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Identification of Non-Listeria and Presence of Listeria in Processing Line Production of Cold-smoked Salmon

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Abstract

This treatise was conducted to investigate *Listeria* presence in one cold-smoked salmon (CSS) processing factory, Al-Sharkia Governorate, Egypt as well as to evaluate detection accuracy of Agar Listeria according to Ottaviani and Agosti (ALOA) using 16S rRNA technique. A total of 50 and 60 samples from final products and their processing line six-point (PLS) have been tested for the occurrence of this bacterium, respectively. The above technique showed that *Bacillus safensis*, *Oceanobacillus picturae* and *B. wiedmannii* have been found in CSS. The presumptive of *Listeria* spp. (*L. monocytogens*) in CSS and PLS were found to be 8 and 16.67%, respectively while presumptive of *Listeria* spp. (*L. innocua*) were 26 and 16.67% respectively. The high occurrence percentage of this pathogen in PLS of factory could be attributed to cross-contamination. As a direct consequence, Listeria spp. control must be implemented through a proper cleaning and sanitation programme as well as a HACCP plan to prevent cross-contamination and spread of these pathogens to final products during the fishery processing.

Keywords: Cold Smoke salmon; Safety; Listeria monocytogenes; pathogenic microorganisms; ALOA detection accuracy

Introduction

Listeria spp. essentially *Listeria monocytogenes* and *Listeria innocua* could be a point under consideration of the fish processing plant and ready-to-eat (RTE) foodstuff. Strategies for improved control of L. monocytogenes in RTE food products are needed to reduce the burden of this serious food-borne pathogen. Although the incidence of human listeriosis is generally low, listeriosis can progress to a severe infection with common case fatality rates of 20-30%. [1]. Worldwide, listeriosis are causing more than 23,000 diseases and approximately 5500 deaths in 2010 [2]. In EU, an increasing trend has been observed in recent years in cases of listeriosis. A total of 2536 cases of human listeriosis were reported, while 19 member countries reported 247 deaths attributable to listeriosis in 2016 [3, 4] "https://www.ecdc.europa.eu/en/publications-data/listeriosis-annual-epidemiological-report-2017"). The third highest mortality rate among foodborne infections in the United States has been listeriosis [5]. Recently, the estimates place *L. monocytogenes* among the top five pathogens responsible for the greatest burden of disease costs and quality-adjusted life-years (QALYs) lost [6, 7]. *L. monocytogenes* is also the most troublesome and costly microorganisms for many food manufacturers due to persistent "house strains", product cross-contaminations and recalls despite extensive Listeria control and testing programs. The need for product redesigns or internal product rejections further increases the economic burden of *L. monocytogenes* to the food industry. The prevalence and numbers of *L. monocytogenes* in various categories of ready-to-eat (RTE) food products taken from retail outlets and food industries over a 5-year period, 30,016 (3.6%) were found positive [8]. A total of 30,016 ready-to-eat food samples were examined for the prevalence of *L. monocytogenes*, and 3.6% were found positive. The highest prevalence was found for ready-to-eat fish and fish products (11.6%), especially for lightly salted and cold-smoked fish products. The prevalence of *L. monocytogenes* in other food groups was low, in the range 0 to 3.9% while a food safety criterion of foodstuff over than 100 CFU/g was exceeded for

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0.3% of RTE [8]. Samples often exceeding the legal safety limit were from the categories of salted and cold-smoked fish products. The high prevalence, 28.6 and 26.5%, respectively, and high numbers of *L. monocytogenes* among salted fish and cold smoked fish products indicate a risk of listeriosis, especially for the high-risk groups. The results of the current study can be used nationally and internationally to update the risk perception of *L. monocytogenes* derived from ready-to-use foods. The prevalence of Listeria is relatively high and it found typically between 10 to 40% in freshly produced cold-smoked [9, 10, 11, 12, 13, 14]. This high prevalence could be due to the low smoking temperature involved during the cold-salmon processing; as these conditions would be ideal for the proliferation of L. monocytogenes if the raw salmon harbored the pathogen or acquired the pathogen from the processing environment. Contamination, survival, and growth of *L. monocytogenes* in cold-smoked salmon represent serious health hazards to consumers and major challenges for salmon processors [15, 16]. Therefore, the aim of this work was to investigate the presence of *Listeria* species in one cold-smoked salmon (CSS) processing factory, Al-Sharkia Governorate, Egypt. Besides Agar Listeria according to Ottaviani and Agosti (ALOA) was estimated for the detection of *Listeria* species in CSS and compared with 16S rRNA gene sequence analysis.

Materials and Methods

Bacterial Strains

Listeria monocytogenes ATCC 19116, *Listeria innocua* ATCC 33090 were obtained from Egyptian Microbial Culture Collection, Microbiological Resource Center (The Cairo MIRCEN: Ain Shams University, Cairo, Egypt). The strains were subculture in Tryptic Soy broth (TSB: Merck 1.05459, Darmstadt, Germany) and checked for purity onto Tryptic Soy agar plates (TSA: Merck 1.05458, Darmstadt, Germany), incubated for 24 h at 37 °C. These strains were used as references strain in this research. Three Gram-stain positive isolates from *Listeria* agar according Ottaviani and Agosti (ALOA), catalase-positive rod isolated from cold smoked salmon were sent to the Reference Laboratory for *Listeria* (Biotechnology unit Reference lab for veterinary quality control on poultry production, Animal health research institute, Dokki, Giza, Egypt) for identification by 16S rRNA gene sequence analysis. The cultures grown on TSA agar (Merck) were used for genotyping analyses. All isolates were maintained at −80 ◦C in TSB broth supplemented with 20 % glycerol (v/v).

Samples

Frozen vacuum cold-smoked salmon (CSS) samples (n=50) were collected from fish processing factory, Al-Sharkia Governorate, Egypt during ten months. The factory was visited ten times during 2019 and the months named T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10. Each month, 5 samples has been bringing at Agricultural Microbiology Laboratory for detection of Listeria. In addition, a total of 60 swabbed samples were examined for Listeria spp. in the same fish processing factory. Overall, 6 contact point samples were examined. In each point a total of 10 samples were examined. The swabbed samples were taken from boxes, processing tables, floors, processing coats and gloves by swapping (5x5 cm2 of area). Each site was swabbed 3 times. All CSS and swabbed samples were put into pre-enrichment broth and transported to the Microbiology Laboratory of Zagazig University, Faculty of Agriculture, Agricultural Microbiology Department in ice box in two h.

Detection and identification of Listeria

ISO, 11290-1/A1 [17] and ISO 11290-2/A1 [18] were used for detection and identification of Listeria spp. in the swapped and cold smoked salmon (CSS) samples. All swapped samples and CSS :25 g) samples were put into pre-enrichment of Fraser half selective supplement (primary enrichment: 225 ml) and homogenized in a stomacher 400 Lab blender (Seward Medical, London, UK) for 2 min then incubated (24 h, 30 ˚C). Following this, a secondary enrichment was prepared by inoculating an aliquot (0.1 ml) of the primary culture into 10 ml Fraser broth (48 h, 30 ˚C). Afterward, a loopful (10 µl) of the primary and secondary enriched cultures were streaked onto Listeria Ottaviani Agosti (ALOA) (Biolife, Milano-Italy) and examined after 24 and 48 h (37 ˚C). When testing cold smoked salmon samples, incubated negative plates for a further 24 ± 2 h. and examined again. The plates were examined for blue/green colonies with and without opaque white halos. Five suspect Listeria spp. colonies from each plate were streaked to purity on tryptone soya agar with yeast extract (24 h., 37 ˚C), the negative plates were incubated for a further 24 ± 2 h. and examined again according to method of ISO [18]. Colonies that showed typical appearance of L. monocytogenes on tryptic soy agar were taken further for Gram-staining, catalase and oxidase tests. Isolates that were rod-shaped and Gram-positive and showed positive for catalase and negative for oxidase were tested for hemolytic ac-

tivity and the Christie, Atkins, Munch-Petersen CAMP tests on sheep blood agar for *L. monocytogenes* confirmation. The haemolysis test was performed for each isolate or strain by stabbing Columbia Blood Agar Plates supplemented with 5% sheep blood (BioMérieux). After incubation at 30 or 37 ˚C for 24 ± 2 h, the reaction was considered positive if an area of β-haemolysis appeared around the colonies. Strains of *L. monocytogenes, L. innocua*, were used as controls. All cultures were tested and identified using the API Listeria identification kit (BioMerieux, Basingstoke, Hants, England) which comprises a gallery of 10 microtubes containing dehydrated substrates for enzymatic or sugar fermentation tests. The API Listeria identification test kit (BioMerieux, Basingstoke, Hants, England) includes an amino acids peptidase substrate (DIM reaction) which is hydrolysed by all *Listeria* species with the exception of Listeria monocytogenes. Kits were used in accordance with the manufacturers for the identification of *Listeria* spp., pure cultures in tryptone soya agar were submitted to a multiplex polymerase chain reaction (PCR) procedure, according to Lawrence and Gilmour [19].

Phylogenetic Analysis Using 16S rRNA

The extraction of genomic DNA by phenol-chloroform from cells treated with mutanolysin (Sigma-Aldrich), lysostaphin (Ambi Products LLC), and achromopeptidase (Sigma-Aldrich) was done as described previously [20, 21]. Initial 16S rRNA gene amplification by PCR and partial 16S rRNA gene sequencing was performed using specific 16S rRNA primers for bacteria, namely, forward primer 8F (5′-GGATCCAGACTTTGATYMTGGCTCAG), as described by Felske et al. [22] but modified by being shortened from the 5′ end, and reverse primer 907R (5′-CCGTCAATTCMTTTGAGTTT), as described by Lane et al. [23]. The total 16S rRNA gene sequence was subsequently extracted from whole-genome shotgun (WGS) data using RNAmmer version 1.2 [24] and compared with those of other taxa of the genus Listeria and Bacillus, available in the GenBank database. Pairwise sequence alignment and calculation of similarity values were administered by a worldwide alignment algorithm, implemented at the EzBioCloud database [25]. The evolutionary history was inferred by the neighbor-joining and maximum likelihood methods using bootstrap values supported 1,000 replications with the software MEGA version 7 [26].

GenBank Accession Numbers

The GenBank accession numbers for the 16S rRNA genes of isolates SM O15, SM K15 and SM C17 are *Oceanobacillus picturae* SM K15-MW485132 and *Bacillus wiedmannii* SM O15-MW485131 and *Bacillus safensis* SMC17-MW485133, respectively, and annotated by the NCBI Prokaryotic Genome Annotation Pipeline.

2.6. Statistical analysis

All the obtained data and numbers from laboratory experiments were entered In Excel program to calculate the averages.

Results

Prevalence of Listeria spp.in cold smoked salmon samples

The cold-smoked salmon samples (n=50) have been obtained from one factory for ten months from January up to October 2019 and named T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10. These samples were tested for the presence of *Listeria* spp., 18 samples (36 %) tested positive. During months T5, T7, T9, and T10 showed the highest percentage of positive samples (Table 1). However, Listeria spp. have not been detected in months T1, T2, T3, and T4. The isolates during months T5, T6, T7, T9 and T10 formed several subclusters with no distinct cluster for any one brand. The similarities between the colonies in the subgroup can be linked to the slaughterhouse from which the processors obtained raw salmon fillets, as the slaughterhouse was popular for processors during months T5, T6, T7, T9 and T10. The number of negative samples lower than 10 CFU/g were found to be 12% while the positive samples higher than 100 CFU/g were 4%. In addition, these samples of cold smoked salmon were tested for the occurrence of *Listeria* spp., 34 % tested positive while *L. monocytogenes* and *L. innocua*, 8 and 26 % tested positive, respectively (Table 3).

Table 1. Occurrence and contamination level of *Listeria* spp. in cold-smoked salmon collected within ten months 2019

Time (n= 5 samples in each month)	Number of positive samples (Using ALOA)	No. of positive samples by colony counting (CFU/g)			
		<10	>10-100	>100-1000	>1000-10000
T1	-	-	-	-	-
T2	-	-	-	-	-
T3	-	-	-	-	-
T4	-	-	-	-	-
T5	2	1	1	-	-
T6	3	2	1	-	-
T7	3	-	2	1	-
T8	3	2	1	-	-
T9	4	1	1	1	1
T10	3	-	2	-	1
Total= (n= 50)	**18 (36%)**	**6 (12%)**	**8 (16%)**	**2 (4%)**	**2 (4%)**

Table 2. Occurrence and contamination level of *Listeria* spp. in processing line plant production of cold-smoked salmon

Sampling point	Number of positive samples (Using ALOA)	No. of positive samples by colony counting (CFU/g)			
		<10	>10-100	>100-1000	>1000-10000
S1	4	3	1	-	-
S2	6	3	2	1	1
S3	5	4	1	-	-
S4	3	2	1	-	-
S5	2	2	-	-	-
S6	-	-	-	-	-
Total= (n= 60)	**22 (36.67%)**	**16 (26.67%)**	**5 (8.33%)**	**1 (1.67%)**	**1 (1.67%)**

Table 3. Occurrence of *Listeria monocytogenes* and *L. innocua* in in cold-smoked salmon collected within ten months 2019

Time (n= 5 samples in each month)	Number of positive samples (Using ALOA)	*Listerias* pp.	
		L. monocytogenes	*L. innocua*
T1	-	-	-
T2	-	-	-
T3	-	-	-
T4	-	-	-
T5	3	-	3
T6	2	-	2
T7	3	1	2
T8	3	١	٢
T9	2	-	2
T10	4	2	2
Total= (n= 50)	**17 (34%)**	**4 (8%)**	**13 (٢6%)**

Prevalence of Listeria spp. in swapped samples

A total of 60 samples from proccing line six-point (Fish, swab of gutted fish on gutting line (S1), Environment, table for deheading (S2), Environment, slicing machine in slicing room (S3), Environment, forceps for picking bones (S4), Environment, conveyor belt manual gutting line (S5) and Environment, surface sorting box after grader (S6) were tested for the presence of *Listeria* spp., 22 samples (36.67%) tested positive. The highest percentage of positive samples showed in the S2 and S3 points (Table 2). However, *Listeria* spp. have not been detected in the S6 points. These tested samples were positive for the occurrence of *L. monocytogenes* and *L. innocua*, 16.67 % positive. S1, S2, S3 and S4 points showed the highest percentage of positive samples (Table 4).

Table 4: Occurrence of *L. monocytogenes* and *L. innocua* in processing line plant production of cold-smoked salmon

Sampling point	Number of positive samples (Using ALOA)	*Listeria*	
		L. monocytogenes	*L. innocua*
S1	4	3	1
S2	6	4	2
S3	5	2	3
S4	3	1	2
S5	2	-	2
S6	-	-	-
Total= (n= 60)	**2، (33.34%)**	**10 (16.67%)**	**10 (16.67%)**

Phenotypic characterization

All three isolates originating from cold smoked salmon samples which grow onto Agar *Listeria* Ottaviani Agosti (ALOA) (Biolife, Milan-Italy), were observed to be bacilli or slightly irregular bacilli, occurring predominantly in pairs/single and clusters/non-cluster, spore forming and motile. The measured values of their cell size ranged from 1.12 to 2.15 µm in diameter based on light microscope (1000x), i.e., cells that are larger than those of *Listeria*. Microscopic and macroscopic morphology and the results of key biochemical and physiological tests relevant for Gram-positive bacilli, namely positive catalase and oxidase activity and growth in the presence of 10 % NaCl and negative growth in the presence of 15% NaCl presumptively identified all three isolates as *Bacillus* spp.

Phylogenetic Analyses

The PCR product of the isolates showed the formation of a single band (Figure 1 d), which indicates that the 16s rRNA gene sequence has successfully amplified PCR at 1500 bp. The results of the sequencing of all isolates were compared with the 16s rRNA gene sequence listed in the GenBank database organized by NCBI. The strain *Bacillus safensis* (GenBank accession number MW485133) had a genetic similarity of 96% with *Bacillus safensis* MT634688, and the relationship between them is described in the phylogenetic trees (Figure 1a). While, the strain *Oceanobacillus pictura* (GenBank accession number MW485131) had a genetic similarity of 100 % with *Oceanobacillus pictura* MN756652.1 and the relationship between them is described in the phylogenetic trees (Figure 1b). Also, the strain *Bacillus wiedmannii* (GenBank accession number MW485132) had a genetic similarity of 100 % with *Bacillus wiedmannii* MT239515.1 and the relationship between them is described in the phylogenetic trees (Figure 1c). The result of identification was differed than conventional analysis because the identification using 16s rRNA gene sequence was more sensitive and valid compared to the morphological, biochemical, and physiological characteristic. The 16S rRNA gene sequences showed Bacillus *safensis* SMC17, *Oceanobacillus picturae* SM K15and *Bacillus wiedmannii* SM O15 gene positive. Group 1, *B. safensis* SM C17 (GenBank accession number MW485133) had 96% reciprocal identity with the 16S rRNA sequence of *B. safensis* MT634688. Group 2 and group 3, *Oceanob. picturae* SMK15 and *B. wiedmannii* SMO15 had 100 % identity with the 16S rRNA sequence of *Oceanob. picturae* MN756652.1 (GenBank accession number MW485131) and *B. wiedmannii* MT239515.1 (GenBank accession number MW485132), respectively.

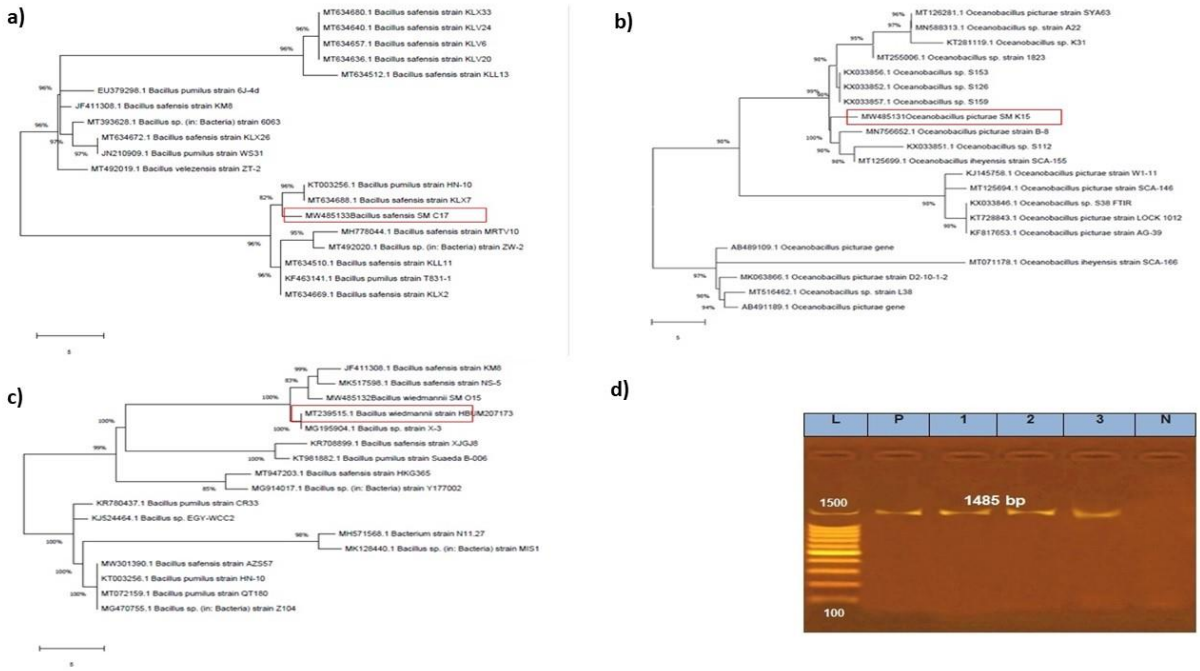

Figure 1: Phylogenetic tree by maximum likelihood model of MEGA X package showing the position of fish bacilli within the genera Bacillus and Oceanobacillus and based on 16S rRNA gene sequence. The sections of the gene sequences correspond to the following gene coordinates of a) *Bacillus safensis* MT634688 (GenBank accession number MW485133), b) *Oceanob. pictura* MN756652.1 (GenBank accession number MW485131), c) *Bacillus wiedmannii* MT239515.1 (GenBank accession number MW485132) and d) Bacterial isolates grow onto Agar Listeria Ottaviani Agosti (ALOA) (Biolife, Milan-Italy),16SrRNA genes, Lane L:100-1500bp Ladder. N: Negative control, P: positive control at (1500bp), Lanes 1 to 3 B. safensis, Oceanob. picturae and B. wiedmannii (16SrRNA) gene positive.

Discussion

With the incidence of listeriosis increasing across Europe and the United States and with consumption of cold-smoked salmon on the rise, vigilance in pathogen surveillance is needed [27]. During ten months from January up to October 2019, the final product of vacuum-packed cold-smoked salmon samples was tested for the presence of *Listeria*. The incidence rate recorded in the current study by traditional methods (36 %) is higher than in Ireland in 2001 when compared to the study conducted on cold-smoked salmon [28, 29, 30]. Data from other countries show that *Listeria* is present in 21% of smoked salmon in the UK [31], 25% of smoked fish in Nigeria [32], and 25% of smoked trout in Spain. Additionally, there are several reported even higher levels (78%) in smoked fish [12, 32, 34], 21% in cold smoked salmon in Italy [35] and 77% in cold smoked salmon in New Zealand [36]. *L. monocytogenes* could be found in raw fish from zero to 30% and salmon [7, 14, 37]. In other study, Acciari et al., [35] found that one hundred fifty-seven samples (20.2%) were contaminated by *L. monocytogenes*, approximately 26 samples at levels >100 CFU/g. The maximum contamination level was 1.3×10^6 CFU/g. The detection frequency of products from different manufacturing locations ranged from 0 to 76.9%. Eight major pulsatile patterns accounted for 70.8% of the isolates. Three of the main spring styles were exclusively from one manufacturer. In universal, products from the same manufacturer showed genetic homogeneity, with a single strongly scattered pulsating pattern. Different manufacturers have typically shown very different levels of final product contamination, emphasizing the importance of raw material suppliers and pro-

cess hygiene management to control *L. monocytogenes* contamination. However, the processing of cold-smoked salmon does not involve any bactericidal step to eliminate *L. monocytogenes*, the contamination at the raw fish level (or the raw fish spreading the pathogen along the production line) will have a significant positive relationship with the presence of this organism in the finished product [15]. Our results are in agreement with the previous works on contamination patterns of *L. monocytogenes* in cold-smoked salmon [38, 39, 40], which confirms the hypothesis that the contamination could be from a few strains that may have been introduced with the raw material, and found a niche in the processing factory, from where they are constantly being shed during the processing, thus contaminating the products [32, 33, 40]. If the raw fillets carried *L. monocytogenes* into the processing factory, the *L. monocytogenes* could have undergone modification to adapt to the new environment and cold-smoking conditions specific to each processor. As cold-smoking is done below 20 ^{0}C and there is no other bactericidal step involved in eliminating the pathogen, there is a possibility that *L. monocytogenes* could have survived through the processing stage. In some samples, multiple strains of *L. monocytogenes* were isolated; this supported the hypothesis that the product does not constitute a particular microenvironment in which only one strain survive. Contamination of food products and the environment by *L. monocytogenes* is a serious threat to those involved in food processing, catering and retailing [41]. It is difficult to produce cold-smoked salmon totally *Listeria*-free. It is, therefore, essential to implement a food safety program at the pre-processing and processing environment, along with new disinfection strategies to control the persistent *L. monocytogenes* strains that would have adapted to the current cleaning strategies. These combined food safety strategies at different levels (raw fish, pre-processing environment and processing factory) will help in controlling the human exposure to this harmful pathogen. Epidemiological investigations must be conducted on a regular basis to determine the primary source of bacterial contamination so that preventive measures can be implemented to reduce the prevalence of *L. monocytogenes* and protect human health. The results obtained from this study demonstrate a relatively high prevalence of *Listeria*, *L. monocytogens* and *L. innocua* in vacuum packed cold-smoked salmon, the percentages were 34, 8 and 26%, respectively when compared to the study in Ireland (13%) conducted in 2001[28]. The presence of this pathogen on cold-smoked salmon represents a serious public health concern, due to the increased consumption of this ready-to-eat food product. The result of identification using 16s rRNA gene sequence was differed than conventional analysis because the identification by these techniques were more sensitive and valid compared to the morphological, biochemical, and physiological characteristic. The utilize of 16S rRNA gene typing that incorporates intragenomic frequency has previously been reported to be useful for molecular subtyping of bacterial species and strains according to several authors [21, 42, 43].

Conclusion

The results indicated that *Listeria monocytogenes* and *L. innocua* was isolated from 8 or 26 % of the cold smoked salmon samples, respectively, while these strains were isolated from 16.67 % of the processing line plant production of cold-smoked salmon samples according to the ISO methods. However, the ability of 16S rRNA partial gene sequencing to rapidly and successfully identify bacterial species showed that three species non-*Listeria* i.e., *Bacillus safensis, Oceanobacillus picturae* and *B. wiedmannii* from selective *Listeria* Ottaviani Agosti (ALOA). The identification of colonies directly from ALOA plates demonstrates the potential of 16S rRNA partial gene sequencing to greatly reduce analysis turnaround time for food samples found to contain Listeria: the ISO method requires 8 days for species-level identification, while the 16S rRNA partial gene sequencing method requires only 5 days (including enrichment and isolation steps). The 16S rRNA partial gene sequencing method would also eliminate the reliance on labor-intensive and somewhat subjective methods currently in use, such as microscopy and the Christie Atkins Munch-Petersen (CAMP) test. Overall, the incorporation of the 16S rRNA partial gene sequencing system described here into the regulatory workflow could reduce the total analysis time and labor involved in the identification of Listeria species in food sam-

ples, while also allowing for molecular subtyping of strains for surveillance and outbreak purposes. Therefore, Listeria spp. and other bacteria must be controlled during processing of fishery products.

Conflicts of interest

"There are no conflicts to declare".

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