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Antimicrobial Blue Light (aBL) as a potential tool to reduce bacterial spoilage in the fishery chain

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ABSTRACT

Along the fishery chain, a high amount of fish is lost for the activity of spoilage microorganisms originating from the environment, human handling and fish themselves. Different techniques are conventionally used to reduce the growth of bacteria: from cold temperature and icing to high concentration of salts, from drying to natural antimicrobial compounds. In this study, the antimicrobial Blue Light (aBL) was considered as an innovative tool. In particular, the irradiation with light at 410 nm inhibited the growth of most bacteria isolated from skin samples of anchovies and sardines chosen for their worldwide commercial importance. Bacterial strains showed a different sensitivity to light treatment: the ones isolated from anchovy were more sensitive than those from sardine. Investigations were performed on *Aeromonas bestiarum*, an emerging foodborne pathogen. Upon irradiation with light at 410 nm (200 J/cm^2), a statistically significant decrease of 3 log units was observed. The same fluence rate successfully inhibited the biofilm formation of *A. bestiarum*, and disrupted 50 % of the adherent biomass of a 24-h old biofilm. The irradiation of *Staphyococcus vitulinus* compromised its viability and the associated proteolytic activity known to contribute to meat spoilage. In vivo experiments showed that aBL caused a remarkable decrease (at least 50 %) of viable counts of bacteria from anchovy and sardine skin samples conserved at 4 °C for one day. In conclusion, these results support the potential use of blue light in reducing the growth of skin microorganisms potentially responsible for loss of food safety, quality and decrease of storage life.

1. Introduction

Over the past 70 years, fishery and aquaculture sectors have acquired an ever-increasing role in the global economy and food supply (FAO, 2022). In fact, aquatic foods offer highly accessible and affordable sources of animal proteins and micronutrients, playing a vital role in the food and nutrition security of many, particularly vulnerable, coastal populations (Hicks et al., 2019; Kawarazuka & Béné, 2010). Unfortunately, production and distribution of aquatic foods are not without problems. There are issues related to harvesting, such as overfishing and habitat degradation (FAO, 2022), as well as issues related to the food safety of the products that reach our tables (Arthur, 2020; Bennett et al., 2021). In this light, along the food distribution chain of fishery industry, up to 25 % of losses can be attributed to microbial growth and related enzymatic activities (Carballo et al., 2020). Recent studies highlighted the role of specific spoilage organisms (SSOs) in seafood spoilage, such as *Shewanella putrefaciens*, *Pseudomonas* spp. and *Photobacterium phosphoreum*. Microbial metabolism results in the production of amines, sulphides, alcohols, aldehydes, ketones, and organic acids with unpleasant and undesirable flavours (Gram & Dalgaard, 2002). In addition to the economic impact, bacterial contaminations represent a risk for the health of consumers. Indeed, pathogenic microorganisms such as *Salmonella* spp. and *Escherichia coli* are transmitted through the consumption of contaminated or poorly preserved fish (Sheng & Wang, 2020). Even a fast-growing industry such as aquaculture suffers the negative impact of bacteriological contaminations with relevant economic losses (Roh, Kim, Kang, & Kim, 2016).

Along the fishery chain, the control of microbial contaminants is obtained through physical and/or chemical approaches. In some countries, dehydration processes are used to preserve freshwater and saltwater fish using hot air or solar dryers. However, mesophilic and coliform bacteria grow within the drying process, especially in solar

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drying operations (Kumar Natarajan, Elangovan, Madurai Elavarasan, Balaraman, & Sundaram, 2021). Salting and cold-smoking processes may be combined to reduce contamination of fish such as salmon (Hwang, 2007). In refrigeration and freezing processes, fluctuations of temperatures can cause chemical and physical changes in muscle proteins with inherent loss of quality in terms of textures, flavours, odours and colours (Sotelo, Pifieiro, & P~rez-Marffn, 1995). Irradiation with ultraviolet light poses safety concerns for operators (Hessling, Spellerberg, & Hoenes, 2017), while disinfectants such as quaternary ammonium salts (QACs) showed to be carcinogenic (Ferk et al., 2007). In addition, traditional storage techniques often cause the loss of nutritional content and the alteration of flavour (Kontominas, Badeka, Kosma, & Nathanailides, 2021).

Economic and health interests are so noteworthy that several innovative preservation methods have been proposed to improve quality and safety of fish products: non-thermal atmospheric plasma (NTAP), pulsed electric fields (PEF), ultrasound, electrolysed water and pulsed light (Speranza et al., 2021). In this regard, in the field of visible light irradiation, recent studies demonstrated that blue-violet light has a great potential in the inactivation of many bacterial food contaminants and pathogens. The technique "aBL" (antimicrobial Blue Light) could lead to great benefits in the preservation of fish and, consequently, advantages for the quality of food as for the health of consumers (Orlandi, Bolognese, Trivellin, Ricci, & Carlucci, 2021; Roh et al., 2016).

A photooxidative stress seems underlying the toxicity induced by blue light, but further investigations are needed to understand these mechanisms. In bacteria, compounds with physiological roles such as porphyrins (protoporphyrin IX, coproporphyrin I, coproporphyrin III), flavins (flavin adenine dinucleotide, nicotinamide adenine dinucleotide) and others could play the role of endogenous photosensitizers (PSs). In aerobic atmosphere, their excitation by light at a specific wavelength induces the arising of singlet oxygen and/or reactive oxygen species (ROS) that cause cellular damage to both Gram-negative and Gram-positive bacteria, as well as yeasts, fungi and parasites (Wang et al., 2016, 2017). Hessling et al. (Hessling et al., 2017) highlighted that the antimicrobial activity of blue light is influenced by several environmental parameters such as oxygen concentration, pH and temperature. In particular, the presence of oxygen appears to be a key element for the production of ROS in response to irradiation. Furthermore, it is necessary to increase the dose of light in proportion to the concentration of microorganisms to photoinactivate (Hessling et al., 2017). In literature, exponential phase bacteria seem more photo-sensitive than those in the stationary phase (Abana et al., 2017), as a possible consequence of a higher production of putative endogenous photosensitizers. In addition, Gad reported that the logarithmic phase cells were more sensitive to photoinactivation by exogenous photosensitizers (Gad, Zahra, Hasan, & Hamblin, 2004). Some microbial strains among Gram-positive and Gram-negative bacteria together with mycobacteria, yeasts, fungi and dermatophytes showed high sensitivity to this treatment (Wang et al., 2017). In addition, the radiometric parameters have to be considered: pulsed light showed to be more effective than continuous emission (Murdoch, Maclean, Endarko, Macgregor, & Anderson, 2012). In addition, Guffey & Wilborn observed that low fluence rates for longer irradiation times seem to be more bactericidal than high fluence rates for short irradiation times (Guffey & Wilborn, 2006), while remarkable anti-sporal and antifungal effects of ultra-high irradiance (UHI) with blue light for short period treatments were recently reported (Lang et al., 2022; Thery, Beney, Grangeteau, & Dupont, 2023).

In this study, the efficiency of light at 410 nm was evaluated for the treatment of European anchovy (*Engraulis encrasicolus*) and sardine (*Sardina pilchardus*) in different steps of the fishery chain. The antimicrobial activity was assayed on samples soon after fishing, or after storage at + 4 °C or – 20 °C for one day to simulate different post-harvest conditions. Preliminary experiments on bacterial biofilms were also included.

2. Materials and methods

2.1. Light source

Irradiation of samples of *Sardina pilchardus*, *Engraulis encrasicolus* or isolated bacteria was performed with LULab light source (University of Padua) that allows a uniform irradiation of a square area of (75 × 75) mm^2 with a head composed by 25 high power LEDs. Each LED is equipped with a specific optic to get the maximum collimation of the light on the target. The device is powered by a specific software-based control system which allows the setting of irradiation times and irradiance values to get the desired fluence rates. In all experiments an irradiance value of 100 mW/cm^2 has been used. Irradiation times of 1, 2, 4, 8, 16, 33, 66 and 132 min were used to reach final fluence values of 6, 12, 25, 50, 100, 200, 400 and 800 J/cm^2, respectively. Since there is no thermal connection between the sample and the lamp, the thermal contribution of the LEDs lamp on the sample is therefore negligible (Martegani, Bolognese, Trivellin, & Orlandi, 2020).

2.2. Fish samples

In this study two fish species were considered: *Sardina pilchardus* and *Engraulis encrasicolus*. Depending on the experiments, fish samples were acquired from market or obtained directly from the fisher and were maintained at + 4 °C or – 20 °C, as detailed in the different experiments.

2.3. Isolation of bacterial strains and presumptive identification by 16S rRNA sequence analysis

LB Agar plates were striked with swabs from single skin specimens of *E. encrasicolus* or *S. pilchardus* and incubated at 37 °C or at room temperature for at least 24 h. Pure cultures were obtained after sequential transfers of mixed cultures. Each isolate was inoculated into 5 ml of LB and grown at a suitable temperature (37 °C or at room temperature for at least 24), centrifuged (4000×*g*, 10 min, room temperature) and, after supernatant removal, the pellet was suspended in 2 ml of LB/glycerol (1:1) for storage in cryogenic vials at - 80 °C.

The identification of bacterial species was achieved by 16S rDNA sequence analysis. Colony PCR was performed as follows: isolated colonies were suspended in 50 μl of water and a volume of 3 μl was added to the PCR reaction (50 μL final volume). The mix was composed as follows: 2 μL of 10 μM FW primer (5′-GAG TTT GAT CCT GGC TCA-3) and 2 μL of 10 μM RV primer (5′-ACG GCT AAC TTG TTA CGA CT-3′), 1 μL of 10 mM dNTPs mixture, 0.5 μL Taq polymerase (5U/uL) and 10 μL of 5X buffer. The primers amplify a DNA fragment of 1507 nucleotides containing all the variable regions of 16S DNA sequence (V1–V9). The thermal cycler was set for the following thermal cycling program: initial denaturation cycle at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 52 °C for 2 min, and 72 °C for 1.5 min, and a final extension step at 72 °C for 5 min. Four PCR reactions were performed for each sample and, after pooling, 10 μL of the collected reaction products were loaded on 1.5 % agarose gel for electrophoretic analysis. The amplified fragments were purified with Euroclone SPINNAker DNA clean up kit according to manufacturer instructions, eluted in H_2O and used for Sanger sequencing (sequencing reaction and chromatogram analyses were performed by Eurofins Genomics srl). A presumptive identification was performed through the alignment with representative sequences from the NCBI 16S BLAST database.

2.4. Microbial strains and culture conditions

Reference strain *P. aeruginosa* PAO1 (Stover et al., 2000) and isolated strains were grown in Luria Bertani (LB) on an orbital shaker at 200 rpm, or in LB agar (15 g/L agar) at 37 °C or at the suitable temperature, as described in the text.

2.5. Photo-spot assay

Photo-spot tests were optimized with *P. aeruginosa* PAO1 to evaluate the microbial photo-inactivation by blue light irradiation (Martegani et al., 2020). Bacterial isolates from *E. encrasicolus* and *S. pilchardus* were inoculated in LB medium and grown at suitable temperature with agitation for at least 24 h. Stationary phase bacterial cultures were 10-fold serially diluted (from 10^{-1} up 10^{-6}) in sterile phosphate buffer (KH_2PO_4/K_2HPO_4 100 mM pH 7.4) in 96 well microplates. Undiluted and diluted cell suspensions (~ 5 μL droplets) were replicated on LB Agar and irradiated under LED emitting at 410 nm (100 mW/cm^2), at increasing light doses (6, 12, 25, 50, 100, 200, 400, 800 J/cm^2). Control samples were incubated in the dark (0 J/cm^2). After irradiation or dark incubation, cells were incubated at 37 °C or at room temperature for at least 24 h. Results were recorded as presence or absence of bacterial growth (spots) upon irradiation at specific fluence rates.

2.6. Photoinactivation of suspended cells

Aeromonas bestiarum SP2, *Staphylococcus vitulinus* SP3, *Pseudomonas aeruginosa* PAO1 were inoculated in 20 ml of LB medium into 100 ml Erlenmeyer flasks and grown overnight at 37 °C with mild agitation. Upon centrifugation (10,000×*g* for 1 min at room temperature) of overnight cultures, supernatants were discarded. After double washing with 1 volume of phosphate buffer, cells were suspended into fresh phosphate buffer at a final concentration of 10^5 cfu/ml. Volumes of 1 ml were irradiated for 9 min, 17 min and 34 min at an irradiance rate of 100 mW/cm^2 into 12 well plates; untreated samples were always included as controls. Viable count was performed inoculating 10 μl droplets of sequential 10-fold dilutions on LB Agar. Experiments were performed at least in triplicate.

2.7. Photo-treatment of Aeromonas bestiarum biofilms

Aeromonas bestiarum was grown in LB medium at 37 °C with agitation (200 rpm). Overnight cultures were 50-fold diluted in 1 ml of fresh medium. Assays were performed in 12-well microplates.

2.7.1. Inhibition of biofilm formation

To evaluate the inhibition of biofilm formation, one 12-well microplate was kept in the dark as control and the other one was irradiated under light at 410 nm (100 mW/cm^2, 200 J/cm^2). Upon dark incubation or irradiation, cells were placed at 37 °C for 24 h to allow biofilm formation. To assess the amount of adherent biomass, crystal violet staining was performed as follows: the planktonic phase from single wells was removed and spectrophotometrically measured at 600 nm. The adherent biomass was gently washed with 100 mM phosphate buffer. Biofilms were stained with 1 ml of 0.1% W/V crystal violet solution for 20 min and, after double washing with deionized water, were dried overnight at room temperature. Spectrophotometric quantification of the adherent biomass was performed upon solubilisation of crystal violet with a 33% V/V acetic acid solution for 10 min. Samples were measured at 590 nm, upon suitable dilution.

2.7.2. Eradication of 24-h old biofilms

The potential of blue light in eradicating formed biofilms was evaluated on 24-h old biofilms of *A. bestiarum*. Overnight cultures were 50-fold diluted in fresh medium and placed into 12-well microplates. After growth at 37 °C for 24 h, planktonic phases were removed and 1 ml 100 mM phosphate buffer solution was added. Upon irradiation (200 J/cm^2) or dark incubation, the optical density of the adherent biomass was measured at 590 nm after crystal violet staining, and planktonic phase was measured as OD600. All experiments were repeated at least three times.

2.7.3. Confocal microscopy analysis

Confocal microscopy analyses have been performed only for biofilm inhibition assays. Experimental procedure is the following. *A. bestiarum* overnight cultures were diluted to 10^5 cfu/ml in fresh LB medium and a volume of 1 ml was inoculated in 12 multi-well microplates containing a glass coverslip. Samples were kept in the dark or irradiated (200 J/cm^2), and further incubated for 24 h at 37 °C. The planktonic phase was removed and adherent cells were stained with fluorochrome 4,4-difluoro-1,3,5,7-tetramethyl-8(2′methoxyphenyl)-4-bora-3a, 4a-diaza-*s*-indacene at 5 μM concentration (Berini et al., 2021; Sunahara, Urano, Kojima, & Nagano, 2007). To allow dye penetration, plates were incubated at 37 °C for 30 min; coverslips were gently washed with 100 mM phosphate buffer and transferred on microscope glass slides for image acquisition through a 63 X objective lens (Confocal light microscopy; Leica Microsystems, Wetzlar Germany).

2.8. Protease assay

Bacteria were grown overnight in LB medium at 37 °C with mild agitation, centrifuged (4000×*g* for 10 min at room temperature) and sequentially ten-fold diluted in 100 mM phosphate buffer. Samples of 10 μl of undiluted and diluted suspensions were inoculated on LB agar containing 5% W/V skimmed milk. Cells were dark incubated or irradiated under light at 410 nm (100 mW/cm^2) at increasing fluence rates (50, 100 and 200 J/cm2). After incubation at 37 °C for 24 h, bacterial growth was observed and relative protease activity was checked according to the size of clear haloes of casein degradation.

To evaluate the effect of blue light on protease activity, overnight cultures were centrifuged (10,000×*g* for 10 min at room temperature). Supernatants possibly containing proteases were collected and centrifuged two more times. Samples were added to 12-well microplates and irradiated under light at 410 nm or kept in the dark as controls. After 9 min, 17 min and 34 min of irradiation to reach fluence values of 50, 100 and 200 J/cm2, respectively, protease activity was tested. To inhibit the growth of residual bacterial cells from supernatants, protease assays were performed on LB Agar containing 100 μg/ml Carbenicillin, 40 μg/ml Gentamycin and 5% W/V skimmed milk. A final volume of 100 μl (ten sequential droplets of 10 μl) of supernatants was spotted on LB milk and after incubation at 37 °C for 48 h, protease activity was checked. All the experiments were repeated at least three times.

2.9. In situ photoinactivation experiments

2.9.1. Irradiation of market fish

Excision of skin specimens close to gills were performed on fish placed on ice using disposable curette punches of 8 mm diameter (~ 50 mm2 area). Three sections were obtained from each single fish and three fish were used from single batches. Samples were dark incubated or irradiated with increasing fluences of light at 410 nm at 100 mW/cm^2 (50, 100, 200 J/cm^2). Irradiated and unirradiated samples were placed at + 4 °C or at - 20 °C for 24 h. Skin samples were placed in 2 ml tubes with 1 ml of sterile water and after stirring for 1 min, 100 μl of suspensions was spread on LB agar. After overnight incubation at room temperature, colonies were counted with OpenCFU Software (free open source).

2.9.2. Irradiation on boat after harvest

Samples of *E. encrasicolus* were placed on ice and kept in the dark or irradiated on boat under light at 410 nm (30 J/cm^2). Samples were stored at + 4 °C for 24 h before microbiological assays. Bacteria were collected from skin areas of ~ 1 cm^2 with cotton swabs, streaked on LB Agar and incubated overnight at 37 °C. Colonies were counted with OpenCFU Software.

Table 1
List of bacterial strains isolated from *E. encrasicolus* and *S. pilchardus*.

Engraulis encrasicolus[a]		*Sardina pilchardus*[b]	
Strain	Presumptive identification	Strain	Presumptive identification
	37°C		**37 °C**
EE1	*Aeromonas* sp	SP1	*Shewanella* sp
EE3	*Acinetobacter baumannii*	SP2	*Aeromonas bestiarum*
EE4A	*Wautersiella* sp	SP3	*Staphylococcus vitulinus*
	Room Temperature	SP4	*Staphylococcus saprophyticus*
EE2	*Enterobacter* sp	SP4ter	*Acinetobacter lwoffii*
EE4B1	Unidentified	SP5	*Psychrobacter* sp
EE4B2	Unidentified	SP6	*Macrococcus caseolyticus*
		SP8	*Bacillus megaterium*
			Room Temperature
		SP9	*Shewanella baltica*
		SP10	*Brochothrix thermosphacta*

The presumptive taxonomic identification, at genus or species level, has been performed by analysis of 16S rRNA sequence.
[a] Homology ≥68 % (~ 450 nucleotides).
[b] Homology ≥96 % (~ 800 nucleotides).

2.10. Statistical analyses

Statistical analyses were assessed by one-way ANOVA in conjunction with Tukey post hoc test.

3. Results and discussion

3.1. Isolation of bacterial strains from anchovy and sardine skin samples

In the fishery field, blue light irradiation represents an innovative antimicrobial approach that could be combined with commonly used techniques to improve food preservation. The investigation of sensitivity to blue light of bacteria originating from fish represents the first step of this study. To this aim, the cultivable component of skin microbiota from samples of *E. encrasicolus* and *S. pilchardus* was considered. The choice of using anchovy and sardine as experimental models is due to the worldwide commercial importance of both species (FAO, 2022).

Since in this study the attention was focused on the mesophilic component of fish skin microbiota, bacteria were isolated after incubation at 37 °C or at room temperature. The first condition could favour the growth of microorganisms originating from human handling, while bacteria growing at room temperature could represent the signature of environmental and fish microbiota. In particular, from the skin of

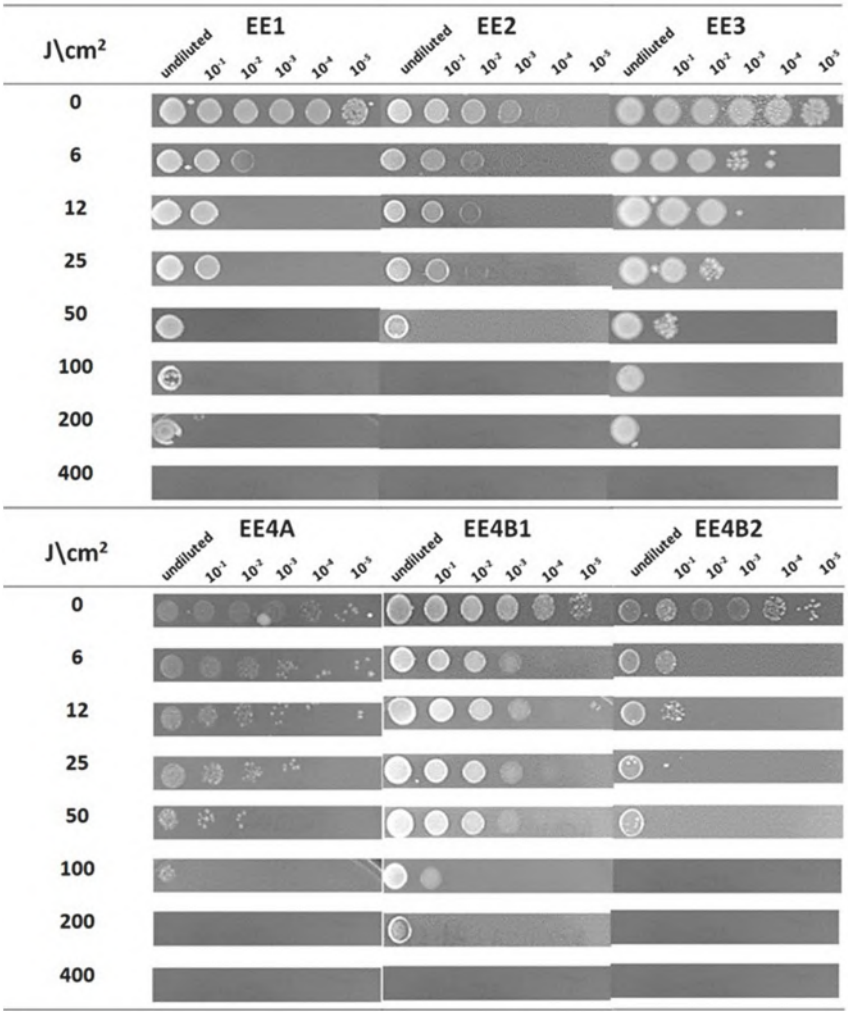

Fig. 1. Sensitivity to blue light irradiation of bacterial strains from *E. encrasicolus*.
Photo-spot tests performed on strains isolated from *E. encrasicolus*. Undiluted and ten-fold serially diluted stationary growth phase samples were inoculated on LB agar and irradiated under light at 410 nm (100 mW/cm^2) at increasing fluence rates (from 6 up to 400 J/cm^2). The corresponding growth spots were observed after overnight incubation at 37 °C.

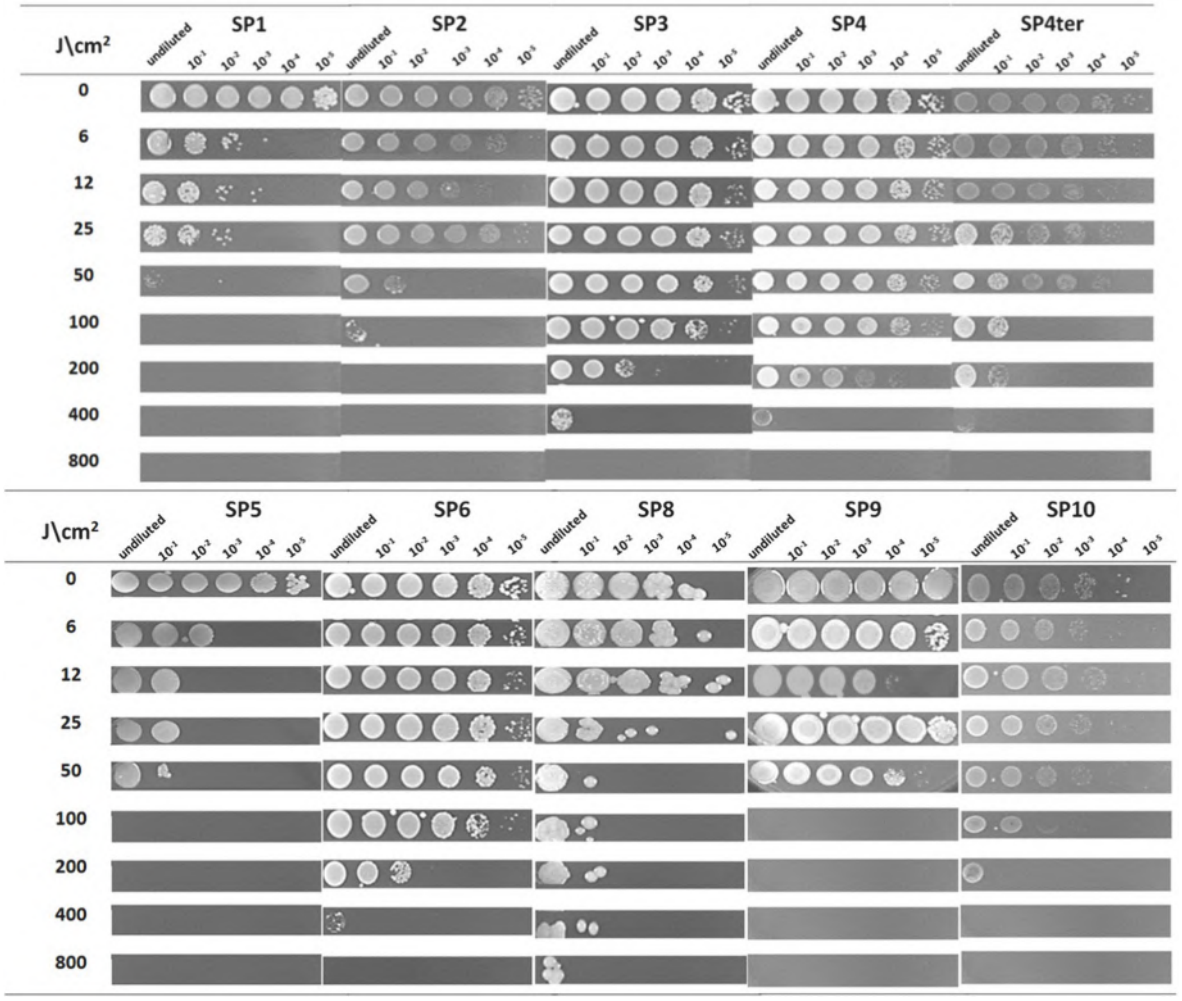

Fig. 2. Sensitivity to blue light irradiation of bacterial strains from *S. pilchardus*.
Photo-spot tests performed on strains isolated from *S. pilchardus*. Undiluted and ten-fold serially diluted stationary growth phase samples were inoculated on LB agar and irradiated under light at 410 nm (100 mW/cm^2) at increasing fluence rates (from 6 up 800 J/cm^2). The corresponding growth spots were observed after overnight incubation at 37 °C.

E. encrasicolus three isolates (EE1, EE3, EE4A) grew at 37 °C and three at room temperature (EE2, EE4B1, EE4B2), respectively. Strains isolated at 37 °C showed a certain degree of identity at 16S rDNA sequence level with Gram-negative species *Aeromonas* sp, *Acinetobacter baumannii* and *Wautersiella* sp. Among strains isolated at room temperature, one belongs to *Enterobacter* sp, while the other two could not be identified (Table 1).

In *S. pilchardus*, eight strains were isolated at 37 °C (SP1, SP2, SP3, SP4, SP4ter, SP5, SP6, SP8) and two at room temperature (SP9, SP10). As summarized in Table 1, five isolates were Gram-negative bacteria (*Shewanella* sp. and *Shewanella baltica*, *A. bestiarum*, *Acinetobacter lwoffii*, *Psychrobacter* sp.) and five Gram-positive bacteria (*Staphylococcus vitulinus*, *Staphylococcus saprophyticus*, *Macrococcus caseolyticus*, *Bacillus megaterium*, *Brochothrix thermosphacta*).

Among the identified microbial species, several are noteworthy for their involvement in compromising food safety and/or consumer health. For example, most bacteria belonging to *Aeromonas* spp are aquatic psychrophilic that colonize the skin of fish and are potentially pathogenic (Pessoa et al., 2022). In particular, *A. bestiarum* was reported to be the etiological agent of infections of carp, causing important economic damages (Kozińska, Figueras, Chacon, & Soler, 2002). Similarly, strains belonging to *Acinetobacter* spp are pathogenic not only for fish, but also for immunocompromised humans (Li et al., 2017). Furthermore, it has been reported that *Acinetobacter lwoffii*, isolated from frozen food, showed high tolerance to different antimicrobial treatments such as dehydration and irradiation (Jawad, Snelling, Heritage, & Hawkey, 1998). Strains belonging to *Wautersiella* spp were isolated from pike in China and identified as disseminators of resistance genes (Zhou et al., 2019). *Shewanella* spp, usually isolated from fish skin and adapted to extreme conditions such as low temperature and high hydrostatic pressure, is known to be involved in meat spoilage (Pekala, Kozińska, Paździor, & Glowacka, 2015). Similarly, *Brochothrix termosphacta* is a psychrophilic microorganism able to deteriorate meat in vacuum-sealed and frozen fish (Borch, Kant-Muermans, & Blixt, 1996). *S. saprophyticus* and *S. vitulinus* were reported as foodborne pathogens in fish and fish products (Fijałkowski, Peitler, & Karakulska, 2016; Santos de Sousa et al., 2017). Notably, *Psychrobacter* spp cause endocarditis and peritonitis in humans (Fondi et al., 2014), and species belonging to *Enterobacter* genus are well known as human pathogens, and only recently as pathogens in aquaculture (Salgueiro et al., 2020).

3.2. Screening of microbial sensitivity to light at 410 nm

To investigate the potential use of blue light as a method to decrease bacterial contaminants in fishery, a preliminary screening by photo-spot test was performed on the panel of Gram-positive and Gram-negative bacterial strains isolated from the skin of the two reference fish models. Bacteria were irradiated with increasing doses of light at 410

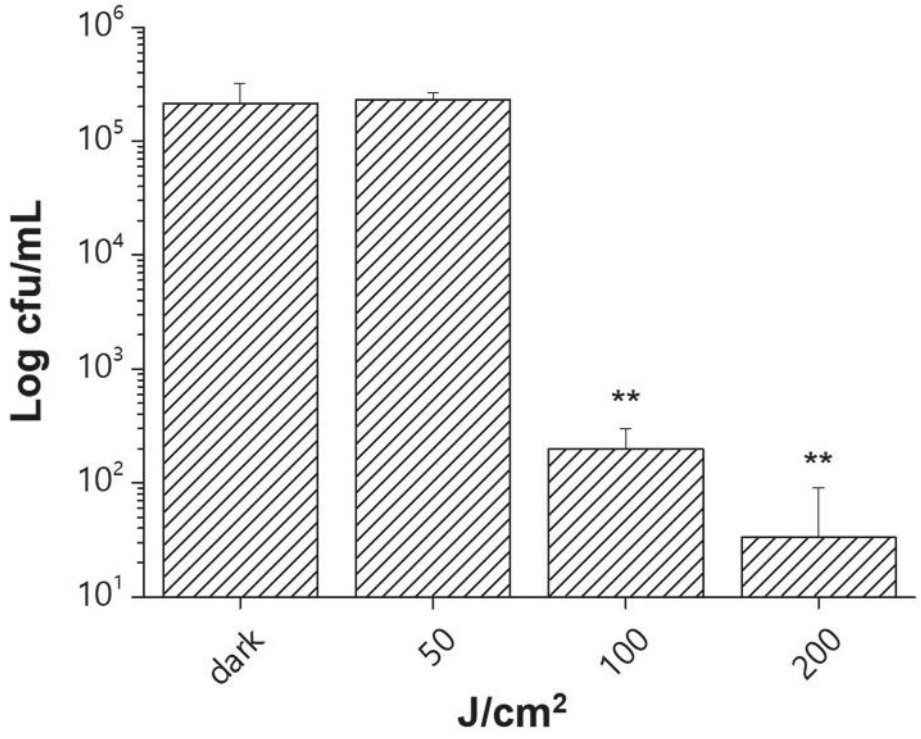

Fig. 3. Effect of aBL against *Aeromonas bestiarum*.
Effect of increasing doses of light at 410 nm on suspensions of *A. bestiarum* at 10^5 cfu/ml. Microbial viability was checked after 24 h at 37 °C; data were collected from three independent experiments. Anova analysis was performed to evaluate significant differences (**$p < 10^{-4}$).

nm (from 6 to 800 J/cm^2) and their relative growth was compared to that of corresponding unirradiated controls (Figs. 1 and 2).

Interestingly, a clear antimicrobial effect against all the tested strains was observed. In details, for anchovy isolates, the treatment with the lowest fluence rates (6 and 12 J/cm^2) was effective in preventing the formation of low-cell density spots. Fluences up to 200/400 J/cm^2 had to be used to inactivate samples with higher cell densities (Fig. 1). Most strains isolated from sardine were more tolerant if compared to those isolated from anchovy: higher fluences were necessary to prevent the growth of bacteria both at the lowest and the highest densities of most microbial samples (Fig. 2). Among the tested strains, the spore forming *Bacillus megaterium* (SP8) resulted the most photo-tolerant. This is in accordance with the work of Maclean showing that an exposure to 1730 J/cm^2 was necessary to achieve a significant reduction of viability in *B. cereus, B. subtilis, B. megaterium*, and *Clostridium difficile* (MacLean, Murdoch, MacGregor, & Anderson, 2013). Since endospores enable bacteria to resist to environmental stressors and drugs or disinfectants, their control in the fishery field represents an important goal.

This preliminary investigation shows that bacteria isolated from the skin of sardine and anchovy are photoinactivated by light at 410 nm in a light-dose, cell-density and strain dependent manner. Importantly, among them, are included *Shewanella* sp. and *Psychrobacter* sp., or faecal

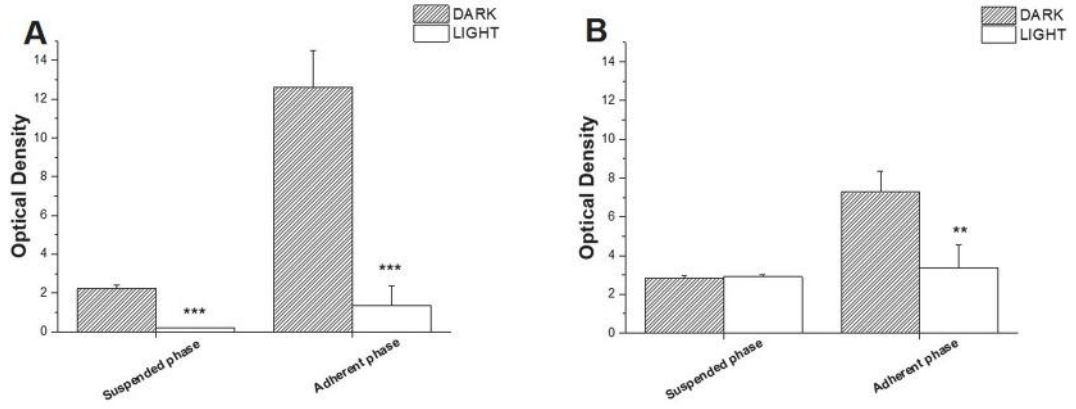

Fig. 4. Effect of blue light on bacterial biofilms.
Effect of aBL on inhibition of biofilm formation (A). Overnight cultures of *Aeromonas bestiarum* were 50-fold diluted in fresh LB medium. Bacteria were dark incubated or irradiated under light at 410 nm (100 mW/cm^2, 200 J/cm^2) and incubated at 37 °C for 24 h to allow biofilm formation. Suspended phases were spectrophotometrically measured (OD600) and adherent phases were quantified by crystal violet staining (OD590).
To evaluate biofilm eradication (B), planktonic phases of 24 h-old biofilms were collected and the adherent biomass was irradiated under light at 410 nm (200 J/cm^2). Adherent and suspended biomasses were evaluated as OD590 and OD600 measurements, respectively.
All experiments were independently repeated at least six times to perform statistical analysis by one-way ANOVA (**, $p < 10^{-4}$, ***$p < 10^{-9}$).

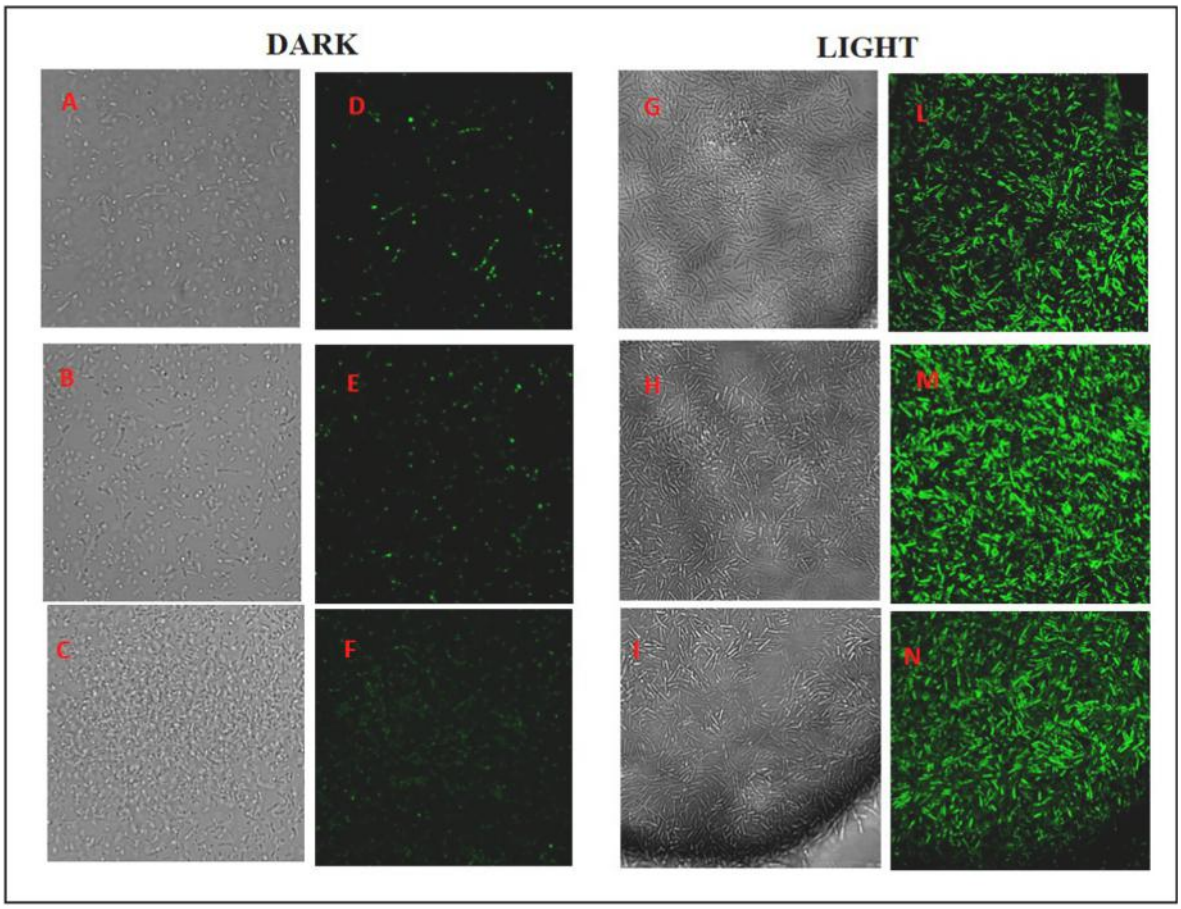

Fig. 5. Confocal analysis of *A. bestiarum* biofilms.
A. bestiarum was grown overnight in LB medium at 37 °C and diluted in the same fresh medium. Samples were kept in the dark or irradiated under light at 410 nm (100 mW/cm^2, 200 J/cm^2). Thereafter, bacteria were incubated at 37 °C for 24 h to allow biofilm formation. Samples were stained with fluorochrome 4,4-difluoro-1,3,5,7-tetramethyl-600 8(2′methoxyphenyl)-4-bora-3a, 4a-diaza-*s*-indacene at 5 μM concentration and images were acquired through a 63 X objective lens (SP5 Confocal light microscopy; Leica Microsystems, Wetzlar Germany). The unirradiated (panels A–F) and irradiated (panels G–N) samples are shown as fluorescent and bright field transmission images, respectively.

contaminants and opportunistic pathogens such as *Enterobacter* sp. that are known as specific spoilage organisms (SSO) (Gram & Dalgaard, 2002). These results are in accordance with previous investigations aimed at evaluating the response to light at 410 nm of the opportunistic pathogen *P. aeruginosa* PAO1 (Martegani et al., 2020).

3.3. Antimicrobial effects of light at 410 nm on Aeromonas bestiarum

Among SSOs, the importance of *Aeromonas* spp as an "emerging foodborne pathogen" is increasing (Hoel, Vadstein, & Jakobsen, 2019). *A. bestiarum* SP2 was isolated from *S. pilchardus* and *Aeromonas* EE1 from *E. encrasicolus*, respectively.

3.3.1. Effect of light on suspended cells

Photoinactivation experiments were performed on suspended cells of SP2 strain (Fig. 3). The killing rate was light-dose dependent: the two highest fluences (100 and 200 J/cm^2) caused a statistically significant decrease of 2 log units ($p < 2.21\ 10^{-5}$) and 3 log units ($p < 5.09\ 10^{-4}$), respectively, compared to the dark controls. Possible differences between irradiation in suspension or in solid phase could be ascribable to the concentration of oxygen that is one of the three actors playing in aBL (Wang et al., 2017).

3.3.2. Effect of light on biofilms

Virtually, all steps of food supply chains could be spoiled by metabolic activities of SSO, with inherent production of hydrolytic enzymes and toxins. A particular concern derives from the ability of bacteria to form organized communities, called biofilms, where microorganisms can grow within a self-produced extracellular matrix. They can give protection from environmental stress, and act as nutrient "suppliers" for pathogenic species such as *E. coli* (Sterniša, Klančnik, & Smole Možina, 2019). This causes the persistence of bacteria at low temperatures, low nutrient concentrations and in the presence of biocides (Lianou, Nychas, & Koutsoumanis, 2020). Biofilm lifestyle is often associated with those bacteria belonging to the genera of *Pseudomonas*, *Aeromonas*, and *Shewanella* as well as those belonging to the family of Enterobacteriaceae and Lactic acid bacteria (LAB), mainly considered as spoilage microorganisms (Gram & Dalgaard, 2002).

The anti-biofilm potential of blue light was investigated in *A. bestiarum*, well-known as a potential fish pathogen causing loss of meat quality (Hoel et al., 2019). Bacterial cells were irradiated (200 J/cm^2) and assayed for their ability to form biofilms in LB medium. Biofilm formation was spectrophotometrically evaluated after crystal violet staining. OD 590 values of unirradiated samples (12.60 ± 1.88) were compared to those obtained from irradiated ones (1.33 ± 1.04), and differences were shown to be statistically significant ($p < 10^{-9}$). A similar impairment ($p < 10^{-9}$) was observed for the planktonic populations (OD 600), ranging from 2.24 ± 0.18 for unirradiated samples to 0.015 ± 0.019 for irradiated ones (Fig. 4A).

As the eradication of formed biofilms is a very difficult goal to achieve, the effect of irradiation (200 J/cm^2) on 24 h-old biofilms of *A. bestiarum* was evaluated (Fig. 4B). In our irradiation experiments, we

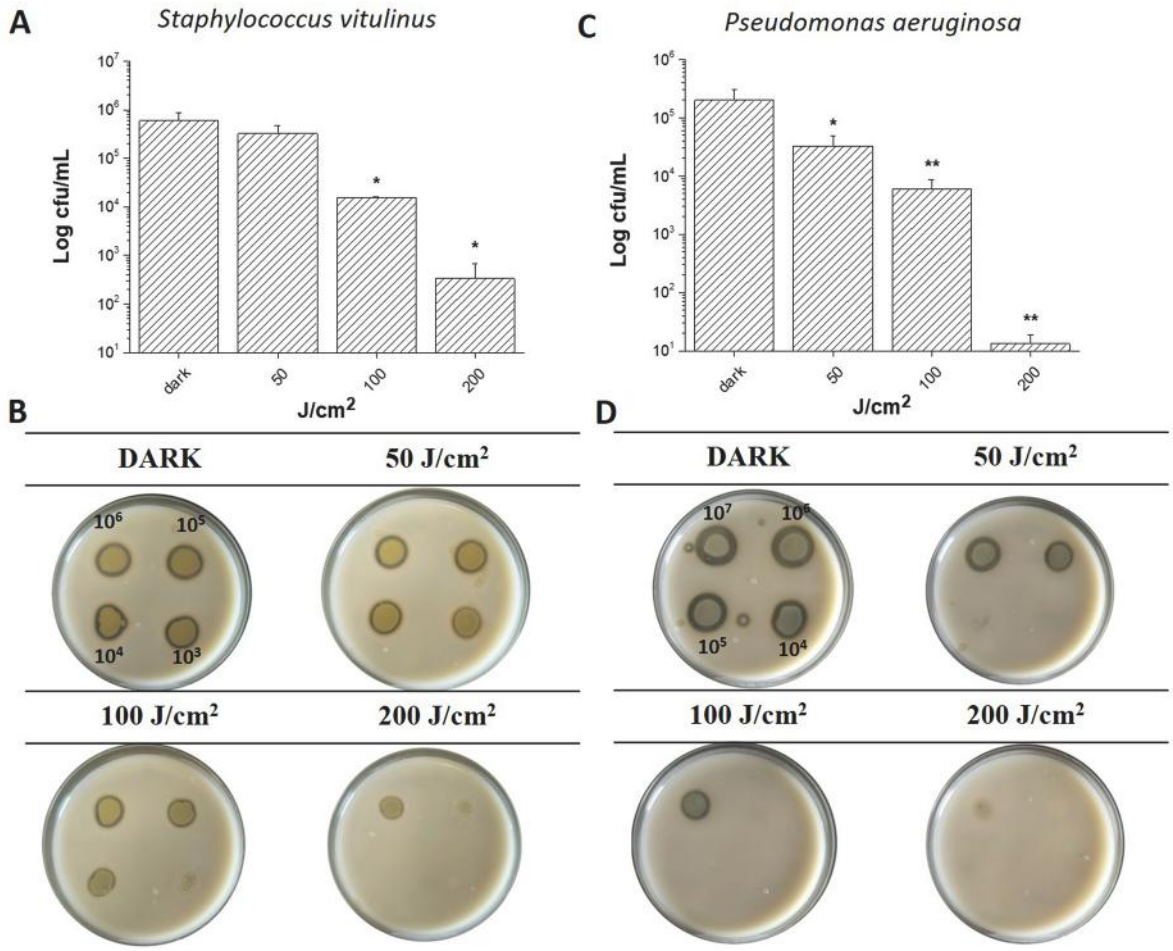

Fig. 6. Photoinactivation of *Staphylococcus vitulinus* and *Pseudomonas aeruginosa*: effect of blue light on proteolytic activity
aBL activity against *Staphylococcus vitulinus* (A) and *Pseudomonas aeruginosa* PAO1 (C). Effect of increasing doses of light at 410 nm on suspensions of *S. vitulinus* and *P. aeruginosa* PAO1 at 10^5 cfu/ml. Microbial viability was checked after 24 h incubation at 37 °C and the data collected at least from three independent experiments. Anova analysis was performed to evaluate the significant differences (*$p < 0.05$; **$p < 10^{-4}$).
Qualitative analysis of irradiation on proteolytic activities of *S vitulinus* (B) and *P. aeruginosa* PAO1 (D), respectively. Droplets of 10 μl of undiluted and ten-fold diluted samples were spotted on milk agar. For each strain, cellular densities are shown only for one plate. Upon dark incubation or irradiation at increasing light doses (50, 100 and 200 J/cm^2), cells were incubated at 37 °C. Reduction of growth spots and of proteolytic activity are observed.

did not observe any change of the planktonic biomass. This could be due to the detachment of cell clusters from sessile phases masking an antimicrobial effect on suspended cells. To support this hypothesis, deeper investigations are needed. Even if no effect was observed on planktonic phase, a statistically significant eradication of ~ 50 % of the adherent biomass was observed ($p < 3 \times 10^{-8}$). These data suggest a potential anti-biofilm activity of light at 410 nm against *A. bestiarum* as previously observed by authors in *P. aeruginosa* PAO1 (Martegani et al., 2020).

Confocal microscopy analyses were performed on *A. bestiarum* biofilms stained with a BODIPY fluorophore (Berini et al., 2021; Sunahara et al., 2007). Bacterial cells were inoculated on glass coverslips and irradiated or kept in the dark and grown in rich medium. After 24 h at 37 °C, the unirradiated samples (Fig. 5A–F) showed a lower number of fluorescent cells compared to the irradiated ones (Fig. 5G-N). Since the chosen fluorophore selectively stains the damaged cells, it could be inferred that blue light irradiation caused an important impairment of adherent cells. Furthermore, the observed bacterial cell elongation could be related to some photooxidative stress elicited by irradiation. Notably, this morphological change is often displayed by bacteria coping with external and internal stresses. For instance, in the model microorganism *E. coli*, it was shown that the oxidative stress inhibited the cell division machinery through the activity of oxyS and long rods were observed (Barshishat et al., 2018).

3.4. Blue light and bacterial proteolytic activity

Extracellular proteolytic enzymes produced by fish contaminants cause meat degradation, skin discoloration and bad smelling or even health concerns once ingested by consumers (Derome & Filteau, 2020; Tavares et al., 2021). In this scenario, the aBL could be possibly exploited to reduce the viability of bacteria that release degradative enzymes and/or directly impair enzymatic activity. Indeed, authors showed the sensitivity to light at 410 nm of purified recombinant *P. aeruginosa* PAO1 catalase A (Martegani et al., 2020).

In this study a proteolytic activity was detected only in *S. vitulinus*, a potential reservoir of virulence and resistance genes for other Staphylococci, such as *S. aureus* (Nemeghaire et al., 2014). Suspensions of *S. vitulinus* at 10^5 cfu/ml were irradiated at 410 nm at increasing light doses and the effect on cell viability and proteolytic activity was evaluated. A significant photoinactivation was obtained in a light dose-dependent manner (Fig. 6A). Decreasing amounts of bacterial cells (from 10^6 to 10^3) were inoculated on milk agar and treated with increasing fluence rates of light at 410 nm (Fig. 6B). In the dark control, a clear proteolytic activity was visible even at the lowest cellular concentration. For samples at 10^5 cfu/ml, irradiation at 50 J/cm^2 caused the disappearance of the proteolytic halo, while a fluence rate of at least 100 J/cm^2 inhibited even cell growth. For samples at 10^8 cfu/ml,

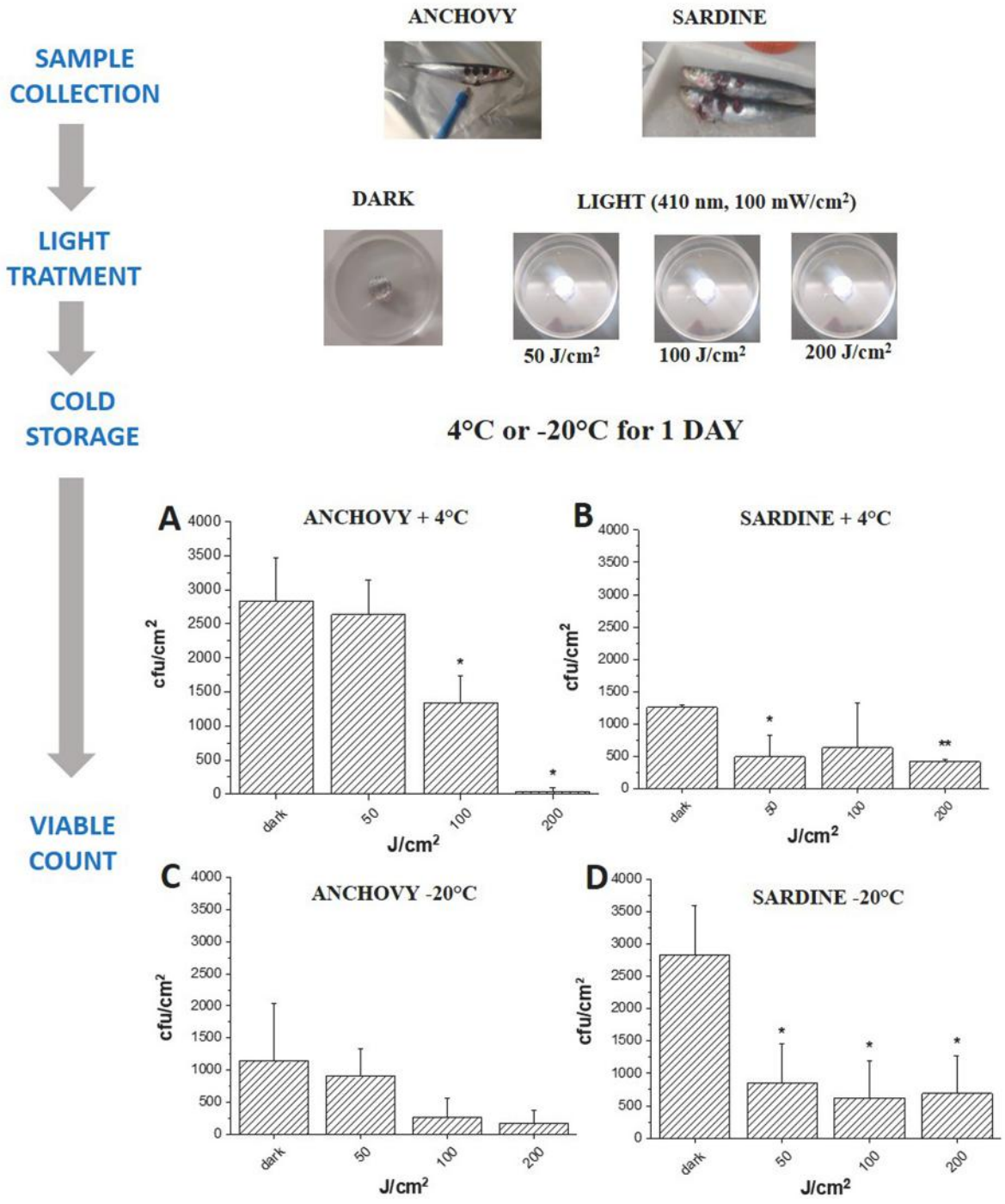

Fig. 7. Photoinactivation of mixed microbial populations from fish skin.
Experimental scheme of photoinactivation of microbial skin bacteria from anchovy and sardine. Comparable round skin sections with equivalent areas were dark incubated or irradiated at 410 nm at increasing light doses of 50, 100 and 200 J/cm^2 and stored at + 4 °C or at −20 °C for one day. After storage, samples were placed in 1 ml of water and stirred, and a suitable volume of bacterial suspension was streaked on LB agar, with subsequent overnight incubation at room temperature. The values of cfu/cm^2 calculated as media ±standard deviation from three samples for each treatment are represented in graph A, C (anchovy) and B, D (sardine). Anova analysis was performed to evaluate the significant differences between irradiated and dark incubated samples (*$p < 0.05$, **$p < 10^{-4}$).

irradiation with 200 J/cm^2 reduced cellular growth and a reduction of the proteolytic halo was clear. A further step in this investigation was to verify the effect of blue light irradiation on the activities of extracellular proteolytic enzymes produced by *S. vitulinus*. An initial attempt to verify their detectability in culture supernatants was unsuccessful (data not shown), while a clear proteolytic activity was observed in culture supernatants of the model strain *P. aeruginosa* PAO1 known for secreting exoproteases such as elastase A, elastase B, large protease, protease IV, alkaline protease, *Pseudomonas* small protease, MucD, and aminopeptidase, all damaging host tissues by degrading hundreds of proteins (Qin et al., 2022). For this reason, the effects of blue light were assayed on PAO1 cells and culture supernatants, respectively. In agreement with previous investigations, *P. aeruginosa* PAO1 was sensitive to irradiations at increasing light doses at 410 nm (Fig. 6C) (Martegani et al., 2020). As observed in *S. vitulinus*, the decrease of proteolytic halos was strictly related to the photoinactivation of cells in a light-dose dependent

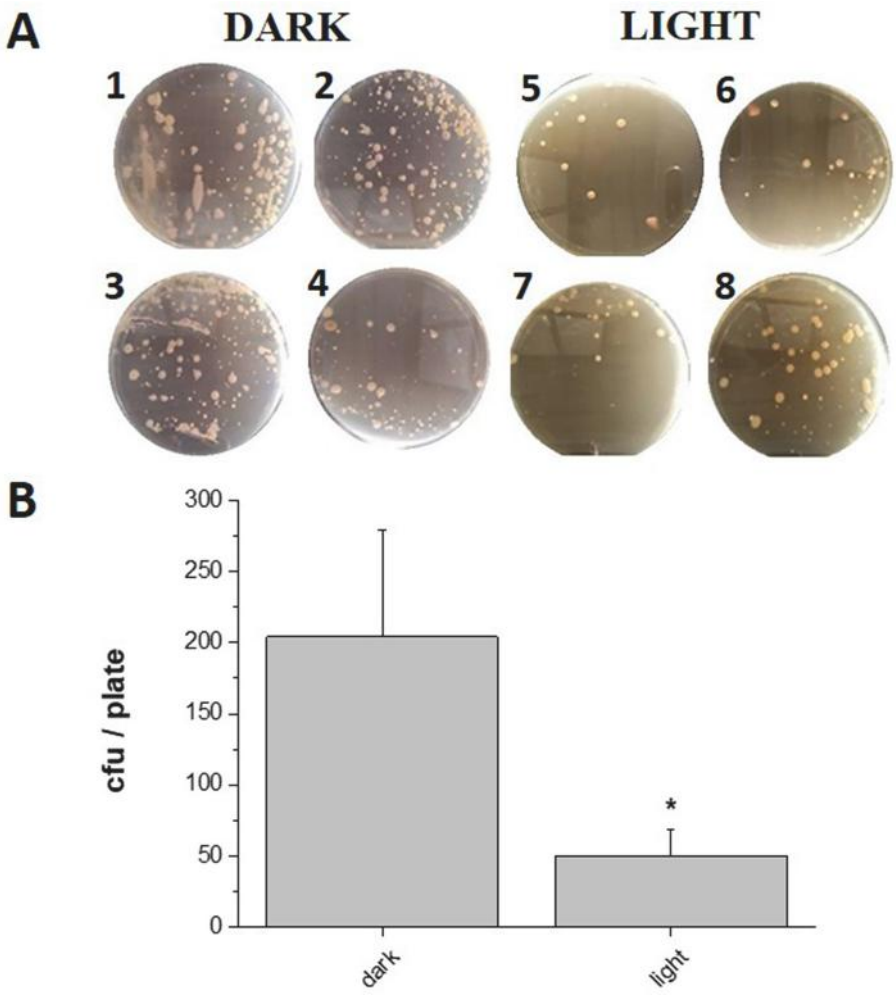

Fig. 8. In situ photoinactivation of skin microorganisms from *E. encrasicolus*.
Anchovies from the Mediterranean Sea (Ortona, Chieti, Italy) were harvested with the LED technology (emitting at 410 nm) to specifically attract phototactic pelagic fish. Samples were dark incubated or irradiated with light at 30 J/cm^2 and stored at + 4 °C for one day. Sterile swabs were used to collect microbiota and streaked on LB agar. In panel A, are shown results after overnight incubation at 37 °C: unirradiated samples (1–4) and irradiated ones (5–8).
Values of cfu/plate are represented in graph B. Anova analysis was performed to evaluate the significant differences between irradiated and dark incubated samples (*$p < 0.05$).

manner (Fig. 6D). When supernatants containing exoproteases were treated with increasing light doses (50, 100 and 200 J/cm^2), no reduction in proteolytic halos were observed (data not shown). The absence of activity of blue light on free proteolytic enzymes could suggest that those proteins lack a potential photosensitizing domain.

In fishery field applications, the impairment of proteolytic activity as a direct consequence of decreased bacterial viability upon irradiation at 410 nm could be remarkable both for food quality and for the health of consumers.

3.5. *In situ photoinactivation of fish skin microbiota*

After the evaluation of photosensitivity of isolated bacterial species, further experiments were performed on complex communities colonizing the skin of anchovy and sardine. The composition of resident microbiota could be affected by many factors such as host species, fish origin, environment, preservation treatments, human manipulation. To minimize the variability of results in microbial viability counting, the experiments on sardines or anchovies were performed on circular sections obtained from areas close to gills of a single fish and triplicates were obtained from fish of the same batch. After dark incubation or irradiation, skin specimens were kept at + 4 °C for 24 h. At the end of the storage, live bacteria were estimated by viable count technique. As summarized in Fig. 7 A, B, the irradiation reduced of at least ~ 50 % the number of colonies compared to the dark controls, suggesting a possible improvement of the antimicrobial effect of low temperature (4 °C).

A different outcome was obtained with samples stored for 1 day at -20 °C. In anchovy, where a lower number of colonies was observed in dark condition as respect to 4 °C, no significant effect of light treatment was observed (Fig. 7 C). In sardine, where a higher number of colonies was present in dark conditions compared to 4 °C, the photoinactivation displayed a significant antimicrobial effect (Fig. 7 D).

Since LED technology (emitting at 410 nm) has been successfully exploited in selective catch of phototactic pelagic species (Ricci et al., 2021), its potential as an antimicrobial tool in the fishery chain deserved much attention. Soon after harvest, anchovies from the Mediterranean Sea (Ortona, Chieti, Italy) were irradiated under LED at 410 nm or kept in the dark and stored at 4 °C for one day before microbiological analyses. As shown in Fig. 8 A, B, irradiation (30 J/cm^2) reduced significantly ($p = 6.78 \times 10^{-4}$) the number of colonies, supporting the antimicrobial potential of blue light irradiation in a model of short fishery chain. In addition, a taxonomical characterization was performed to deeply investigate the effect of light on microbial diversity. Bacterial strains isolated from unirradiated and irradiated anchovies are

Table 2
List of presumptive genus or bacterial species associated to isolates obtained from *E. encrasicolus* [a].

Strains isolated from unirradiated fish	Strains isolated from irradiated fish
Acinetobacter baumannii	*Acinetobacter septicus*
Microbacterium oxydans	*Pantoea agglomerans*
Micrococcus yunnanensis	*Staphylococcus hominis*
Roseomonas cervicalis	*Arthrobacter* sp
Staphylococcus hominis	*Bacillus pumilus*
Acinetobacter calcoaceticus	*Rothia terrae*
Micrococcus sp	

[a] The presumptive taxonomic identification, at genus or species level, has been performed by analysis of 16S rRNA sequence (homology 71–94 %).

summarized in Table 2.

Analyses of 16S rDNA sequences showed an identity with *Acinetobacter* spp, *Staphylococcus* spp and *Bacillus* spp and with strains often isolated from soil, plants and water such as *Rothia terrae* (Z.-X. Liu et al., 2013), *Arthrobacter* spp (Chen, Xiao, Wang, Ae, & Wang, 2005), *Pantoea agglomerans* (Gutiérrez-Barranquero, Cazorla, Torés, & De Vicente, 2019), *Micrococcus* spp (Kumar, Fulekar, Hiranmai, Kumar, & Kumar, 2020) *Microbacterium oxydans* (Kim et al., 2013). It is noteworthy that *S. hominis*, probably acquired through human manipulation, has been reported as natural probiotic of human skin with strong and broad activity against other Gram-positive pathogens (Y. Liu et al., 2020). In particular, strains belonging to *Staphylococcus* spp and *Acinetobacter* spp were detected in both untreated and treated samples. This could be in agreement with their high photo-tolerance displayed in photo-spot tests (Fig. 2).

3.6. Conclusions

The results of this study support the potential use of antimicrobial Blue Light in the fishery field to control the growth of spoilage microorganisms belonging to fish skin microbiota. Most bacterial strains isolated from *E. encrasicolus* and *S. pilchardus* showed to be sensitive to light at 410 nm in a light dose-dependent manner. This technique was also successful in inhibiting the formation of biofilms of *A. bestiarum,* and reducing adherent cells of formed biofilms. Importantly, bacteria that release hydrolytic enzymes such as proteases involved in meat spoilage were sensitive to light treatment.

The combination of blue light irradiation with traditional conservation methods, such as icing, could reduce bacterial contamination at different steps of the supply chain. This technology could be applied soon after fishing, as shown in purse seine (PS), at working bench of fish shop, as in-home refrigerators. Deeper analyses should investigate if sublethal doses of blue light could select tolerant variants in order to optimize the radiometric parameters aimed at enhancing food quality and extending the shelf-life of fish. Accordingly, also the effect of irradiance values should be included in future investigations.

Therefore, the dual objective of blue light in PS to attract fish and slow down their decay could represent an optimized integrated strategy, with the aim of improving the food security and quality of the fishery products, as well as the sustainability of the entire supply chain. In addition, as tested in this study, the aBL treatment in the post-harvesting phase would be very important for small pelagics, which are very sensitive to deterioration, often the target of pest parasites, dangerous to human health, e.g., *Anisakis pegreffii*, (Cipriani et al., 2016; "Scientific Opinion on Risk Assessment of Parasites in Fishery Products," 2010). In particular, the climate change could affect temperature, which is an important factor in the post-harvest conservation (Getu, Misganaw, & Bazezew, 2015). Therefore, future studies should be addressed to explore the potential relationships between the performances of the aBL treatment and the environmental temperatures along the fish supply chain.

CRediT authorship contribution statement

Viviana T. Orlandi: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Fabrizio Bolognese:** Writing – review & editing, Methodology, Investigation. **Nicola Trivellin:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Giulia Cipriano:** Writing – review & editing, Investigation. **Pasquale Ricci:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Roberto Carlucci:** Writing – review & editing, Project administration, Investigation, Funding acquisition.

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.

Data availability

Data will be made available on request.

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