fig. 1).

Hake covered with the film + probiotic (HFP) showed an increment of the lactic flora at day 2 of storage, which corresponds to the increase of the total flora (30 ºC) (Fig. 1b, 1f). The increment of lactic acid bacteria could be due, potentially, to the passage of probiotic bacteria (both, *L. paracasei* and *B. lactis*) from film to muscle. At the end of the storage, control hake (H) registered lower counts (≈ 5 log units) than probiotic film lot (HFP) ($p \leq 0.05$). A delay in the detection of Enterobacteriaceae was observed in lots containing probiotics (HFP, HFTP) at the early stages of the storage, but then these lots resumed growth (Fig. 1g). Some authors observed an important improvement in the production and safety of the Hungarian salami by the use of probiotic bacteria cultures (the same used in the present work) (Pidcock et al., 2002). In that case, salami was fermented with the probiotic bacteria at 25 ºC, followed by maturation at 15 ºC; whereas in our study the conditions of fish storage were at 4 ºC. This low temperature

may not favour the growth of the probiotic bacteria, and therefore the bacteria might not be in the best condition to exert its effect.

The films containing green tea extract (HFT and HFTP batches) were the most effective to delay the microbial growth. Green tea extract is likely the principal responsible for the inhibitory activity, especially for those counts of psychrotropic organisms (total bacteria at 15 ºC, H_2S-microorganisms, *Pseudomonas* sp., and luminescent colonies) (Fig 1). The most sensitive microorganisms were the luminescent colonies, presumptive *P. phosphoreum* (Fig. 1d), since the green tea films inhibited completely its growth. Lactic acid bacteria remained more or less constant, except in lot HFT at the end of the storage (Fig. 1f). For green tea batches, the decrease in counts was more noticeable in H_2S-producing bacteria and total flora (15 ºC), with reductions up to 4 logarithmic cycles ($p \leq 0.05$). Differences of 4 log cycles were also found between the control and HFTP hake for enterobacteria at 10 days ($p \leq 0.05$) but this effect is lost over time. It could be that the effect of tea might last a period of time and lose activity during the whole storage. In this sense, the strains might adapt to antimicrobial compounds of green tea and develop resistance to these compounds, or even the green tea films could not be able to inhibit the growth by the effect of the bacterial proliferation.

The antimicrobial activity of the green tea extract (*Wu Lu Mountain*) added to the film was shown against Gram-negative bacteria, such as *P. phosphoreum*, *S. putrefaciens*, *Pseudomonas fluorescens* and *Vibrio parahaemolyticus* (López de Lacey, 2012). In other study, green tea extracts (both aqueous and ethanolic ones) showed inhibition on Gram-positive microorganisms (including *Staphylococcus aureus*, *Bacillus cereus* and *L. monocytogenes*), but not on Gram-negative microorganisms (*E. coli* or *Salmonella enterica*), (Chiu and Lai, 2010). These authors found that when green tea extract was incorporated to edible coatings (based on tapioca starch/decolorized hsian-tsao leaf gum), an antimicrobial activity on Gram-positive bacteria was also found since the active compounds could migrate from the coating and increase the inhibition area.

Thus, the green tea extracts may inhibit the growth of both Gram-negative and Gram-positive bacteria, but this effect is highly dependent on the species being tested.

Catechins, a group of polyphenols present abundantly in green tea extract, have been reported as responsible for the antimicrobial action of tea infusions against Gram negative and Gram positive bacteria (Almajano, Carbó, Jiménez, & Gordon, 2008; Singh Arora, Jeet Kaur, & Kaur, 2009; Sivarooban, Hettiarachchy, & Johnson, 2008). Ku, Hong & Song (2008) showed that the incorporation of catechin into the *Gelidium–corneum* edible film increased the degree of inhibition of *E. coli* O157:H7, with an increase in the catechin concentration. Several effects of catechins on the bacteria, proposed by different authors (Cushnie & Lamb, 2011), could provoke the inhibition or the reduction of the bacterial growth such us: alteration of the cell morphology (Sivarooban, et al., 2008), inhibition of essential enzymes (Gradišar, Pristovšek, Plaper, & Jerala, 2007; Navarro-Martínez et al., 2005), inhibition of energy metabolism (Chinnam et al., 2010) or the production of hydrogen peroxide by oxidation of catechins (Arakawa, Maeda, Okubo, & Shimamura, 2004). According to Shimamura, Zhao and Hu (2007) the inhibition caused by the attack of (-)-epigallocatechin-3-gallate (EGCG) to the lipid bilayer of bacterial cell membrane may be minor in Gram-negative bacteria since this membrane is protected by an outer cell wall. Our results showed that the sensitivity of Gram-negative bacteria to natural catechins presented in green tea incorporated into an edible film could be variable (Fig. 1c-e, 1g).

Previous studies have demonstrated that green tea does not affect the growth of *Bifidobacterium* B94 and *Lactobacillus* L26 (López de Lacey, 2012). For this reason, the counts of lactic acid bacteria in both lots containing bacteria with tea (HFTP) or without tea (HFP) should have been similar. However, >2 log units of difference was found (the lower counts corresponding to HFPT) (Fig. 1f). Thus, it might be possible that green tea film could affect in some way the growth of lactic acid bacteria of hake, but not affect the probiotic inoculated (Fig. 1f).

Except for lactic acid bacteria, lots covered with green tea films presented the lowest microbial counts at the end of storage ($p \leq 0.05$). In addition, the joint presence of probiotic bacteria in the tea film (HFTP) could have a certain additive effect on delaying microbial growth (Fig 1a, e and g). In this sense, Su, Henriksson, Nilsson and Mitchell (2008) observed a clear synergistic effect between *L. acidophilus* L10, *B. animalis* B94 and *L.casei* L26 and a green tea extract ("TEAVIGO™") in relation to the inhibition of growth *in vitro* of *S. aureus* and *Streptococcus pyogenes.* Although previous references on the joint incorporation of tea extract and probiotics in an edible film are not known, the relationship between probiotics and other polyphenolic compounds is established. Thus, flavonoids have been reported to have effects on the gut microflora as an increase in the count of beneficial bacterias (*Lactobacillus rhamnosus*), enhance adherence of this bacterium on the gut wall and inhibition of the proliferation of enteropathogens like *S. aureus* or *S. thyphimurium* (Parkar, Stevenson & Skinner, 2008).

3.2. pH

Changes in pH of hake are shown in Figure 2a. Hake (H) used in the experiment had an initial pH of 7.02. This pH remains constant until day 7, reaching pH>7.7 at 15 days ($p \leq 0.05$). Similar behaviour was observed in HF and HFP lots (although the pH progression in HFP lot was slower that in HF lot). On the contrary, the hake covered with green tea film, with or without probiotic bacteria (HFT, HFTP), maintained the initial levels of pH ~7 at the end of storage period. This result can be a consequence of the bacterial counts observed in these samples (lower 5-6 log CFU/g) since the bacterial activity was reduced, and therefore could produce minor quantities of some volatile base compounds, which can provoke an increment of pH (Reddy, Villanueva, & Kautter, 1995).

3.3. Total volatile basic nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N)

The initial TVB-N was 9.95 mg/100 g fish (Fig. 2b). The TVB-N values increased gradually throughout the conservation in all the samples, except in the hake covered with the green tea film without (HFT) or with probiotic (HFTP), markedly reduced by about 75 and 62 %, respectively, comparing with the control at day 15 of storage. It is also worth noting that green tea lots maintained the levels of TVB-N below the limit of acceptability (30 to 35 mg/100 g) set by the European Union regulation (EC, 1995).

The initial TMA-N in hake was 9.43 mg/100 g fish (Fig. 2c). The major reduction in these values was also noticed in the HFT and HFTP lots at the end of the storage, with decreases around 80 % compared with the control hake (H).

In both indexes (TVB and TMA), the behavior is similar until 7-10 day, from which the lots that do not contain green tea extract in their film formulation presented an exponential rise of the amine volatile concentration. This fact was consistent with the lessening of H_2S-producing bacteria and luminescent colonies, which were also observed from day 10. Thus, green tea film seems to be the cause of the reduced levels of TVB-N and TMA-N, caused mainly by the delayed or reduced growth of bacteria such us *S. putrefaciens* and *P. phosphoreum,* which are involved in the production of basic compounds (TVB-N and TMA-N) in fish (López-Caballero et al., 2001, 2002).

3.4. Colour

All the samples maintained stable values of lightness (L*) throughout the conservation and although significant small differences were founded in some cases, they do not differ by more than 10% (data not shown). The low values of redness (a*) are according to the low pigment (carotenoids and hemopigments) content (Sánchez-Zapata, Pérez-álvarez, Fernández-López & Barber-Valles, 2010), characteristic of hake. The H and HF lots did not present modifications ($p \leq 0.05$) of the tendency towards yellowness value (b*). The application of the green tea film did not alter

appreciably the colour in the fish despite the polyphenols present in green tea. This colour was stable throughout the storage ($p \leq 0.05$) (data not shown).

In summary, the application of green tea films on hake delayed the growth of microorganisms in fish and therefore reduced the spoilage indexes. The probiotic strains could pass to the fish, producing an increment of lactic acid bacterial counts, even in the presence of green tea extract. Probiotics alone temporarily reduced the H_2S-producing organisms by reducing their growth rate, but they had a smaller effect over reducing the chemical spoilage indicators. Thus, it can be concluded that films with green tea and probiotic films could extend shelf-life of hake at least for a week and, at the same time, it could be a way to incorporate beneficial probiotic bacteria to the fish.

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Figure legends

Figure 1. Bacterial counts in hake during chilled storage (4 ºC). (H), hake-film (HF), hake-film + probiotic (HFP), hake-green tea film (HFT) and hake-green tea film + probiotic bacteria (HFTP).

Figure 2. pH**,** total volatile base nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N) of hake during chilled storage (4 ºC). Hake (H), hake-film (HF), hake-film + probiotic (HFP), hake-green tea film (HFT) and hake-green tea film + probiotic bacteria (HFTP).

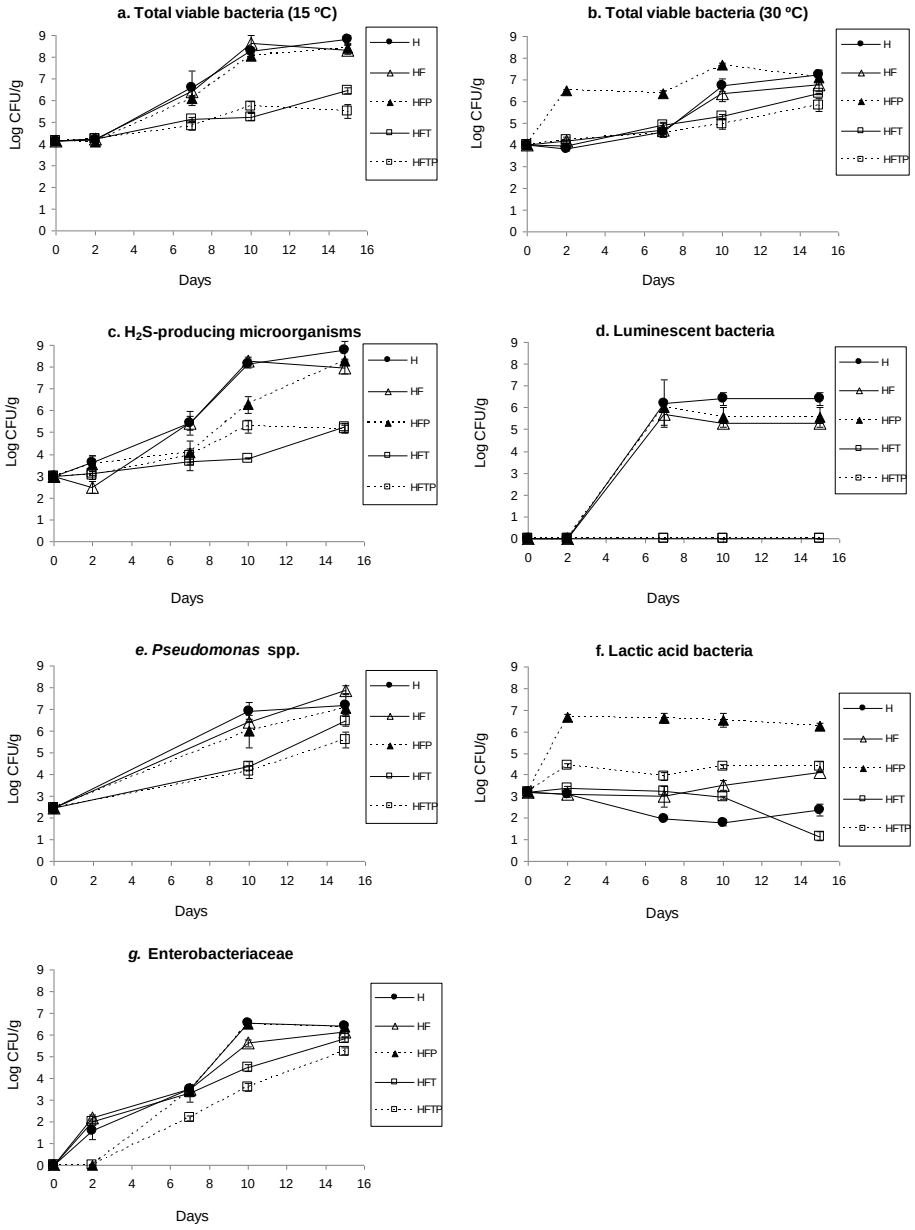
a. Total viable bacteria (15 ºC)
b. Total viable bacteria (30 ºC)
c. H2S-producing microorganisms
d. Luminescent bacteria
e. Pseudomonas spp.
f. Lactic acid bacteria
g. Enterobacteriaceae
Log CFU/g
Days
H
HF
HFP
HFT
HFTP

Figure 1

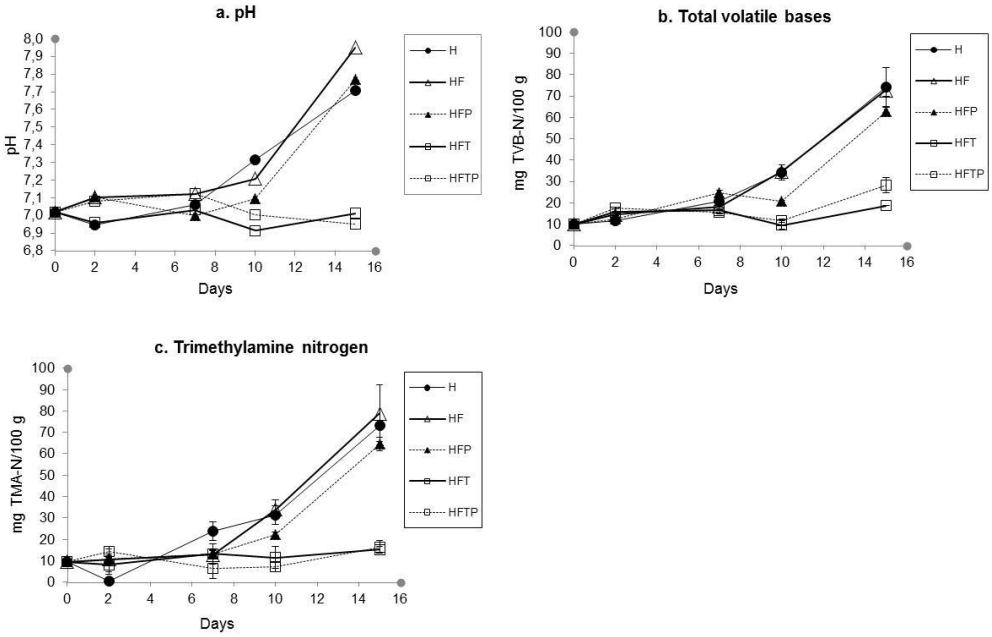
a. pH
pH
Days
H
HF
HFP
HFT
HFTP
b. Total volatile bases
mg TVB-N/100 g
Days
H
HF
HFP
HFT
HFTP
c. Trimethylamine nitrogen
mg TMA-N/100 g
Days
H
HF
HFP
HFT
HFTP

Figure 2.