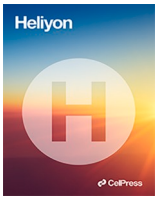
Heliyon 10 (2024) e32170

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

A challenge test on *Pseudomonas* spp. as spoiling microorganism in fish fillets

Giulia Alberghini [a], Nesrine Ben Mhenni [b], Vincenzo Di Leva [c], Riccardo Forzano [c], Riccardo Miotti Scapin [a], Placido Matteo Pappalardo [a], Federica Giacometti [a], Valerio Giaccone [a,*]

[a] *Department of Animal Medicine, Productions and Health, School of Agricultural Sciences and Veterinary Medicine (MAPS), University of Padua, Legnaro, 35020, Italy*
[b] *Bioresources, Integrative Biology and Valorization Lab (BIOLIVAL), Higher Institute of Biotechnology of Monastir, University of Monastir, Monastir, 5000, Tunisia*
[c] *Fiorital SPA, Venezia, 30135, Italy*

ARTICLE INFO

Keywords:
Growth potential
Hygiene criterion
Vacuum packaging
Regulation EC n.2073/2005

ABSTRACT

Fish fillets are highly susceptible to spoilage, with *Pseudomonas* spp. bacteria being among the main culprits. To maintain products' quality and safety, it is important to control the load of these microorganisms and understand their growth potential in fish fillets. However, setting up challenge tests might be hard due to the difficulty of differentiating intentionally inoculated bacteria from those already present on the fillets. To overcome this obstacle, a pilot study using *Pseudomonas aeruginosa*, a clinically significant bacterial species that is rare in food, was conducted. Vacuum-packed Northern cod, salmon, and plaice fish fillets were experimentally inoculated and subjected to trials at both refrigeration (4 °C) and thermal abuse temperatures (from +4 °C to +6 °C and then to +8 °C). The results showed that the growth potential of *Pseudomonas aeruginosa* in all the fish fillets was less than 0.5 Log_{10} CFU/g. This confirms that vacuum packaging could reduce the multiplication of *Pseudomonas* spp. in the fish fillets and underlines as it is crucial to have fish fillets containing initial loads of *Pseudomonas* between 10^4-10^5 CFU/g or lower at the beginning of the shelf life in order to control the deterioration rate of the product. This study provides a basis for developing further challenge tests for *Pseudomonas* spp. in the fish industry and highlights the importance of controlling initial loads of *Pseudomonas* to prevent product deterioration during the shelf life.

1. Introduction

Food deteriorates in 90 % of cases due to excessive microbial proliferation and only in 10 % of cases due to strictly chemical reactions such as, for example, the oxidation of lipids. The population dynamics of the microbial community in food is influenced by the

* Corresponding author. Department of Animal Medicine, Productions and Health, School of Agricultural Sciences and Veterinary Medicine. University of Padua. Legnaro, 35020, Italy.

E-mail addresses: giulia.alberghini@studenti.unipd.it (G. Alberghini), benmhenninesrine@gmail.com (N. Ben Mhenni), vincenzo.dileva@fiorital.com (V. Di Leva), riccardo.forzano@fiorital.com (R. Forzano), riccardo.miottiscapin@unipd.it (R. Miotti Scapin), placidomatteo.pappalardo@studenti.unipd.it (P.M. Pappalardo), federica.giacometti@unipd.it (F. Giacometti), valerio.giaccone@unipd.it (V. Giaccone).

https://doi.org/10.1016/j.heliyon.2024.e32170
Received 19 July 2023; Received in revised form 20 May 2024; Accepted 29 May 2024
Available online 30 May 2024

food itself and by the environmental conditions in which it is stored. Fish products are rich in non-protein nitrogenous compounds (NPN), i.e. low molecular weight compounds that microorganisms can use more easily than proteins; this leads to a greater speed and intensity of their deterioration compared to other products of animal origin, with consequent unpleasant odours and flavours due to the formation of trimethylamine [1–5]. For these reasons it is necessary to pay attention to the microbial loads of the main specific agents of alteration of fish products.

Storage conditions and handling practices are the most important factors in determining the shelf life of all species of fish. If the fish product is handled carefully, the temperature and others environmental conditions at which it is held controls microbial growth kinetics, the rate of bacterial spoilage and enzyme breakdown in foods [6,7]. Temperature increases the effect of other factors and it is the factor most likely to fluctuate [7]. Moreover, at temperatures above 4 °C, the relative rate of spoilage counts up very fast even if only of 2 °C of difference [6].

It is consolidated, in literature, that psychrotrophic Gram-negative bacilli, of which the major representative is *Pseudomonas* spp., are the main Specific Spoiling micro-Organisms (SSO) of fresh fish products stored at refrigeration temperature [3,8,9]. When they multiply excessively in fish fillets, they cause deterioration and make the products unfit for human consumption with the consequence that they must therefore be removed from the market pursuant to article n.14 of the European Regulation (EC) n. 178/2002 [10]. It is generally accepted that the SSO load beyond which deterioration of fresh fish is evident is 8–9 Log_{10} CFU/g but in some cases the organoleptic characteristics of fish preserved in air, vacuum and modified atmosphere packaging (MAP) are unacceptable as early as 6–7 Log_{10} CFU/g [1,11].

However, the problem is not so much establishing the maximum load not to be exceeded at the end of the shelf life but the maximum load at the time of production (also called "threshold value") or in any case in the hours immediately following production, in order to not exceed, at the end of the shelf life, the maximum acceptable value (10^6–10^7 CFU/g) beyond which the product could start to deteriorate to an appreciable sensory extent. From previous studies conducted on fillets of salmon, plaice and Northern cod, an averaged values of *Pseudomonas* spp. of the order of 10^4–10^5 CFU/g but not infrequently as far as 1–2 Logarithmic degrees more were reported immediately after packaging [12]. The filleting operations and the general hygiene of the place of processing of fish products can also contribute to the increase in the number of *Pseudomonas* [13].

For a fishing industry it is therefore worth including *Pseudomonas* spp. as a self-monitoring process hygiene criterion. In European Regulation (EC) n. 2073/2005 [14], article n.2 (point d) is defined as a process hygiene criterion "*a criterion indicating the acceptable functioning of the production process. Such a criterion is not applicable to products placed on the market. It sets an indicative contamination value above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law*". The "indicative contamination" that is mentioned in the definition corresponds to the maximum acceptable load at the time of production. To date, the most effective tool to get that data is the challenge test. It is an experimental food inoculation test aimed at studying the growth of microorganisms specially inoculated in a food matrix [15]; all this then translates into a datum: the growth potential.

In the European food legislation and, in particular, with the EC Regulation n. 2073/2005 (article n.3 paragraph 2 and annex II), the challenge test has assumed a very great importance. The design and implementation of any challenge test must follow the standard ISO 20976–1:2019 [16] in order to be validatable and legally protective. Challenge test can be designed for any type of bacterium, mold or yeast that does not form mycelium [15,16]. However, in most cases, pathogens, generally not present in foods, are considered and therefore their isolation resulted easy to be performed. Differently to *Listeria monocytogenes*, for which indications and guidelines are found in the literature by ANSES document "*EURL Lm technical guidance document for conducting shelf life studies on L. monocytogenes in ready-to-eat foods*" [17] or EN SANCO/1628/2008 *"on Listeria monocytogenes shelf life studies for ready-to-eat foods"* [18], there is no specific document on *Pseudomonas* genus.

The present study had the aim of conducting a challenge test using one of the major and most frequent food spoilage bacterial genus, as well as the main SSO of fishery products [3,8,9]. The most issue is the determination of *Pseudomonas* contamination that is attributable to the pre-existing background contamination in the fillet, versus the inoculum experimentally spiked in the study. To overcome this issue, literature provided the cooking of the food or the creation of a fish muscle agar [19], but each proposed substrate does not have the same characteristics as the original food so it could affect the results. Therefore, among the overall *Pseudomonas* species, *Pseudomonas aeruginosa* was chosen as microorganism to be experimentally inoculated given it is considered a leading human opportunistic pathogen as well as not widespread in food [2,4,20–22]. Even if some Authors stated that *P. aeruginosa* has been underestimated as a foodborne pathogen in various food groups such as water, milk, meat, fruits and vegetables, the data firmly support that it is not widely distributed in fish fillets [21,22]. This suggestion is confirmed by the findings observed in a previous study, namely an average value of *Pseudomonas* spp. (10^4–10^5 CFU/g) found in fresh fish fillets of the same fish species considered in the present study and coming from the same fish industry as well as the absence of *P. aeruginosa* on salmon, plaice and Northern cod fillets among more than 50 strains isolated and subjected to species identification [12]. Therefore *P. aeruginosa* was chosen as microorganism to be spiked in challenge test because it is rare on fish fillets and well distinguishable from others species belonging to the genus *Pseudomonas* by selective and differential media.

2. Materials and methods

For the design of the challenge test the ISO 20976–1:2019 standard "*Challenge test to study growth potential, lag time and maximum growth rate*" was used as a basis [16].

Three different commercial references of fish fillets (salmon, Northern cod and plaice) were subjected to challenge tests at three different times. Only one batch for each investigated fish species was considered for each reference according to the indications of ISO 20976–1:2019 which states that when the chemical-physical parameters do not have significant variations, a single batch is sufficient.

In fact, the historical data collected by Food Business Operator (FBO) were considering in relation to pH and water activity (a_w), which are supported by data in literature which, at least as regards pH, confirmed non-significant standard deviations [23,24]. To better define the storage parameters of this experimental study, the indications on the labels previously provided by the food industry were of primary importance. It was useful to know the shelf life of the fillets (6 days for plaice fillets, 8 days for salmon and Northern cod) and the storage temperature between 0 and 4 °C. Moreover, 10^4–10^5 CFU/g is the average value of *Pseudomonas* spp. found in fresh fish fillets of the same species considered in the present study and coming from the same fish industry [12].

Each challenge test can ideally be divided into two phases: in the first phase the experimentally inoculum is carried out and in the second phase (the duration of which depends on the shelf life of the product) the analyses are performed on a predetermined number of samples and in at least 5 pre-established times during the shelf life of the food.

2.1. First phase

2.1.1. Selection of Pseudomonas aeruginosa strains

Three reference strains of *P. aeruginosa* were used, namely ATCC (*American Type Culture Collection*) 9027, 27,853 and 15,442. The use of more than one strain is intended to compensate for differences in resistance (growth/survival) which naturally exist between individual strains [16,25,26]. Wild strains were not used because they have never been found in previous analysis which concerned the isolation and species identification (biochemically via Biolog®) of more than 50 *Pseudomonas* strains [12].

2.1.2. Suspension culture

For the inoculation a suspension of *P. aeruginosa* was used: the stock culture was previously induced at the VBNC (Viable But Not Culturable cell) stage as the non-spore-forming microorganisms in foods are mainly found in this stage as they are affected by the influence of various factors which FBO exploits to keep microbial proliferation under control, for example oxygen removal and temperature [1,27]. In the VBNC state the microorganisms tend to become much more resistant to stresses than their actively multiplying counterparts. The reason could be found in the gene regulation and in particular in the presence of a gene called "sigma factor" which, when activated, activates a series of other genes in cascade with the result that proteins and molecules are synthesized, giving the microorganism greater resistance to unfavourable environmental conditions such as heat, pH and a_w drop [1,27]. Bacteria in the VBNC state, as first discovered by Colwell and collaborators for *Vibrio cholerae* [28], can resurrect and then revert back to the cultivable state when conditions become favourable [27,29].

A mix of three *P. aeruginosa* strains was used in challenge test to inoculate the fish fillets. For each reference strain of *P. aeruginosa* a suspension was created: the target strain was inoculated in Brain Heart Infusion (BHI) broth and incubated for 24 h at 37 °C to obtain a suspension with cells in exponential growth phase. Subsequently we induced a cold thermal shock was induced by incubating the suspensions at 4 °C for three days. Three hours before the inoculations, the suspensions were further transferred to the room temperature of about 25 °C.

The final suspension to be inoculated was obtained by mixing together equal volumes of the three previously created suspensions. It is important to know the exact concentration of the suspension and, due to the methods and times with which it was obtained, it was reasonably assumed that its concentration was around 10^7–10^8 CFU/ml; to confirm this, the serial dilutions were prepared by plating the suspension on *Pseudomonas* Isolation Agar medium (Liofilchem®) and incubating the plates in the thermostat at 35 ± 2 °C for 18–48 h. Subsequently, 1 mL from the first dilution was put into 99 ml of physiological solution (0.9 % NaCl) to have a hypothesized concentration of 10^4–10^5 CFU/ml in 100 ml. This last suspension of 100 ml was the one used for the inoculations (*see 2.1.4*).

2.1.3. Preparation of samples

The fish fillets used in the challenge test were collected from a fish industry located in the Venetian area (Italy). The fish species subjected to the challenge test were salmon (*Salmo salar*), Northern cod (*Gadus morhua*) and plaice (*Pleuronectes platessa*). They were vacuum packed (skin packaging) on the same morning of the analysis. Differently from salmon and plaice, the Nordic cod had arrived frozen at the fish industry and was thawed before filleting. The fish fillets arrived within 1 h at the food microbiology laboratory (Department of Animal Medicine, Productions and Health, University of Padua), the fish fillets arrived in about 1 h in expanded polystyrene boxes with eutectic plates under refrigeration conditions. A total of 45 packs for each batch of salmon, cod and plaice arrived respectively on November and December 2022, and January 2023. Once arrived to the laboratory, the packages were weighed, opened, and the contents were transferred, under a laminar flow biological hood and maintaining the highest possible sterility, into bags having the same gas permeability as the original ones. On average, the weights of plaice packs were 300 g, while those of Northern cod and salmon ranged between 200 and 250 g.

2.1.4. Inoculations

For each fish species, 24 test units were considered and inoculated (3 test units for sampling point) with the bacterial suspension created (*see 2.1.2*) while 8 control units (1 control unit for sampling point) were inoculated with sterile saline. Therefore, for each reference, 32 units were considered.

In all cases the volume of inoculum was always the same, i.e. a maximum of 1 % of the volume or mass of feed (so the pH and a_w of the matrix did not vary). The volume chosen to inoculate in each test unit was 2 ml since the fish fillets were between 200 and 300 g. By doing this, according to the ISO 20976–1:2019 standard [16], it was possible to reach a concentration between 50 and 10,000 CFU/g in each single fish fillet. Since *Pseudomonas* multiplies on the surface as it needs oxygen [1], the dispersion of the inoculum was done on the surface of the fillets and took place with the aid of 2 ml pipettes and a Falcon® automatic pipettor. To be certain of the homogeneity

of the inoculum on the surface of the food, when the counts at T0 were done, the calculation of the standard deviation between the 3 data of the test units was required. If the SD calculated at T0 was <0.3 Log_{10} CFU/g it therefore meant that the inoculation had taken place correctly, dispersing the suspension homogeneously on the surface of the fillets [16]. The set-up of the control units was necessary to determine the level of natural contamination of the target microbial population, as well as the intrinsic factors of the food.

2.1.5. Closure of the packages

The creation of the vacuum took place by means of a chamber vacuum machine (Orved S. p.A) which at the same time thermo-sealed the polypropylene bags and therefore recreated the same packaging conditions (skin packaging) as when the samples arrived at the laboratory. Finally, each sample was signed with an alphanumeric identification code that uniquely identified it. Through the different identification codes it was possible to distinguish the test units from the control units.

2.2. Second phase

2.2.1. Storage and sampling points

The samples were initially kept in a fridge at a temperature of 4 °C. Starting from day T2, the samples were divided into those intended for simulating thermal abuse and those that would remain at a constant temperature of 4 °C. At T2, twelve samples were transferred from 4 °C to 6 °C, and at T7, the remaining four samples were moved to 8 °C to simulate, respectively, thermal abuse in the warehousing/retail and home conservation phases. The choice of these temperatures was dictated by some recent studies reporting the average temperature of Italians' domestic refrigerators: most Italians are mistakenly convinced that the temperature of home refrigerators fluctuates between 4 and 5 °C. This is not the case, the temperature inside is on average 50–80 % higher [30,31]. Therefore, where the label indicates a storage temperature of 0°/4 °C, keeping the food even at 6 °C is considered thermal abuse.

A challenge test must be planned based on the shelf life of the product [1] so, as it was reported on the labels, for Northern cod and salmon it was 8 days while for plaice it was 6 days. The analyses were carried out at five predetermined moments (sampling points) of the shelf life of the product with equal time intervals between the sampling points, excluding weekends: T0, T2, T4, T7 and T9 where "T0" corresponded to the day of the inoculations and also of the first analysis and so, for example, the 2 in "T2″ meant 2 days after the first analysis. To simulate potential temperature and time abuse, all three types of fish were kept up to 9 days after inoculations and so 1 day (Norther cod, salmon) and 3 days (plaice) beyond their labelled shelf life.

2.2.2. Conducting analyses

The analyses were carried out following the ISO 6887–1:2017 [32] for the preparation of the test samples, the initial suspension and the decimal dilutions for the microbiological analysis. The aim of the analysis was to quantify the presence of *P. aeruginosa*. In the control unit, in addition to the quantification of *P. aeruginosa* possibly present in the fillets, the total microbial load was quantified. Moreover, pH and a_w were measured in duplicate respectively by Mettler Toledo® Seven Compact S220 pH-meter and Aqua-Lab CX-2®). The means of the two pH values and the two a_w values were calculated.

For each sample, a total of 20 g of fillet were taken from various points and placed in a sterile bag for Stomacher®. Then, 180 ml of sterile physiological solution was added to the bag and everything was homogenized in the Stomacher® 400 Circulator. From the generated suspension, serial dilutions 1:10 were started by taking 1 ml and placing it in 9 ml of physiological solution (NaCl 0.9 %). Seeding was done by spatula on ready-to-use Petri dishes with *Pseudomonas* Isolation Agar medium (Liofilchem®), a selective and differential medium. Said plates were then incubated aerobically in the thermostat at 35 ± 2 °C for 18–48 h. The control unit suspension was also seeded by inclusion in Plate Count Agar medium (Merck®) and the plates were incubated in the incubator at 31 °C ± 2 °C for 48–72 h. For the count of *P. aeruginosa*, ISO 7218: 2013 [33] was followed. The quantification limit of the test was *P. aeruginosa* < 100 CFU/g. Greenish-yellow pigmented colonies were counted and, as ISO 20976–1:2019 mentions, it was not mandatory to perform confirmatory tests on typical colonies as part of the technical enumeration procedure. For the Total Viable Count, the ISO 4833–1:2022 [34] was followed, which is a specific standard for counting microorganisms capable of growing and forming colonies on the surface of a solid medium after aerobic incubation at 30 °C.

2.3. Statistical analysis

The arithmetic mean (μ) and standard deviation (SD or σ) of pH- and a_w-values were calculated. Then SAS 9.4 (Copyright © 2002–2012 by SAS Institute Inc., Cary, NC, USA) was used to perform the analysis of variance in order to evaluate the significance.

As for TVC in control units and *P. aeruginosa* in test units, the results identified as CFU per gram were then converted to the Logarithm based on 10. Then for *P. aeruginosa,* in each analysis time, the arithmetic mean (μ) and standard deviation (SD or σ) value of the three test units was calculated. A deeper evaluation of the standard deviation value of the first three test units recorded at T0 was necessary in order to verify the acceptability of the entire challenge test: if SD was greater than the limit of 0.3 Log_{10} CFU/g it identified a non-homogeneity of the inoculation/contamination and therefore it would have been senseless to continue the challenge test with the previously inoculated samples. Furthermore, also in this case the SAS 9.4 analysis of variance model was used considering the effect of species and time; this in parallel for the samples at +4 °C and those in thermal abuse. Finally, the growth potential of *P. aeruginosa* was calculated. It represents a measure of how much bacteria can potentially grow in a food sample over a certain period. It was calculated using the following formula: $\Delta = Log_{max} - Log_{i}$ [16] where:

- Δ is the growth potential, measured in Log_{10} colony-forming units per gram CFU/g;

- Log $_{max}$ is the highest average value obtained from the samples considering all analysis times (T0, T2, T4, T7, T9);
- Log $_{i}$ is the initial (T0) average value obtained from the samples.

If Log $_{i}$ is the highest value of all sampled test units, then the growth potential is zero because there is no potential for further bacterial growth.

3. Results and discussion

Based on the challenge tests, the standard deviations calculated among the 3 loads of suspension at T0 (*see Appendix B*) of each reference were respectively of 0.15, 0.16 and 0.04 Log_{10} CFU/g for the salmon, plaice and Northern cod batches. Being the SD always $<$0.3 Log_{10} CFU/g, all the inoculations (salmon, Northern cod and plaice) took place correctly, dispersing the suspension homogeneously on the surface of the fillets [16]. In addition, the search for *P. aeruginosa* in the control units never gave qualitatively positive results. This therefore contributes to the veracity of the overall experimental results.

The *P. aeruginosa* loads observed in the different fish species stored both at refrigeration (4 °C) and in thermal abuse (6–8 °C) temperatures, are reported in Appendix B (Table B.1., Table B.2., Table B.3.) and represented in Figs. 1, 3 and 5. Instead, the Total Viable Counts (TVC) of the control units are in Appendix A (Table A.1., Table A.2., Table A.3.).

The behaviours of the TVC (Figs. 2, 4 and 6) are associated with the graphs of *P. aeruginosa* loads detected in the test units to compare the trends of the specific bacterial considered and the background microbial community.

In plaice fillets, Figs. 1 and 2 clearly show as both TVC and *P. aeruginosa* loads were higher in samples kept in thermal abuse than in those stored at refrigeration temperature. A slight increase of *P. aeruginosa* is observed between T0 and T2, followed by a continuum decrease from T2 to T7 and remained stable at T9. No significant difference was observed between storage at 4 °C and 6/8 °C.

In salmon fillets, *P. aeruginosa* slightly decreased between T0 and T9 whereas an increase of TVC is observed in both storage conditions (Figs. 3 and 4).

In Northern cod fillets (Figs. 5 and 6), a slight decrease was observed during the storage at both temperature conditions. The general trend was however similar between the two conditions and was of decrease. Concerning the TVC, it remained stable in refrigerated samples whereas an increase was observed in case of thermal abuse (Fig. 6). From the previously described statistical analysis of the data (*see 2.3*), in Northern cod, the time was found to be significant but not the species. TVC is significantly increasing over time, especially starting from T7.

The calculated growth potential, namely the measure of how much bacteria can potentially grow in a food sample over a certain period, was:

- For the salmon batch at +4 °C: 1.85–1.85 = 0.00 Log_{10} CFU/g;
- For the thermal abuse salmon batch: 1.85–1.85 = 0.00 Log_{10} CFU/g;
- For the Northern cod batch at +4 °C: 3.43–3.39 = 0.04 Log_{10} CFU/g;
- For the heat abused cod lot: 3.39–3.39 = 0.00 Log_{10} CFU/g;
- For the plaice batch at +4 °C: 2.75–2.41 = 0.34 Log_{10} CFU/g;
- For the thermal abuse plaice batch: 2.73–2.41 = 0.32 Log_{10} CFU/g.

All the growth potentials calculated were all lower than 0.5 Log_{10} CFU/g confirming that *P. aeruginosa* did not significantly growth on the vacuum-packed salmon, plaice, and Northern cod fillets during their shelf life, regardless of storage conditions. This indicates that the risk of spoilage caused by this bacterial species could be classified as low. However, the growth potential calculated for plaice stored at 4 °C and in thermal abuse was slightly higher [0.34 and 0.32 Log_{10} CFU/g, respectively] than in the other samples tested, indicating a slightly higher potential of multiplication and spoilage by this bacterial species in those conditions. The highest concentration values of *P. aeruginosa* were recorded within the shelf life of the plaice fillets indicated by the producer (for the calculation of plaice growth potential, T9 was excluded because it was too far beyond the 6-day shelf life of the product; however, analyses were

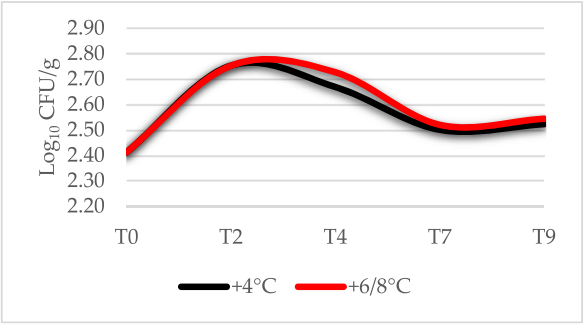


Fig. 1. Mean log CFU/g values of *P. aeruginosa* during the vacuum storage at both refrigeration (4 °C) and thermal abuse (6–8 °C) temperatures in plaice test units.

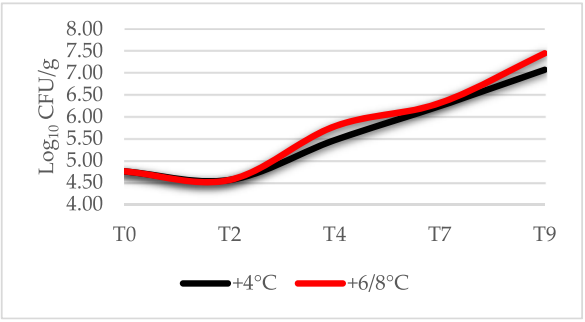


Fig. 2. Mean log CFU/g values of Total Viable Counts during the vacuum storage at both refrigeration (4 °C) and thermal abuse (6–8 °C) temperatures in plaice control units.

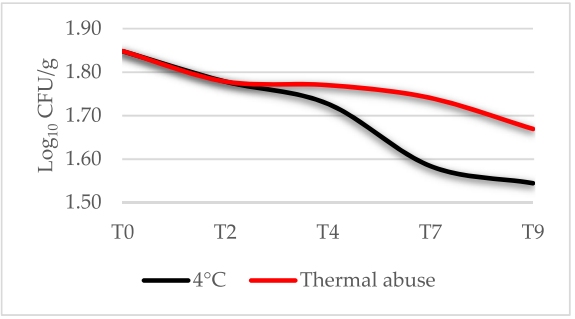


Fig. 3. Mean log CFU/g values of *P. aeruginosa* during the vacuum storage at both refrigeration (4 °C) and thermal abuse (6–8 °C) temperatures in salmon test units.

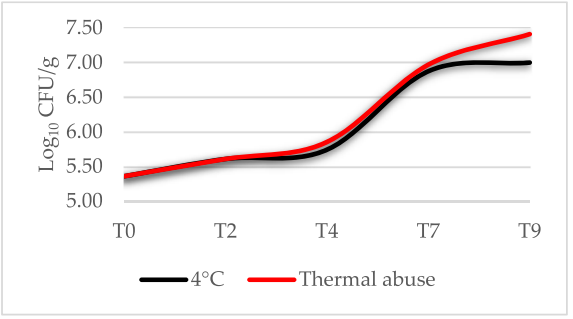


Fig. 4. Mean log CFU/g values of Total Viable Counts during the vacuum storage at both refrigeration (4 °C) and thermal abuse (6–8 °C) temperatures in Salmon control units.

carried out to see the trend of the microorganism in the product in parallel with cod and salmon).

The growth potential values for *P. aeruginosa* in all remaining samples were around 0 Log_{10} CFU/g. These results were expected given that the fillets were vacuum packed and *Pseudomonas* bacteria need oxygen as it is the electron acceptor in their metabolic reactions [1]. This is consistent with previous studies, such as Gram and Huss [3], who found that vacuum packing reduces the growth of *Pseudomonas* spp. However, in the plaice (at 4 °C) between T0 and T2 and in the Northern cod (at 4 °C) between T2 and T4 there is evidence of a growth of *P. aeruginosa*. This can be explained by the fact that the bacteria of the genus *Pseudomonas*, being psychrotrophs, have an optimal temperature range between 25 and 30 °C but are able to manage to double even between 0 and 3 °C [1,9]. It can also be said that, based on what emerged from the statistical analysis, Northern cod always had significantly higher *P. aeruginosa* load values than the other two fish species. This significance was higher for fillets under refrigeration (Fig. 7) than for those under thermal abuse (Fig. 8).

The growth rate of Pseudomonadaceae strongly depends on the substrate in which they are as well as on extrinsic and process factors [1,3,35]. The substrate is characterized by certain so-called "intrinsic" factors which include, in addition to the redox potential and the presence of natural antimicrobials, the pH value and the a_w in the food and available for the microbial metabolism. The importance of pH and a_w values is clearly expressed in EC Regulation n.2073/2005 where it deals with *Listeria monocytogenes* in

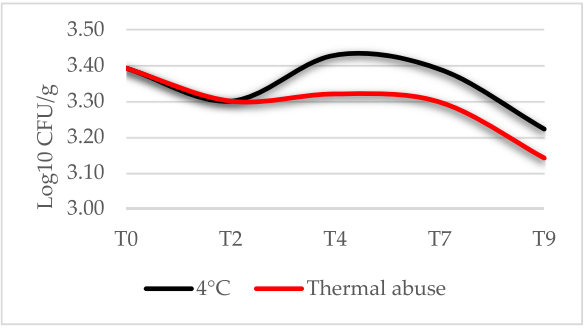


Fig. 5. Mean log CFU/g values of *P. aeruginosa* during the vacuum storage at both refrigeration (4 °C) and thermal abuse (6–8 °C) temperatures in Northern cod test units.

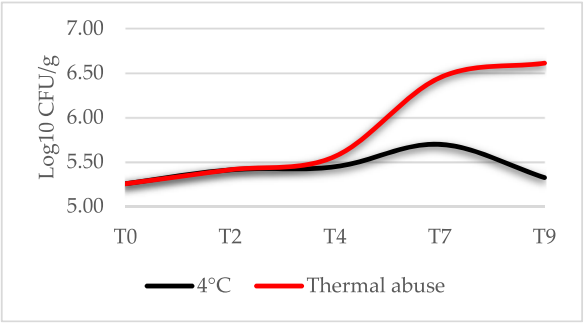


Fig. 6. Mean log CFU/g values of Total Viable Counts during the vacuum storage at both refrigeration (4 °C) and thermal abuse (6–8 °C) temperatures in Northern cod control units.

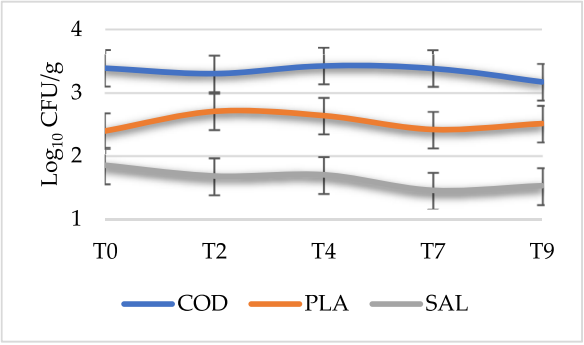


Fig. 7. Estimated means and relative standard errors (SE) of *P. aeruginosa* loads in samples maintained at refrigeration temperature. Twice the SE values were used for the representation to obtain a 95 % confidence interval.

ready-to-eat products.

Pseudomonas is a genus of normoduric bacteria so they multiply well at pH values between 5.8 and 7 and fail to duplicate if the substrate drops below 4.5. In the control units analysed, following a statistical analysis of the data (*see 2.3*), the species was found to be significant. The pH of cod increased, that of salmon remained fairly constant, in plaice decreased (Fig. 9). So, the Northern cod was significantly different from the other two which were equal in general mean value.

The other fundamental parameter is a_w: the minimum value for the growth of *Pseudomonas* is between 0.950 and 0.935 [1]. It should be noted (Fig. 10) that the minimum recorded value (0.950) was reached between T7 and T9 in plaice fillets. For a_w both time and species were found to be statistically significant. In order to evaluate the interaction between time and species, in plaice a_w values significantly decreased in both conservation regimes and this decrease was predictable in relation to the progressive loss of liquid from the fillets, liquid which accumulated in the package in increasing quantities towards the end of the shelf life and in the following period between T7 and T9. In plaice the liquid loss occurred more than in salmon and Northern cod.

It is generally accepted that the SSO load beyond which the organoleptic characteristics of fish preserved in air, vacuum and MAP

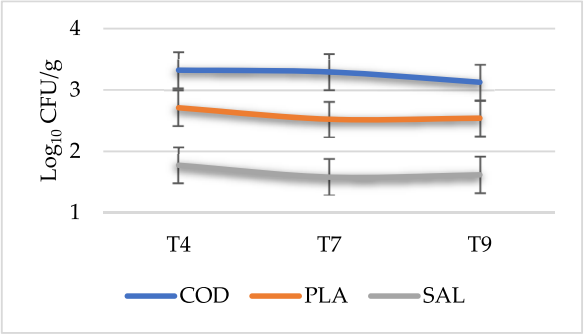


Fig. 8. Estimated means and relative standard errors (SE) of *P. aeruginosa* loads in samples maintained at abuse temperature (only 6/8 °C). Twice the SE values were used for the representation to obtain a 95 % confidence interval.

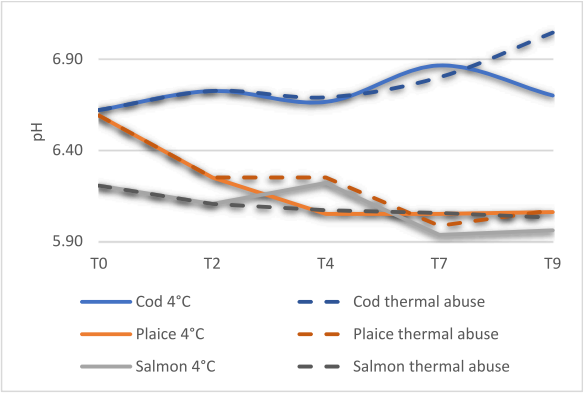


Fig. 9. pH average values in control units.

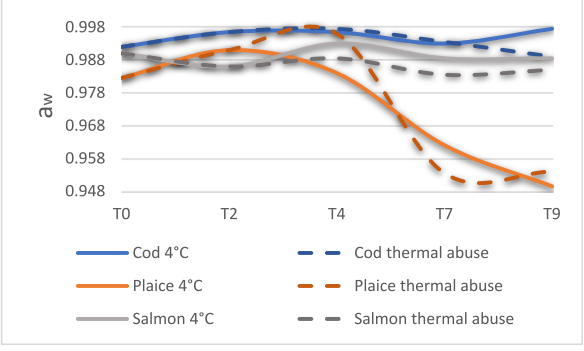


Fig. 10. Water activity average values in control units.

are unacceptable is 6–7 Log_{10} CFU/g [1,11]. Therefore, knowing the maximum value of *Pseudomonas* CFU/g at the end of shelf life and that the growth potential is < 0.5 Log_{10} CFU/g, it is reasonable to establish an initial threshold value of <6 Log10 CFU/g for *Pseudomonas* spp. load at the time of production or in the hours immediately following. This value can be suggested as a process hygiene criterion in a fishing industry in addition to those already established by EC Regulation n. 2073/2005 [14]. Previous studies have shown that salmon, plaice, and cod fillets have average values of *Pseudomonas* spp. ranging from 10^4-10^5 CFU/g immediately after packaging, with occasional values going up to 1–2 Logarithmic degrees higher [12]. This means that particular attention must be paid by FBO to the hygiene of the environment and of the tools during the filleting and fillet handling operations. This especially during the spring and summer months when temperatures are favourable for *Pseudomonas* growth [12,36].

4. Conclusions

The design of this study allowed to extend the data collected for *P. aeruginosa* to the entire *Pseudomonas* genus. The fact that *P. aeruginosa*, which is rarely isolated in fish products and in foods in general [2,12,20–22], was used as the basis for this study could be used by fish industry as an useful strategy for the implementation of challenge tests on *Pseudomonas* following the ISO 20976–1:2019 standard [16]. This is particularly relevant given that *Pseudomonas* is a spoiling bacterium and one of the main sources of SSO in fish products [3,8,9].

The overall findings indicate that the load of *Pseudomonas* at the beginning of the shelf life is crucial to maintain product quality and safety. Additionally, our results reveal that the growth potential of *Pseudomonas* in vacuum-packed plaice, salmon, and Northern cod fillets is always less than 0.5 Log_{10} CFU/g. This confirms the importance of vacuum preservation to inhibit the growth of this bacterium. A threshold value at the beginning of the shelf life was recommended to be preferably between 10^4-10^5 CFU/g which is the average value found in fillets of these species of fish in the non-summer period with hygiene standards applied in the food industry as established by EC Regulations 852 and 853/2004 [12,37,38]. By using this threshold value, vacuum-packed salmon and Northern cod fillets can maintain acceptable quality conditions until the end of their shelf life (in this case 8 days) and are safe for human consumption. For plaice fillets, which have a shorter shelf life (6 days), it is prudent to maintain the initial load below the recommended threshold to ensure product safety and quality.

Our study has important implications for the food industry, especially for fish processing companies. The findings can serve as a basis for FBO to design effective control measures and contribute to improving food safety and quality. Overall, the results of this first challenge study provide a basis for further research and assay development on specific seafood spoiling microorganisms (in this case *Pseudomonas*) using the natural matrix and not substitutes such as cooked fish or fish agar.

Data availability statement

Data included in article will be made available on request.

Funding

This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Giulia Alberghini: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nesrine Ben Mhenni:** Writing – original draft, Formal analysis. **Vincenzo Di Leva:** Resources, Project administration. **Riccardo Forzano:** Resources. **Riccardo Miotti Scapin:** Methodology, Formal analysis. **Placido Matteo Pappalardo:** Formal analysis. **Federica Giacometti:** Writing – review & editing. **Valerio Giaccone:** Writing – review & editing, Validation, Resources, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A

Table A.1
Physico-chemical and microbiological values referring to plaice control units.

TIME	°C	μ pH	σ pH	μ a_w	σ a_w	TVC Log CFU/g	*P. aeruginosa* Log CFU/g
T0	4	6.59	0.04	0.983	0.002	4.75	<2
T2	4	6.25	0.01	0.991	0.001	4.55	<2
T4	4	6.05	0.01	0.984	0.008	5.46	<2
T4	6	6.25	0.07	0.996	0.003	5.78	<2
T7	4	6.05	0.13	0.962	0.000	6.24	<2
T7	6	5.99	0.04	0.954	0.004	6.31	<2
T9	4	6.06	0.03	0.950	0.002	7.07	<2
T9	8	6.07	0.03	0.954	0.001	7.45	<2

Table A.2
Physico-chemical and microbiological values referring to salmon control units.

TIME	°C	μ pH	σ pH	μ a_w	σ a_w	TVC Log CFU/g	*P. aeruginosa* Log CFU/g
T0	4	6.21	0.01	0.990	0.003	5.36	<2
T2	4	6.11	0.06	0.986	0.001	5.61	<2
T4	4	6.22	0.03	0.993	0.007	5.74	<2
T4	6	6.07	0.07	0.989	0.003	5.85	<2
T7	4	5.94	0.03	0.989	0.002	6.88	<2
T7	6	6.06	0.02	0.984	0.004	6.97	<2
T9	4	5.96	0.03	0.989	0.002	7.00	<2
T9	8	6.03	0.04	0.985	0.001	7.41	<2

Table A.3
Physico-chemical and microbiological values referring to Northern cod control units.

TIME	°C	μ pH	σ pH	μ a_w	σ a_w	TVC Log CFU/g	*P. aeruginosa* Log CFU/g
T0	4	6.62	0.03	0.992	0.003	5.25	<2
T2	4	6.73	0.04	0.997	0.001	5.41	<2
T4	4	6.67	0.01	0.997	0.000	5.44	<2
T4	6	6.69	0.05	0.998	0.004	5.56	<2
T7	4	6.87	0.01	0.993	0.002	5.70	<2
T7	6	6.80	0.07	0.994	0.005	6.45	<2
T9	4	6.70	0.02	0.998	0.003	5.32	<2
T9	8	7.05	0.01	0.989	0.007	6.61	<2

Appendix B

Table B.1
P. aeruginosa loads in plaice both at refrigeration (4 °C) and in thermal abuse (6–8 °C) temperature.

Sampling time	Test unit	*P. aeruginosa (*Log CFU/g)	
		Plaice	
T0	I	2.51	
	II	2.47	
	III	2.20	
		μ = 2.41 σ = 0.16	
T2	I	2.90	
	II	2.81	
	III	2.40	
		μ = 2.75 σ = 0.27	
		Refrigeration (4°C)	**Thermal abuse**
T4	I	2.70	2.54
	II	2.81	2.78
	III	2.40	2.81
		μ = 2.67 σ = 0.21	μ = 2.73 σ = 0.15
T7	I	2.60	2.70
	II	2.65	2.44
	III	2.00	2.43
		μ = 2.50 σ = 0.36	μ = 2.52 σ = 0.15
T9	I	2.40	2.60
	II	2.48	2.48
	III	2.65	2.54
		μ = 2.52 σ = 0.13	μ = 2.54 σ = 0.06

Table B.2
P. aeruginosa loads in salmon both at refrigeration (4 °C) and in thermal abuse (6–8 °C) temperature.

Sampling time	Test unit	*P. aeruginosa (*Log CFU/g)
		Salmon
T0	I	1.70
	II	1.85
	III	2
		μ = 1.85 σ = 0.15
T2	I	2.04
	II	1.70

(*continued on next page*)

Table B.2 (*continued*)

Sampling time	Test unit	*P. aeruginosa* (Log CFU/g)	
	III	1.30	
		μ = 1.78 σ = 0.37	
		Refrigeration (4°C)	**Thermal abuse**
T4	I	1.90	1.74
	II	1.60	1.85
	III	1.60	1.73
		μ = 1.73 σ = 0.17	μ = 1.77 σ = 0.07
T7	I	1.48	1.00
	II	1.88	1.74
	III	1.00	2.00
		μ = 1.58 σ = 0.44	μ = 1.74 σ = 0.52
T9	I	1.70	1.30
	II	1.48	1.74
	III	1.40	1.81
		μ = 1.54 σ = 0.16	μ = 1.67 σ = 0.28

Table B.3
P. aeruginosa loads in Northern cod both at refrigeration (4 °C) and in thermal abuse (6–8 °C) temperature.

Sampling time	Test unit	*P. aeruginosa* (Log CFU/g)	
		Northern cod	
T0	I	3.41	
	II	3.34	
	III	3.41	
		μ = 3.39 σ = 0.04	
T2	I	3.43	
	II	2.90	
	III	3.57	
		μ = 3.30 σ = 0.35	
		Refrigeration (4°C)	**Thermal abuse**
T4	I	3.33	3.31
	II	3.51	3.42
	III	3.43	3.24
		μ = 3.43 σ = 0.09	μ = 3.32 σ = 0.09
T7	I	3.47	3.33
	II	3.27	3.24
	III	3.41	3.31
		μ = 3.39 σ = 0.10	μ = 3.30 σ = 0.05
T9	I	3.27	3.04
	II	3.39	3.28
	III	2.85	3.06
		μ = 3.22 σ = 0.28	μ = 3.14 σ = 0.13

References

[1] V. Giaccone, G. Colavita, Principi di Microecologia degli Alimenti, Point Vètèrinaire Italie Autore (2015). ISBN 9788895995748.
[2] N.C. Gillespie, A numerical taxonomic study of Pseudomonas-like bacteria isolated from fish in southeastern Queensland and their association with spoilage, J. Appl. Bacteriol. 50 (1981) 29–44.
[3] L. Gram, H.H. Huss, Microbiological spoilage of fish and fish products, Int. J. Food Microbiol. 33 (1) (1996) 121–137, https://doi.org/10.1016/0168-1605(96)01134-8.
[4] F.F. Parlapani, D.A. Anagnostopoulos, E. Karamani, A. Mallouchos, S.A. Haroutounian, I.S. Boziaris, Growth and Volatile organic compound production of *Pseudomonas* fish spoiler strains on fish juice agar model substrate at different temperatures, Microorganisms 11 (1) (2023), https://doi.org/10.3390/microorganisms11010189.
[5] Z. Zhang, R. Wu, M. Gui, Z. Jiang, P. Li, Identification of the specific spoilage organism in farmed sturgeon (*Acipenser baerii*) fillets and its associated quality and flavour change during ice storage, Foods 10 (2021), https://doi.org/10.3390/foods10092021.
[6] J.P. Doyle, Seafood Shelf Life as a Function of Temperature (1989), https://doi.org/10.4027/sslft.1989. (Accessed 2 April 2024).
[7] T. McMeekin, J. Bowman, O. McQuestin, L. Mellefont, T. Ross, M. Tamplin, The future of predictive microbiology: strategic research, innovative applications and great expectations, Int. J. Food Microbiol. 128 (1) (2008) 2–9, https://doi.org/10.1016/j.ijfoodmicro.2008.06.026. ISSN 0168-1605.
[8] Brian Austin, The bacterial microflora of fish, revised, Sci. World J. 6 (2006) 931–945, https://doi.org/10.1100/tsw.2006.181.
[9] B.G. Shaw, J.M. Shewan, Psychrophilic spoilage bacteria of fish, J. Appl. Bacteriol. 31 (1) (1968) 89–96, https://doi.org/10.1111/j.1365-2672.1968.tb00344.x.
[10] Commission Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, Official Journal L031 (2002). https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32002R0178&from=IT. (Accessed 2 April 2023).
[11] A. Silbande, S. Adenet, J. Smith-Ravin, J.J. Joffraud, K. Rochefort, F. Leroi, Quality assessment of ice-stored tropical yellowfin tuna (*Thunnus albacares*) and influence of vacuum and modified atmosphere packaging, Food Microbiol. 60 (2016) 62–72, https://doi.org/10.1016/j.fm.2016.06.016.
[12] N. Ben Mhenni, G. Alberghini, V. Giaccone, A. Truant, P. Catellani, Prevalence and antibiotic resistance phenotypes of *Pseudomonas* spp. in fresh fish fillets, Foods 12 (5) (2023) 1–12, https://doi.org/10.3390/foods12050950.
[13] D. Taliadourou, V. Papadopoulos, E. Domvridou, I.N. Savvaidis, M.G. Kontominas, Microbiological, chemical and sensory changes of whole and filleted Mediterranean aquacultured sea bass (*Dicentrarchus labrax*) stored in ice, J. Sci. Food Agric. 83 (13) (2003) 1373–1379, https://doi.org/10.1002/jsfa.1553.

[14] Commission Regulation (EC) No 2073/2005 of 15 November 2005 on Microbiological Criteria for Foodstuffs, Official Journal of the European Union L338 (22 December 2005). https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R2073from=EN. (Accessed 19 June 2023).
[15] L. Lanni, V. Morena, A.S. Marchese, G. Destro, M. Ferioli, P. Catellani, V. Giaccone, Challenge test as special tool to estimate the dynamic of *Listeria monocytogenes* and other foodborne pathogens, Foods 11 (1) (2022) 1–22, https://doi.org/10.3390/foods11010032.
[16] ISO 20976-1, Microbiology of the food chain – Requirements and guidelines for conducting challenge tests of food and feed products. Challenge tests to study growth potential, lag time and maximum growth rate, ICS: 07.100.30 (2019).
[17] ANSES "EURL Lm Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to L. monocytogenes, version 4 of 1 July 2021" Retrieved from: https://eurl-listeria.anses.fr/en/minisite/listeria-monocytogenes/mandate Accessed 19 June, 2023.
[18] EN SANCO/1628/2008 ver. 9.3 (26112008), Commission Staff Working Document Guidance Document on *Listeria Monocytogenes* Shelf-Life Studies for Ready-To-Eat Foods, under Regulation (EC) No 2073/2005 of 15 November 2005 on Microbiological Criteria for Foodstuffs, EU Commission, Brussels, Belgium, 2008. https://www.fsai.ie/uploadedFiles/EU_Guidance_listeria_monocytogenes.pdf. (Accessed 2 April 2023).
[19] M. Chandrasekaran, P. Lakshmanaperumalsamy, D. Chandramohan, Fish flesh agar medium - a suitable experimental medium for the detection of spoilage bacteria, Antonie Leeuwenhoek 51 (2) (1985) 219–225, https://doi.org/10.1007/BF02310014.
[20] M. Duman, M. Mulet, S. Altun, I.B. Saticioglu, B. Ozdemir, N. Ajmi, J. Lalucat, E. García-Valdés, The diversity of *Pseudomonas* species isolated from fish farms in Turkey, Aquaculture 535 (2021), https://doi.org/10.1016/j.aquaculture.2021.736369.
[21] E. Heir, B. Moen, A.W. Åsli, M. Sunde, S. Langsrud, Antibiotic resistance and phylogeny of *Pseudomonas* spp. Isolated over three decades from chicken meat in the Norwegian food chain, Microorganisms 9 (2) (2021) 1–19, https://doi.org/10.3390/microorganisms9020207.
[22] X. Li, N. Gu, T.Y. Huang, F. Zhong, G. Peng, *Pseudomonas aeruginosa*: a typical biofilm forming pathogen and an emerging but underestimated pathogen in food processing, Front. Microbiol. 13 (2023), https://doi.org/10.3389/fmicb.2022.1114199. Frontiers Media S.A.
[23] M. Åsli, M. Rødbotten, G. Vogt, S. Afanasyev, T. Mørkøre, Quality assessment of lightly salted atlantic salmon fillets injected with brine solutions containing sodium bicarbonate, J. Food Res. 2 (3) (2013) 110, https://doi.org/10.5539/jfr.v2n3p110.
[24] R. Malcolm Love, The post-mortem ph of cod and haddock muscle and its seasonal variation, J. Sci. Food Agric. 30 (4) (1979) 433–438, https://doi.org/10.1002/jsfa.2740300414.
[25] S. Hilau, S. Katz, T. Wasserman, R. Hershberg, Y. Savir, Density-dependent effects are the main determinants of variation in growth dynamics between closely related bacterial strains, PLoS Comput. Biol. 18 (10) (2022), https://doi.org/10.1371/journal.pcbi.1010565.
[26] C. Sekse, J. Bohlin, E. Skjerve, G.E. Vegarud, Growth comparison of several *Escherichia coli* strains exposed to various concentrations of lactoferrin using linear spline regression, Microb. Inf. Exp. 2 (1) (2012), https://doi.org/10.1186/2042-5783-2-5.
[27] K. Dong, H. Pan, D. Yang, L. Rao, L. Zhao, Y. Wang, X. Liao, Induction, detection, formation, and resuscitation of viable but non-culturable state microorganisms, Compr. Rev. Food Sci. Food Saf. 19 (1) (2020) 149–183, https://doi.org/10.1111/1541-4337.12513.
[28] M. MacDonell, R. Colwell, Phylogeny of the vibrionaceae, and recommendation for two new genera, *listonella* and *shewanella*, Syst. Appl. Microbiol. 6 (2) (1985) 171–182, https://doi.org/10.1016/s0723-2020(85)80051-5.
[29] J.D. Oliver, Recent findings on the viable but nonculturable state in pathogenic bacteria, 2010 Jul, FEMS Microbiol. Rev. 34 (4) (2010) 415–425, https://doi.org/10.1111/j.1574-6976.2009.00200.x. Epub 2009 Nov 24. PMID: 20059548.
[30] G. Tantillo, A. Giuffrida, A. Guidi, G. Manfreda, E. Novelli, 29th national congress of the Italian association of veterinary food hygienists (AIVI) | bari, 11-13 september 2019, Italian Journal of Food Safety (2019), https://doi.org/10.4081/ijfs.2019.8553.
[31] R. La Pira, Fa troppo caldo nei frigoriferi. La temperatura media è di 7,4°C: bisogna rivedere la scadenza dei prodotti, Il fatto alimentare (4 November 2022), 2022, https://ilfattoalimentare.it/frigoriferi-temperatura-data-scadenza-prodotti.html. (Accessed 8 March 2024).
[32] ISO 6887-1:2017 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions. ICS: 07.100.30.
[33] ISO 7218:2007/Amd 1:2013 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations — Amendment 1. ICS: 07.100.30.
[34] ISO 4833-1, Microbiology of the Food Chain — Horizontal Method for the Enumeration of Microorganisms — Part 1: Colony Count at 30 °C by the Pour Plate Technique, 2022. ICS: 07.100.30.
[35] A. Bekker, L. Steyn, G. Charimba, P. Jooste, C. Hugo, Comparison of the growth kinetics and proteolytic activities of chryseobacterium species and *pseudomonas fluorescens*, Can. J. Microbiol. 61 (12) (2015) 977–982, https://doi.org/10.1139/cjm-2015-0236.
[36] Z. Baña, N. Abad, A. Uranga, I. Azúa, I. Artolozaga, M. Unanue, J. Iriberri, J.M. Arrieta, B. Ayo, Recurrent seasonal changes in bacterial growth efficiency, metabolism and community composition in coastal waters, Jan, Environ. Microbiol. 22 (1) (2020) 369–380, https://doi.org/10.1111/1462-2920.14853. Epub 2019 Nov 24. PMID: 31713276.
[37] Commission Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs, Official Journal L131/1 (30 April 2004). https://eur-lex.europa.eu/legal-content/it/TXT/?uri=CELEX:32004R0852. (Accessed 19 June 2023).
[38] Commission Regulation (EC) No 853/2004 of the european parliament and of the council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs, Official Journal L 139/55 (30 April 2004). https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32004R0853&from=it. (Accessed 19 June 2023).