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Full Length Research Paper

Factors impacting on the microbiological quality and safety of processed hake

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Problems with the safety and shelf life of export hake have been raised by the Namibian fishing industry. This prompted an investigation into the factors that may have an impact on the microbiological quality and safety of processed hake. Samples were collected along the processing line; the general microbiological quality (mesophylic and psychrotrophic aerobic plate counts), total *Vibrio* species and common fish spoilage bacterial counts were performed. The results constantly showed relatively high counts for the psychrotrophic and spoilage bacteria, indicating that most of these bacteria already formed part of the incoming fish. Hake is headed and gutted on board of fishing vessels and delivered to the factory only after 7 – 8 days for final processing. It is likely that this practise of heading and gutting the hake may have a negative effect on microbiological quality of the final product. A sharp increase in the mesophilic and sucrose fermenting *Vibrio* species counts were observed after filleting. It has been suggested that this contamination could be due to biofilms present in the distribution system for the treated sea-water used during processing. Although, sea-water could be an alternative source of water for marine fish processing plants, the treatment and the quality of the water needs to be carefully managed.

Key words: Hake fish, aerobic plate counts, *Vibrio* species, mesophiles, psychrophiles, spoilage bacteria.

INTRODUCTION

Fishing is the second largest export industry after mining and earned about 25% of the total export value for Namibia in 2002 (Meyn, 2005). Of these exports, hake constituted about 45% of the total export value of the Namibian fishing industry by 1998 (Ministry of Fisheries and Marine Resources, 2004). Hake is initially processed off shore where the head and intestines are removed on board of vessels and the fish is kept frozen for 7 - 8 days before being delivered for processing into fillets at the land based facilities. At the processing plant, the fish is first defrosted before being sliced into fillets by the filleting machine (Baader, Germany) followed by trimming of the fillets and rinsing with water before final packaging and freezing for export. All exported fish including hake is subjected to microbiological tests to ensure compliance with the EU Directive EU 91/493/EEC (Iyambo, 1995) in order to ensure both the quality and safety of the product. As part of the evaluation, total viable counts (TVC), total coliforms, faecal coliforms, *Vibrio* species, *Staphylococcus aureus* and *Escherichia coli* levels have to be determined.

Concerns have been raised by the fishing industry with regards to the microbial quality of hake since premature spoilage and fillets loosing their firmness have been noted. Deterioration of the processed product is part of the global problem that causes huge economic losses (Huis in't Veld, 1996; Gram and Dalgaard, 2002).

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Table1. Fish samples analysed.

Sample	Description	Number of samples analysed
H and G	Headed and gutted hake fish stored on ice	5 x 4
ABM-S	Fillets taken after filleting using sea water	5 x 4
ABM-F	Fillets taken after filleting using fresh water	5 x 4
	Laminated, folded hake fillets	5 x 4
FP	Fillets with skin on	5 x 4
	Mascato packs	5 x 4

Although spoilage could be due to endogenous enzymes (Chang et al., 1998; Ordóňez et. al., 2000; Chytiri et al., 2004), it is widely found that bacteria play an important role (Gram, 1992; Gennari et. al., 1999; Try-finopoulon et. al., 2002). It was reported that the number and identity of the initial fish microflora and those present after processing play an important role in determining the shelf life of the fish (Gram and Huss, 1996; Koutsoumanis and Nychas, 1999). In temperate regions fish spoilage is caused by a range of gram negative bacteria including *Shewanella putrefaciens*, *Photobacterium phosphoreum, Pseudomonas*, *Aeromonas*, *Acinetobacters*, *Psychrobacter*, *Flavobacterium*. and *Brochothrix thermosphacta* (Tryfinopoulon et al., 2002; Chytiri et al., 2004; Gennari et al., 1999). *Moraxella*, *Corynebacterium*, *Pseudomonas, Micrococcus* and *Shewanella* predominate under cold storage in seafood harvested from both temperate and tropical regions (Gram and Huss, 1996; Ordóňez, et. al. 2000).

Another concern for the producers and export countries alike is the safety of the hake harvested from Namibia. Questions with regard to the presence of pathogens such as pathogenic *Vibrio* species have been raised. Should *V. cholerae* or any of the other pathogenic *Vibrio* spp. (*V. cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*) be detected on the hake, by importing countries, they will reject the whole consignment and that will result in huge economic losses to the fishing industry. Human vibriosis is typically acquired through consumption of contaminated seafood. These pathogens may be present due to either the ubiquity of the causative agents in aquatic environments (Harriague et al., 2008; De Paola et al., 2003) or through contamination during processing.

The aim of this study was to determine and highlight potential factors that may lead to the deterioration of the microbial quality of hake during processing and impact on the safety of the final product. The microbiological quality of the hake was assessed at three sampling points during processing. Apart from the incoming fish, fillets after processsing by the filleting machine (ABM) and the finished product after packaging were also sampled. The filleting machine was targeted as a possible source of contamination due to its compact mechanical nature that could prove difficult during cleaning and sanitation. The initial samples were taken at a time when sea-water was used for washing the fish during filleting. A second limited sampling programme was performed later when fresh water was used for the same purpose. The results from these two sampling periods were also compared.

MATERIALS AND METHODS

Sampling

During the first sampling period, hake samples were taken from three points along the processing line. These samples included the headed and gutted fish (H and G) kept on ice after delivery from the fishing boat after processing by the filleting machine (ABM) and the finished product after packaging and ready for freezing (FP). All samples were collected in food sampling bags, transported to the laboratory and either analysed immediately or kept frozen at -20°C until it is analysed within 24 h. Frozen samples were thawed into the refrigerator at 2 - 5°C for not more than 18 h (ICMSF, 1978a).

During the first sampling period, treated sea-water was used for defrosting incoming fish and the washing of the fish after filleting. A total of 120 hake samples were analysed: 20 headed and gutted fish, 20 samples after the filleting machine (ABM-S) and 60 finished products (FP). The FP samples were either the laminated and folded hake fillets (LFHF), fillets with the skin on or the Mascato packs. The samples consisted either of a whole fish or fillets with a weight of about 300 g. The sample descriptions and number of samples are summarized in Table 1. During a smaller follow-up study, twenty (20) fillets were re-sampled at the ABM point. At this time only fresh water was used for washing of the fillets after they were fillited (ABM-F). Each sample was analysed in five replicates.

Mesophilic and psychrotrophic plate counts

The total aerobic plate count was performed according to the method described by Kaysner et al. (1992) and ICMSF (1978a) for psychrotrophic and mesophilic counts, respectively. A resuscitation step was included to aid recovery of potential stressed or damaged cells including those present in the frozen samples. For resuscitation, 25 g of fish tissues were transferred to a stomacher bag containing 225 ml of 0.1% peptone water (PW) (Oxoid) and hand minced for one minute at room temperature (22°C) to obtain a homogenous suspension. Serial decimal dilutions of homogenates were prepared up to 10^{-6} and plated on plate count agar (PCA) (Oxoid) and sea water agar (SWA) (Farmer and Hickman-Brenner, 1991) using natural purified sea-water in place of artificial sea-water. Plates were incubated at 35°C for 24 h (mesophilic count) and 22°C for 72 h (psychrotrophic count), respectively. All colonies were counted.

Sucrose fermenting *Vibrio* species

Tissues (25 g) were aseptically excised, minced in 225 ml alkaline peptone water (APW) pH 8.4 (Kaysner et al., 1992; ICMSF, 1978a) and incubated at 22°C for 6 h according to Farmer and Hickman-Brenner (1991) to aid in the recovery of any damaged cells. Serial decimal dilutions of the homogenates were prepared in APW up to 10^{-3}. 100 µl aliquots of each dilution were spread in duplicate on thiosulphate citrate bile salts sucrose (TCBS) agar plates (Oxoid), and incubated at 35°C for 24 h (Kaysner et al., 1992; ICMSF, 1978a). Colonies that appeared on TCBS agar and counted were large, smooth and yellow, with flattened centres and translucent peripheries.

Sucrose non-fermenting *Vibrio* species

Fifty grams (50 g) of fish tissue, aseptically excised, was hand minced in 450 ml 3% NaCl (Kaysner et al., 1992). Serial decimal dilutions were prepared in 3% NaCl up to 10^{-4}. Aliquots of the serial dilutions (10 ml) were inoculated into 10 ml double strength glucose salt teepol broth (GSTB) (Kaysner et al., 1992) and incubated at 35°C for 4 - 6 h. Aliquots of the serial dilutions in GSTB (0.1 ml) were surface plated onto TCBS (Oxoid), and incubated at 35°C for 24 h. Round, bluish or green colonies 2 - 3 mm in diameter (Kaysner et al., 1992; Arias et al., 1998) were recorded.

Enterobacteriaceae

Fish tissue (10 g) was aseptically excised, mixed with 90 ml of buffered peptone water (BPW) in sterile polythene bags and hand minced. The samples were decimally diluted in series up to 10^{-4} in BPW. After thorough mixing, dilutions were incubated at 35°C for 6 h (ICMSF, 1978b). Aliquots (1 ml) of the dilutions were transferred in duplicates to sterile 90 ml petri dishes. 15 ml of cooled violet red bile glucose (VRBG) agar (Oxoid) was added and immediately mixed with the sample. After the agar had set, a second layer (10 ml) of VRBG agar was added, allowed to set and the plates were incubated at 35°C for 24 h (Chouliara et al., 2004; Paleologos et al., 2004) after which the number of pink colonies were recorded.

Pseudomonas, S. putrefaciens* and *Aeromonas

For these analyses, 25 g of fish tissue were placed in sterile stomacher bags containing 225 ml basal medium (BM) (Baumann and Baumann, 1991) and hand minced. Homogenates were incubated at 22°C for 6 h to aid in the recovery of any damaged cells. Thereafter, serial dilutions were made in BM up to 10^{-4}. Aliquots (0.1 ml) each of dilution were transferred to cetrimide fusidin cephaloridin agar (CFC) (Oxoid) supplemented with supplement SR 103, (Oxoid) for culturing potential pseudomonads (Chytiri et al., 2004; Chouliara et al., 2004; Paleologos et al., 2004). The CFC plates were incubated at 20°C for 2 days. Small grey round colonies on CFC were scored as *Pseudomonas* spp.

For *S. putrefaciens*, 1 ml of the same dilutions was added to 10 ml of molten (45°C) iron agar (IA) (Oxiod), poured into 90 ml petri plates and allowed to set. After settling, a further 10 ml of IA was added as a seal and allowed to set, the plates were then incubated at 20°C for 4 days. Black colonies forming on IA were scored as presumptive *S. putrefaciens* colonies (Chytiri et al., 2004). Average logs of counts/gram fish for five replicates were determined for each test.

For *Aeromonas*, 0.1 ml aliquots of the dilutions enriched in APW as described for *Vibrio* species enrichment were spread in duplicate onto *Aeromonas* agar (oxoid) to which supplement SR 151 (Oxoid) was added. Plates were incubated at 25°C for 48 h according to Farmer et al. (1991). Pale green colonies were scored as presumptive *Aeromonas* spp. Average logs of counts/gram fish for five replicates were determined for each test.

Statistical analysis

A number of statistical methods were used. In order to be able to compare the data from the different stages during processing, average logs of counts were first determined for all the microbial analyses and the standard deviations were calculated using Microsoft excel. ANOVA for randomised complete block design and the least significant difference technique to separate means were also used. In some instances, the nested hierarchal approach was used in cases where the factors were nested.

RESULTS

The microbial data for the sampling period when sea-water was used in the processing plant are shown in Figure 1. At the beginning of the processing line (H and G), the mean mesophilic aerobic plate count was log 3.73 cfu/g, increasing to log 6.50 and 6.24 cfu/g after the filleting machine (ABM) and in the final products (FP), respectively. The increase in mesophilic counts by nearly 3 logs along the processing line suggests either loss of temperature control or exogenous contamination along the processing line. The mean psychrotrophic aerobic plate counts remained fairly constant during processing (Figure 1). The measured counts for the incoming fish (H and G) was log 7.03cfu/g. At ABM-S and FP the recorded psychrotrophic counts were log 6.85 and 6 18cfu/g, respectively.

No bacteria grew on TCBS after APW enrichment of the incoming fish (H and G). At the filleting machine (ABM-S) and in the final product, the level of sucrose fer-menting bacteria rose sharply with an average log of 5.63 and 6.61 cfu/g, respectively. This increase in potential *Vibrio* levels was indicated by exposing hake to some form of contamination during processing. These results were recorded at a time when treated sea-water was used for processing. No non sucrose fermenting *Vibrio* species with the characteristic appearance of *Vibrio parahaemolyticus* were detected in any of the samples.

Enterobacteriaceae counts on the fish were the same at the beginning (H and G) and at the end of the processing line (FP) with levels of log 5.00 and 5.03 cfu/g, respectively. A lower value of log 3.20 cfu/g was noted at the intermediate stage (ABM-S). High counts of *Pseudomonas,* similar to those observed for the psychrotrophic counts, were observed throughout processing. The mean levels were log 7.48 cfu/g (H and G), log 6.34 (ABM-S) and log 6.62 cfu/g (FP). The *S. putrefaciens* counts decreased during processing and were log 6.17 cfu/g (H and G), log 5.68 cfu/g (ABM-S) and log 4.5 cfu/g (FP). The *Aeromonas* levels remained fairly constant at log 5.69

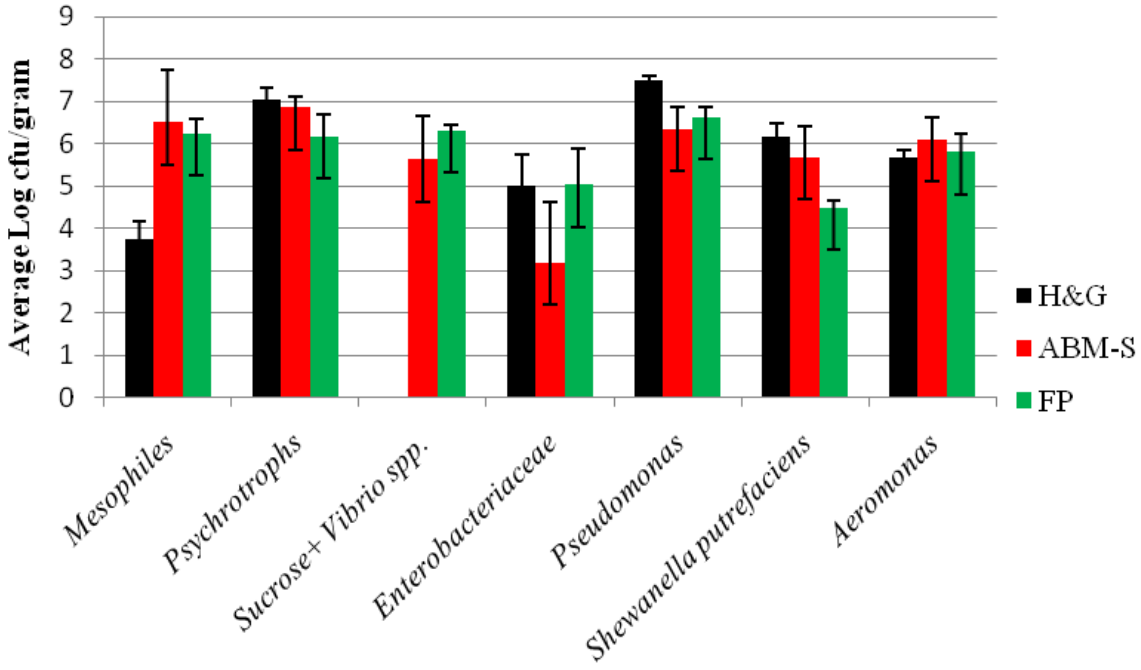

Figure 1. Total viable bacterial counts on hake. H and G = Headed and gutted, ABM-S= after filleting machine with sea water, FP= Finished packed hake fish.

Table 2. Bacterial counts of hake at three stages along the processing line when sea water (H and G, ABM-S; FP) or freshwater (ABM-F) was used when filleting the fish.

	H and G (CFU/g)	ABM-S (CFU/g)	FP (CFU/g)	ABM-F (CFU/g)
Mesophiles	3.73	6.5	6.24	3.41
Psychrotrophs	7.03	6.85	6.18	5.11
Sucrose fermenting Vibrio spp.	ND	5.63	6.31	ND
Enterobacteriaceae	5	3.2	5.03	2.26
Pseudomonas	7.48	6.34	6.62	0.63
Shewanella putrefaciens	6.17	5.68	4.5	3.14
Aeromonas	5.69	6.11	5.8	3.35

ND = Not detected.

cfu/g (H and G), log 6.11 cfu/g (ABM-S) and log 5.8 cfu/g (FP).

As the use of treated sea-water to wash the fish after filleting was discontinued, another set of samples was taken. In most cases, the bacterial levels differed significantly from those measured during the first sampling period (Table 2). The mean mesophilic counts recorded was log 3.41 cfu/g, 3 logs lower than the previous counts obtained when sea-water was used at this point. The count of *Enterobacteraiceae* was log 2.26 cfu/g, that of Pseudomonads was log 0.63 cfu/g, *S. putrefaciens* was log 3.14 cfu/g and *Aeromonas* was log 3.35 cfu/g. All of these results were significantly different from the original levels ($p = 0.001$).

DISCUSSION

The microbial quality of processed fish is usually determined by a number of factors, including the levels of microbes on the raw product, the microbial contamination during processing and the exposure of the product to conditions that will allow for the multiplication of the existing microbes on the product. High mesophilic counts in marine fish are usually indicative of the existence of such conditions and may signal a potential spoilage or health hazard as many spoilage and pathogenic bacteria are mesophilic (ICMSF, 1978a). Total viable aerobic counts on seafood are normally in the ranges of ca. 10^4 -10^6cfu/g on the skin, 10^4-10^7 cfu/g in the gills (Gennari, et al 1999)

and 10^4 – 10^6 cfu/g in the intestines (Austin and Austin, 1987).

In this study, the mean viable mesophilic count was log 3.73 cfu/g on the raw product (H and G) but increased dramatically to a value of higher than log 6 cfu/g at the intermediary stage (ABM) and in the finished product (FP). The levels determined for the headed and gutted fish, kept on ice on board of the fishing vessels for several days before the fish was delivered to the factory, compared well with the findings of other studies (Pastoriza et al., 1996; Cakli et al., 2006; Tzikas et al., 2007).

The mean psychrotrophic counts obtained in this study remained high at nearly the same level throughout all the stages of processing. High psychrotrophic counts observed could have originated from the natural flora on hake that multiplied from the time of the fish catch to the time of delivery to the factory indicating potential problems on board of the fishing vessels. The psychrotrophic counts are usually representative of normal spoilage organisms such as *Pseudomonas* and *Shewanella* spp (Gram and Dalgaard, 2002) that can grow at refrigeration and ambient temperatures. This was confirmed by Ordóňez et al. (2000) who also showed that *Pseudomonas* and *Shewanella* were the predominant spoilage bacteria on gutted hake stored on ice. In this study, the *Pseudomonas* counts of log 7.48 cfu/g in the incoming fish and log 6.62 cfu/g in the final hake products were similar to those measured for the total psychrotrophic counts. These values may indicate a short product shelf life. *Aeromonas* spp. could also form part of the psychrotrophic bacteria and have been isolated from a number of marine and fresh water fish species (Papadopoulou et al., 2007). They are also fish spoilage organisms and may produce H_2S. In this study counts ranged from log 5 to log 6 cfu/g and hake spoilage due to this group of bacteria can therefore, not be excluded.

S. putrefaciens is typically one of the predominant microflora of ice stored fish from temperate regions (Chytiri et al., 2004; Gennari et al., 1999; Paarup et al., 2002). In this study, the levels of sulphate reducing bacteria (SRB) including *S. putrefaciens* in H and G and FP hake were log 6.17 and log 4.50 cfu/g, respectively. Some reduction in the levels of *S. putrefaciens* was noted as the fish moved along the processing line. Despite this reduction, the levels are still of concern and it should be noted that the method of keeping fish on board for several days before delivery to the factory for final processsing may have a negative effect on microbiological quality and could lead to spoilage.

Some of the bacterial counts were higher than what was reported in literature (Vennemann, 1991; Tsikas et al., 2007). This could be linked to a recovery step that was included in the analysis of some of our samples. This step was included as Tsikas et al. (2007) observed a lag phase in the growth of total viable bacteria count performed on Mediterranean horse mackerel and blue jack mackerel muscle done after 4 to 6 days of fish storage on ice. Within fish processing environments, bacteria are also continually exposed to stressing situations such as chill temperatures and the presence of sanitizers that cause sublethal injury to bacteria. An enrichment step often assists with the recovery of these bacteria (ICMSF, 1978b; Foegeding and Ray, 1992). Human pathogens are typically mesophilic bacteria with an optimum growth range between 30 - 45°C (Forsythe and Hayes, 1998). An increase in the mesophilic count is therefore, of potential health concern. *Enterobacteriaceae* are widely distributed in aquatic environments including marine waters (Papadopoulou et al., 2007) and could be one of the reasons for the observed increase. High counts of *Enterobacteriaceae* typically indicate potential faecal contamination (ICMSF, 1978a). During this study, the *Enterobacteriaceae* initial counts for hake were log 5.00 cfu/g (H and G), and similar counts were observed in the finished products. These counts were similar to those obtained by Ordóňez et al. (2000) on hake steaks before storage, and by Economou et al. (2007) in tuna fish which were kept at 20°C. Himelbloom et al. (1991) found lower (10^2 cfu/g) *Escherichia coli* counts on Alaskan finfish. In thIs study, it was therefore demonstrated that the levels were within the expectable norms, and faecal contamination of the processed fish was not suspected to be the reason for the deterioration in the mesophilic counts.

The sharp increase in mesophilic counts observed during processing was still a cause for concern as this trend corresponded with a similar increase in the levels of sucrose fermenting *Vibrio* spp. No sucrose fermenting *Vibrio* species were detected in the incoming hake (H and G), but this group of bacteria suddenly appeared at high levels in the ABM-S and FP samples. Further investigations indicated that this sharp increase in potential *Vibrio* spp. could be as a result of the introduction of the bacteria by means of the treated sea-water used during processing. There were indications that the major source of these bacteria was not inefficient treatment of the raw water but the subsequent formation of biofilms in the distribution network in spite of the presence of residual chlorine (Shikongo-Nambabi et al., 2010). Identification of the isolated sucrose fermenting bacteria confirmed that these strains were not *V. cholerae* but *Vibrio alginolyticus* and that they did not pose any immediate health risk to any of the consumers (Data not shown).

The impact of sea-water was further investigated when the factory was refurbished and started to use fresh water as the major source of water during processing. No sucrose fermenting *Vibrio* species were detected in any of the products tested (Table 2). The differences observed for all the microbial parameters were of statistical significance indicating a positive impact on the overall quality of fish. This improvement should significantly increase the shelf life as well as the safety of the hake processed in the plant.

Conclusion

The microbial quality as observed during the initial study period raised a number of concerns and warranted a closer investigation to ascertain that good manufacturing practises are strictly adhered to from the time the fish is caught up to the point of processing of the final product. The results indicated that some deterioration in quality could be due to contamination during processing while others may have originated with the fish supplied to the plant since all fish samples analysed during this study were not freshly caught.

This study has shown that *Pseudomonas*, *S. putrefaciens* and *Aeromonas* and typical spoilage organisms, form part of the bacterial population on the hake. *Pseudomonas* and *Aeromonas* were present at the same level while *S. putrefaciens* levels were slightly lower. The results indicated that these organisms already formed part of the incoming fish and that process did not increase their levels dramatically. It is likely that the method of keeping fish on board and the fishing vessels for several days before delivery to the factory for final processing may have a negative effect on microbiological quality and could lead to spoilage.

Comparison of viable bacterial counts obtained from the three stages along the processing line has revealed higher mesophilic counts in hake after filleting. Of particular interest was the sucrose fermenting *Vibrio* species that were not detected in the incoming (H and G) fish, but were detected in high numbers (ca. log 6.31 cfu/g) when sea-water was used to wash the hake fillets before trimming and packaging. A link was made to the treated seawater used during processing as the most likely source of contamination. This was confirmed when a significant improvement was observed when fresh water was used to wash fish at the same point during processing. Although sea-water could be an alternative source of water for marine fish processing plants, the treatment and the quality of the water needs to be carefully managed.

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Abbreviations

AA, Aeromonas agar; **ABM-F,** after filleting machine hake fillets washed with fresh water; **ABM-S,** after filleting machine hake fillets washed with sea-water; **APW,** alkaline peptone water pH 8.4; **APHA,** American Public Health Association; **BM**, basal medium; **CFC**, cetrimide fusidin cephaloridin agar; **EU,** European Union; **FP,** hake fish finished products; **H and G,** headed and gutted hake fish; **ICMSF**, international commission on microbiological specifications for foods of the international association of microbiological societies; **LFHF,** laminated and folded hake fillets; **PCA,** plate count Agar; **PW,** peptone water; **SWA,** sea water agar; **TVC,** total viable counts; **TCBS,** thiosulphate citrate bile salts sucrose agar; **VRBG,** violet red bile glucose agar.

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