Food Control 164 (2024) 110614

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Comparative analysis of commercial cleaning and disinfection formulations and protocols for effective eradication of biofilms formed by a *Pseudomonas fluorescens* strain isolated from a poultry meat plant

Natalia Merino, Carlota García-Castillo, Daniel Berdejo, Elisa Pagán, Diego García-Gonzalo, Rafael Pagán *

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

ARTICLE INFO

Keywords:
Biocides
Detergents
Biofilms
Pseudomonas
Cleaning and disinfection
Aerosolization

ABSTRACT

Pseudomonas spp. is the main genus of spoilage bacteria for meat stored under aerobic chilling conditions. Given their ability to adapt to various environmental conditions and to form biofilms, they are common among residential microbiota in the food industry. Biofilms are complex, structured microbial communities encased in a self-produced exopolysaccharide matrix. Resident biofilm bacteria show increased resistance against disinfectants and dynamic or hostile environments, and are therefore a persistent source of food contamination. Hence, there is a need to design and implement control strategies that are effective against biofilms in the food industry. In this study, we tested a series of commercial cleaning and disinfection agents applied by immersion or aerosolization against robust biofilms of a *Pseudomonas fluorescens* strain isolated from a poultry meat plant by comparing the sessile cell counts before and after the procedure. The single application of a) an alkaline or an enzymatic detergent, b) a disinfectant based on peracetic acid and hydrogen peroxide, or c) a detergent-disinfectant based on tertiary alkylamines or sodium hypochlorite, failed to completely eradicate the biofilm. However, two-step cleaning and disinfection procedures involving the application of a detergent (either alkaline or enzymatic at 50 °C) followed by the application of the disinfectant based on peracetic acid and hydrogen peroxide at the manufacturer's recommended concentrations did result in the complete eradication ($p \leq 0.05$) of the robust biofilm. In addition, cleaning and disinfection procedures applied by aerosolization showed a notable efficacy ($p \leq 0.05$) against surface-dried cells and sessile cells of *P. fluorescens*. Therefore, this approach could be regarded as a potentially viable alternative to commonly applied immersion techniques.

1. Introduction

Pseudomonas spp. is the main genus of spoilage bacteria of food stored under chilling aerobic conditions, including meat, seafood, and dairy products (Odeyemi et al., 2020). The spoilage potential of *Pseudomonas* spp. relies on their ability to produce extracellular enzymes that degrade nutrients, resulting in organoleptic alterations of food (Cousin et al., 2001). For example, slime forms on meat and off-odors develop when glucose and lactate are depleted and *Pseudomonas* spp. begin to metabolize nitrogenous compounds, such as amino acids (Nychas et al., 2008).

Pseudomonas spp. occur ubiquitously, as they have the capacity to adapt to a wide variety of environmental conditions. Since they can be found in soil, water, plant surfaces, as well as an array of raw materials associated with the food continuum, these bacteria have many routes that allow access to food processing environments. Additionally, the ability of pseudomonads to grow at low temperatures and sparse nutrient levels, combined with their ability to form biofilms, would explain their establishment as one of the important residential bacteria found in the food industry (Møretrø & Langsrud, 2017).

Biofilms are complex, structured microbial communities encased in a self-produced exopolysaccharide (EPS) matrix that confers protection to the cells inside the biofilm (sessile cells). Compared to their planktonic counterparts, these sessile cells are thus more resistant to disinfectants

* Corresponding author. Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), C/ Miguel Servet 177, 50013, Zaragoza, Spain.

E-mail address: pagan@unizar.es (R. Pagán).

https://doi.org/10.1016/j.foodcont.2024.110614
Received 28 February 2024; Received in revised form 21 May 2024; Accepted 29 May 2024
Available online 30 May 2024
0956-7135/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

and the stresses imposed by dynamic or hostile environments (López et al., 2010). Food processing lines provide an ideal environment for biofilm formation due to their complexity, their lengthy production cycles, the vast areas they provide for microbial growth, and the availability of nutrients (Lindsay & von Holy, 2006; Yuan et al., 2021). Biofilms formed on food contact surfaces may create a persistent source of food contamination, leading to economic losses due to food spoilage and to serious hygienic problems when pathogenic microorganisms are present in the biofilm (Sofos & Geornaras, 2010). It is noteworthy that biofilms produced by *Pseudomonas* spp. have the potential to entrap and protect pathogenic microorganisms (Caraballo et al., 2020). In addition, *Pseudomonas* spp. are often present in multi-species biofilms involving pathogenic microorganisms (Quintieri et al., 2021), in particular, the non-pathogenic species *P. fluorescens* can enhance the adhesive and biofilm-forming capacity of the pathogen *Listeria monocytogenes* (Maggio et al., 2021; Puga et al., 2018). For these reasons, appropriate cleaning and disinfection (C&D) procedures should be regularly implemented in order to eradicate biofilms and prevent them from forming in the first place.

Cleaning is the operation through which detergents are applied to eliminate remaining food deposits where bacteria could otherwise survive and form biofilms. Specific detergents are chosen depending on the type of food being manufactured, the type and nature of soiling or spoilage, the surfaces' physicochemical properties, and the overall potential for microbial growth on surfaces and processing equipment (Troller, 1993). Although the food industry has traditionally employed alkaline and acid detergents, they are now being replaced by enzymatic detergents due to the low toxicity and excellent biodegradability of the latter, along with their potential to remove biofilms (Carrascosa et al., 2021). Enzymes have the capacity to attack the EPS matrix that surrounds and protects sessile cells (Borges et al., 2020), thereby making it easier for the disinfectant agents to penetrate the matrix and carry out their bactericidal action (Nahar et al., 2018). However, the application cost of enzymatic detergents, as well as their requirements in terms of temperature and contact time, are more elevated than those of traditional detergents (Carrascosa et al., 2021).

Although detergents can destabilize the EPS matrix of biofilms, they lack bactericidal activity. Disinfection is thus also required to ensure microbial inactivation (Gram et al., 2007). The food industry has extensively used sodium hypochlorite and peroxyacetic compounds (peracetic acid and hydrogen peroxide) because of their pronounced oxidizing capacity and their proven efficacy against biofilms. Alkyl amines, chlorine dioxide, and quaternary ammonium mixtures are also habitual components of disinfection programs (X. Liu, Yao, et al., 2023; Srey et al., 2013).

Regular C&D procedures consist in applying liquid cleaning and disinfectant agents with intermediary rinsing steps. However, some areas or certain processing equipment components may be difficult to reach using this method (Møretrø et al., 2019). Aerosolization is an alternative disinfection procedure in which a liquid disinfectant is dispersed in the form of a fine mist (Oh et al., 2005). This method of application has been tested in the food industry for product decontamination, as well as for air and surface decontamination of working areas (Masotti et al., 2019). It has also proven its efficacy against biofilms (Park et al., 2012). Aerosolization could thus be an effective alternative to traditional disinfectant application methods. The ISO standard 17272:2020 is used to evaluate the disinfectant activity of automated procedures in which a disinfectant is distributed by air diffusion. Nevertheless, that standard exclusively examines disinfectant activity against dried cells adhering to non-porous surfaces (hereafter referred to as surface-dried cells) and does not take biofilms into account. Given the potential aggregation of surface-dried cells into biofilms (Otter et al., 2015), disinfectant efficacy tests should also take biofilms into account.

The aim of the current study was: a) to compare the efficacy of several different commercial cleaning and disinfection agents against the biofilm of a *Pseudomonas fluorescens* strain previously isolated from the poultry meat plant, and b) to assess the efficacy of two application protocols (immersion and aerosolization) against surface-dried cells and the biofilm of a *P. fluorescens* strain isolated from the poultry meat plant.

2. Materials and methods

2.1. Isolation of Pseudomonas spp. strains from a chicken burger production line

Several food products (primarily, chicken meat and chicken burgers) as well as surfaces from different equipment (including formula table, grinder, kneader, forming hopper and conveyor belt) were sampled during processing throughout a burger production line in a Spanish chicken and turkey meat producing firm. Once the samples were collected, stock dilutions were prepared, and *Pseudomonas* CFC/CN agar (base agar) enriched with Cephalothin, Fucidin, Cetrimide (CFC) selective supplement was used to isolate *Pseudomonas* spp. (plates were incubated at 25 °C for 24–48 h). Once isolated, colonies with varying morphologies were selected in order to assess their ability to form biofilms. For a more comprehensive understanding of the experimental design, sample collection process, and culturing method for *Pseudomonas* spp., please refer to Merino et al. (2021).

2.2. Growth conditions of isolated Pseudomonas spp. strains

The twelve isolated *Pseudomonas* spp. strains were kept in cryovials with glycerol (20% v/v) at −80 °C. From those stocks, plates of tryptone soya agar with 0.1% yeast extract (Oxoid, Basingstoke, United Kingdom; TSAYE) were prepared on a weekly basis. To obtain bacterial subcultures, test tubes containing 5 mL of tryptone soya broth with 0.1% of yeast extract (Oxoid, TSBYE) were inoculated with one single colony and incubated under aerobic conditions for 12 h at 25 °C and 130 rpm in an orbital shaker (Heidolph Vibramax 100, Schwaback, Germany) inside an incubator (Trade, Raypa, Barcelona, Spain). After the incubation time, flasks containing 10 mL of TSBYE were inoculated with 10 μL of the bacterial subculture to obtain an initial concentration of $\sim 10^5$ colony-forming units per mL (CFU/mL), then incubated under the same conditions for 24 h to obtain a stationary phase culture ($\sim 4 \times 10^8$ CFU/mL).

2.3. Assessment of biofilm formation ability of isolated Pseudomonas spp. strains

Our biofilm formation protocol consisted of static incubation in 316L in-house manufactured stainless steel 24-well plates. The central wells were inoculated with 2 mL of diluted stationary phase cultures in TSBYE (1:100), and the external ones with 1 mL of sterile distilled water (SDW) to avoid dehydration during incubation. Once inoculated, plates were incubated for 96 h at 25 °C under static conditions. Finally, visual inspection and microscopy (Eclipse E400, Nikon, Japan) were used to determine which strains were able to form biofilms.

2.4. De novo sequencing of the biofilm-forming Pseudomonas sp. strain

The only *Pseudomonas* sp. strain possessing the ability to form biofilms was taxonomically identified. First, DNA extraction was performed following the manufacturer's instructions of the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), and DNA concentration was measured using a Qubit fluorometer, following the Qubit 1x dsDNA HS assay kit protocol (Thermo Fisher Scientific, Massachusetts, United States). Then, the Illumina NovaSeq platform was utilized for *de novo* sequencing (150 bp paired-end reads), and raw reads were processed using the TORMES 1.3.0 pipeline (Quijada et al., 2019). First, Trimmomatic (Bolger et al., 2014) was employed for quality filtering, and reads which passed the quality control were assembled *de novo* using the SPAdes assembler (Bankevich et al., 2012) with default settings. Taxonomic identification

was performed by Kraken2 (Wood et al., 2019), and multilocus sequence typing was performed with the MLST software (https://github.com/tseemann/mlst) and the PubMLST database (Jolley et al., 2018). Additionally, the draft genome was screened for antibiotic resistance and virulence factors using ABRIcate (https://github.com/tseemann/abricate) against the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013) and the Virulence Factor Database (VFDB) (Chen et al., 2005).

Bioinformatic analysis revealed that the *Pseudomonas* strain belonged to the species *P. fluorescens*; no antibiotic resistance genes or virulence factors are associated with this strain.

The unassembled reads were deposited in the Sequence Read Archive (SRA) of NCBI (BioProject ID: PRJNA1079903); and the accession numbers is SAMN40094511.

2.5. Characterization of *P. fluorescens* biofilm formation

To describe the formation of the *P. fluorescens* biofilm over time, we applied plate count technique and optical density readings. After each incubation period (24, 48, 72, and 96 h), the supernatant of each well was removed and 2 mL of SDW were added twice as a washing step to remove non-attached cells. Then, 2.5 mL of phosphate buffer saline (Sigma-Aldrich, PBS) with 1% of Tween 20 (Sigma-Aldrich) were added to each well, and biofilms were carefully scraped with sterile loops to obtain biofilm suspensions. Afterwards, each well was sonicated (Digital Sonifier S450D, Branson Ultrasonic Corporation, Connecticut, United States) for 5 s at 10% amplitude (21 μm; 20 kHz) to disaggregate the sessile cells. We conducted additional experiments (plate counting and microscopy observation) to ensure that sessile cells were disaggregated and not inactivated by sonication (data not shown). After the disaggregation step, aliquots of each well were serially diluted in PBS, pour-plated in TSAYE, and incubated for 48 h at 25 °C, to determine the viable sessile cell counts. The optical density of each well was measured at 595 nm using a spectrophotometer (Libra S12, Biochrom, Cambridge, UK) to assess biofilm biomass.

2.6. Assessment of the efficacy of C&D procedures applied against *P. fluorescens* biofilms by immersion

The bactericidal activity of various C&D agents was tested against mature *P. fluorescens* biofilm. These agents included detergents, disinfectants, and combinations of detergents and disinfectants (Table 1), kindly provided by OX-CTA S.L. (Cuarte, Huesca, Spain).

One-step C&D procedures (Table 2) and two-step C&D procedures (Table 3) were carried out following the manufacturer's recommendations. After the biofilm washing step, 2.5 mL of the selected C&D agent were added to each well. As C&D agents were applied at specific doses, we carried out dilutions in SDW. In addition, some procedures were applied at 50 °C in a thermostatic bath (Ultrasonic cleaner 031, Ultracleaner). Once the procedure had been applied, the remaining biofilm was quantified using the plate count technique (Section 2.4.). We measured the efficacy of each C&D procedure by comparing the sessile cell counts before and after the procedure. For procedures at 50 °C, a control with SDW at 50 °C was included.

Additionally, to assess the effect of temperature on the application of enzymatic procedures, we tested the enzymatic agents fabricated by OX-CTA S.L. (ENZYOX, ENZYOX EC, and ENZYOX PLUS), along with other enzymatic agents fabricated by Itram Higiene (Barcelona, Spain) (ENZY JET, ENZY CIP, BIO JET and BIO COLD), at 22 °C.

Table 1
Description of the cleaning and disinfection agents assessed in this study.

Commercial name	Description
OX-VIRIN	Disinfectant based on peroxyacetic compounds (hydrogen peroxide and peracetic acid)
AMINAS OX1	Detergent-disinfectant based on tertiary alkylamines
SCAN 650 LK	Detergent-disinfectant based on sodium hypochlorite
OX-NETAL 4	Alkaline foaming detergent based on potassium and sodium hydroxide
ENZYOX	Enzymatic foaming detergent based on amylase, lipase and protease
ENZYOX EC	Enzymatic non-foaming detergent based on amylase, lipase and protease
ENZYOX PLUS	Enzymatic cocktail based on amylase, lipase and protease, used as an additive for enzymatic detergents

Table 2
One-step cleaning and disinfection procedures applied (by immersion) to *Pseudomonas fluorescens* mature biofilm formed in 316L stainless steel 24-well plates.

Code	C&D agent	Doses	Time	Temperature	Washing
VR	OX-**VIR**IN	1%	20 min	22 °C	No
AM	**AM**INAS OX1	1%	20 min	22 °C	Yes
SC	**SC**AN 650LK	3%	20 min	22 °C	Yes
NT	OX-**NET**AL 4	3%	20 min	22 °C	Yes
EN + P.0.2	**EN**ZYOX ENZYOX **P**LUS	4% **0.2%**	20 min	50 °C	Yes
EN + P.20	**EN**ZYOX ENZYOX **P**LUS	4% **20%**	20 min	50 °C	Yes
EN.EC + P.0.2	**EN**ZYOX **EC** ENZYOX **P**LUS	3% **0.2%**	20 min	50 °C	Yes
EN.EC + P.20	**EN**ZYOX **EC** ENZYOX **P**LUS	3% **20%**	20 min	50 °C	Yes

*Codes were created using the bold letters of the C&D procedure and the applied doses, if necessary.

Table 3
Two-step cleaning and disinfection procedures applied (by immersion) to *Pseudomonas fluorescens* mature biofilm formed in 316L stainless steel 24-well plates.

Code	C&D agent	Step	Doses	Time	Temperature	Washing
NT-VR	OX-**NET**AL 4	First step	3%	20 min	22 °C	Yes
	OX-**VIR**IN	Second step	1%	20 min	22 °C	No
NT-AM	OX-**NET**AL 4	First step	3%	20 min	22 °C	Yes
	AMINAS OX1	Second step	1%	20 min	22 °C	Yes
EN + P.0.2-VR	**EN**ZYOX ENZYOX **P**LUS	First step	4% **0.2%**	20 min	50 °C	Yes
	OX-**VIR**IN	Second step	1%	20 min	22 °C	No
EN + P.20-VR	**EN**ZYOX ENZYOX **P**LUS	First step	4% **20%**	20 min	50 °C	Yes
	OX-**VIR**IN	Second step	1%	20 min	22 °C	No
EN.EC + P.0.2-VR	**EN**ZYOX **EC** ENZYOX **P**LUS	First step	3% **0.2%**	20 min	50 °C	Yes
	OX-**VIR**IN	Second step	1%	20 min	22 °C	No
EN.EC + P.20-VR	**EN**ZYOX **EC** ENZYOX **P**LUS	First step	3% **20%**	20 min	50 °C	Yes
	OX-**VIR**IN	Second step	1%	20 min	22 °C	No

*Discontinuous line divides the first and second steps within a procedure.
**Codes were created using the bold letters of the C&D procedure and the applied doses, if necessary.

2.7. Evaluation of the efficacy of C&D procedures by following two different application methods against surface-dried cells and biofilms of *P. fluorescens*

The efficacy of two different application methods (immersion and aerosolization) against surface-dried cells and biofilms of *P. fluorescens* was assessed and compared.

On the one hand, sterile 316L stainless steel discs of 4 cm diameter and 3 mm thickness (in-house manufacturing) were placed in Petri plates and inoculated with 50 μL of the stationary phase culture containing 0.3 g/L of bovine albumin (Sigma-Aldrich) as an interfering substance. The inoculum was spread on the disc surface, and the discs were dried for 2 h at ambient temperature in a biological safety cabinet. After ensuring that the cells had dried on the surface, we applied various C&D procedures (Table 4) by immersion or aerosolization.

On the other hand, biofilms were formed in 316L stainless steel 24-well plates (section 2.5.) and on 316L stainless steel discs. For this purpose, sterile discs were placed in a tilted position inside sterile containers (VWR, Barcelona, Spain) and inoculated with 18 mL of a stationary phase culture diluted in TSBYE (1:100) so that they remained submerged in the culture. The biofilm formed after an incubation of 96 h at 25 °C under static conditions. The liquid culture was then removed by aspiration and the biofilm was rinsed twice with SDW before the application of several different C&D procedures (Table 4) by either immersion or aerosolization.

Aerosolization procedures were carried out following the ISO standard 17272:2020 protocol. The diffusion device OX-DISAIR BASIC (OX-CTA S.L.) was used to distribute the disinfectant OX-VIRIN (particle size: 5 μm) for a period of 54 min in an enclosure of 105 m^3 where four discs (contaminated either with surface-dried cells or biofilms) were placed in specific positions (one vertically positioned facing the diffusion device, one vertically positioned facing away from the diffusion device, one horizontally positioned facing the floor, and one horizontally facing the ceiling). After the diffusion period, a contact time of 20 min was allowed before entering the enclosure to collect the discs.

Immersion procedures were carried out as described in Section 2.5. Nevertheless, whereas in the case of surface-dried cells, 18 mL of the C&D agent were added to each Petri plate with the disc; in the case of biofilms, 2.5 mL were added to the central wells of the 24-well plates, and 25 mL were added to each container with the disc.

Once the procedures had been applied, either by aerosolization or by immersion, the remaining surface-dried cells or sessile cells were quantified using the standard plate count technique (Section 2.4.). In the case of surface-dried cells, swabs were used to recover cells with no sonication step required. Conversely, a disaggregation step was necessary in the case of biofilms, which involved the transfer of biofilm-containing discs to different wells of 24-well plates in order to carry out the sonication step. Finally, the efficacy of each C&D procedure was assessed by comparing the relative cell counts before and after the aforementioned described procedures.

2.8. Statistical analysis

All results were obtained from at least three independent experiments carried out on different working days with different bacterial cultures. Graphics are displayed as the mean ± standard deviation, using GraphPad Prism software (v.8.4.2.679, Boston, Massachusetts, USA). Data were analyzed and submitted to comparison of averages using analysis of variance (ANOVA) followed by post-hoc Tukey test with GraphPad Prism, and differences were considered significant if $p \leq 0.05$.

3. Results and discussion

3.1. *P. fluorescens* biofilm formation

As a preliminary step in assessing the efficacy of various C&D procedures against the *P. fluorescens* biofilm, we examined its formation over time by using standard plate counts to determine viable cell numbers combined with optical density measurements to evaluate total biomass (Fig. 1).

As can be observed in Fig. 1A, a viable cell density of $>10^9$ CFU/mL was obtained after only 24 h of incubation, and remained constant throughout the entire incubation period. This result differed from the biofilm formation process observed by Wang et al. (2018), who noted a gradual increase in the concentration of *P. fluorescens* sessile cells over time. Regarding biomass production (Fig. 1B), a significant increase ($p \leq 0.05$) was observed for optical density (OD_{595}) measurements of the wash buffer used to remove the biofilms throughout the incubation time. Specifically, the mean OD_{595} readings for the suspensions of removed biofilm constituents after the initial 24 h was 1.50. However, over the next 72 h the OD_{595} increased to a maximum value of 4.61. Previous authors observed a similar increase in biomass production during incubation time in two strains of *P. fluorescens* isolated from the dairy industry (Rossi et al., 2018). However, J. Liu, Feng, et al. (2023) noted that the maximum optical density value had already been reached after only 24 h of incubation, followed by a decrease in biomass production rate over time. These differences in biofilm formation may be due to differences among strains and the conditions of biofilm formation, including factors such as material, temperature, and contact time.

Regarding visual characteristics (Fig. S1), the biofilm formed by the strain of *P. fluorescens* developed a thick floating pellicle at the air-liquid interface, similar to the biofilm described by J. Liu, Feng, et al. (2023). Not only was a thickening of the pellicle observed over the course of the incubation, but also an increased presence of wrinkles, indicative of a higher maturity level of the biofilm (Trejo et al., 2013).

The biofilm produced by *P. fluorescens* under the conditions implemented in the present study was considered to be a suitable model system for investigating the effectiveness of different C&D procedures due to; 1) since the biofilm was formed by a wild *Pseudomonas* strain isolated from a poultry meat plant it has real-world implications, and 2) its high concentration of viable sessile cells, high biomass production, and advanced maturity level, inherently made it a particularly resilient biofilm, thus providing a "worst case" scenario under which the

Table 4
Cleaning and disinfection procedures applied via different techniques (immersion and aerosolization) on *Pseudomonas fluorescens* surface-dried cells and biofilms.

Code	C&D procedure	Application technique	Step	Doses	Time	Temperature	Washing
VR.I	OX-**V**I**R**IN	**I**mmersion	Unique step	1%	20 min	22 °C	No
VR.A	OX-**V**I**R**IN	**A**erosolization	Unique step	1%	20 min	22 °C	No
NT.I	OX-**N**E**T**AL 4	**I**mmersion	Unique step	3%	20 min	22 °C	Yes
NT.I-VR.I	OX-**N**E**T**AL 4	**I**mmersion	First step	3%	20 min	22 °C	Yes
	OX-**V**I**R**IN	**I**mmersion	Second step	1%	20 min	22 °C	No
NT.I-VR.A	OX-**N**E**T**AL 4	**I**mmersion	First step	3%	20 min	22 °C	Yes
	OX-**V**I**R**IN	**A**erosolization	Second step	1%	20 min	22 °C	No

*Discontinuous line divides the first and second steps within a procedure.

**Codes were created using the bold letters of the biocide procedure, the application technique, and the applied doses, if necessary.

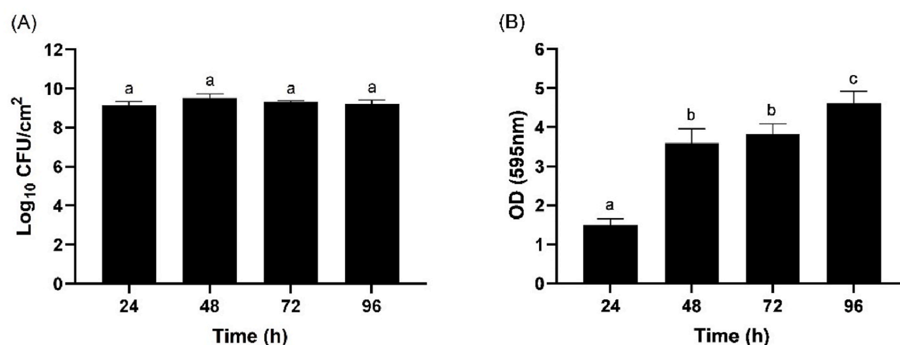

Fig. 1. Microbial counts of sessile cells (A) and biomass (OD_{595nm}) (B) of *Pseudomonas fluorescens* biofilms formed in 316L stainless steel 24-well plates throughout the incubation time. Bars represent the mean and standard deviation of at least three independent assays. Different letters over the bars represent statistically significant differences ($p < 0.05$) among the means of each group as determined by one-way ANOVA followed by Tukey's multiple pairwise comparisons post hoc test.

disinfectants could be compared.

3.2. Assessment of the efficacy of C&D agents applied by immersion against P. fluorescens biofilms

To assess the efficacy of various C&D procedures, we used mature biofilms of *P. fluorescens* after 96 h of incubation.

Fig. 2 shows the counts of viable sessile cells before and after the application of one-step procedures with disinfectants or detergent-disinfectants.

After a 20 min application of the peroxyacetic disinfectant (VR) at 1% (equivalent to 0.25% of peroxide hydrogen and 0.05% of peracetic acid), the reduction achieved for viable cells counts was found to be only 1.64 Log, a value consistent with that determined in the investigation conducted by Santos Rosado Castro et al. (2021), where the application of peracetic acid at 300 mg/L (0.03%) for 20 min only resulted in a 2.28 Log decrease in viable sessile cells of *P. fluorescens*. Conversely, however, Martín-Espada et al. (2014) applied a disinfectant based on peracetic acid at 1.61% (equivalent to 0.004% of peracetic acid) for a 15 min exposure period and achieved an inactivation of over 5 Log cycles of *Pseudomonas aeruginosa* sessile cells. Nevertheless, the conditions under which the biofilms were produced in the aforementioned study were considerable different than those employed here, as well as the initial densities of sessile cells, which were significantly greater in the present study. These factors likely accounted for the heightened efficacy of this disinfectant reported in the earlier study.

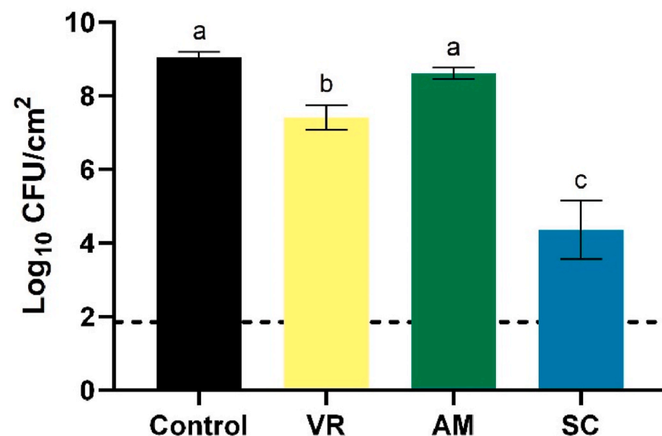

Fig. 2. Microbial counts of sessile cells in biofilms of *Pseudomonas fluorescens* formed in 316L stainless steel 24-well plates after 96 h of incubation (control) and after one-step procedures (VR, AM and SC).
VR – 1% OX-VIRIN at 22 °C for 20 min.
AM – 1% AMINAS OX1 at 22 °C for 20 min.
SC – 3% SCAN 650LK at 22 °C for 20 min.
Bars represent the mean and standard deviation of at least three independent assays. Horizontal dotted line represents the limit of detection. Different letters over the bars represent statistically significant differences ($p < 0.05$) among the means of each group as determined by one-way ANOVA followed by Tukey's multiple pairwise comparisons post hoc test.

In contrast, the application of two different detergent-disinfectants led to divergent results. A treatment with the amine-based product (AM) at 1% for a period of 20 min did not significantly reduced the viable sessile cells ($p > 0.05$), while the treatment with the sodium hypochlorite-based product (SC) at 3% (equivalent to 0.15% of active chlorine) for 20 min resulted in a 4.69 Log reduction ($p \leq 0.05$). These results differ from the study carried out by Kocot and Olszewska (2020), where tertiary alkyl amines at 2% for 5 min reduced the number of viable *P. aeruginosa* biofilm by 3 Log, while a chlorine-based disinfectant at 0.18% (equivalent to 0.10% of active chlorine) only achieved a reduction of approximately 2 Log cycles. Nevertheless, the high efficacy we observed of the sodium hypochlorite-based product (SC) was consistent with another study where the application of 200 mg/L (0.02%) of sodium hypochlorite for 20 min inactivated more than 5 Log cycles of *P. fluorescens* sessile cells (Wang et al., 2018).

Although none of the C&D agents applied in one step managed to completely eradicate the *P. fluorescens* biofilm, the remarkable effectiveness ($p \leq 0.05$) of the sodium hypochlorite-based product (SC), when compared with the other C&D agents, suggested that a combination of cleaning and disinfection products might be essential. As previous studies have demonstrated that the application of a detergent and a disinfectant with an intermediary washing step is more effective than their simultaneous application, we decided to test several different C&D procedures involving the application of a detergent and a disinfectant in two separate steps against the *P. fluorescens* biofilm (Figs. 3 and 4).

Fig. 3 shows the counts of viable sessile cells before and after the application of the alkaline detergent (NT) alone or followed by the application of the peroxyacetic disinfectant (VR).

The sole application of the alkaline detergent (NT) at 3% for 20 min already led to a significant reduction ($p \leq 0.05$) of the counts of sessile cells by 4.48 Log cycles, but the combination of the alkaline detergent with the peroxyacetic disinfectant including an intermediary washing step (NT-VR) successfully achieved a reduction below the detection limit (>7.0 Log cycles) ($p \leq 0.05$). This result was in agreement with our expectations, namely that the application of this alkaline detergent, capable of destabilizing EPS by hydrolyzing and dissolving organic soil (Antoniou & Frank, 2005), facilitates the penetration of the disinfectant into the biofilm, thereby allowing the disinfectant to reach and inactivate sessile cells. In the present study, the disintegration of the pellicle could be visualized after the application of the alkaline detergent (NT). This result is consistent with the study carried out by Antoniou and

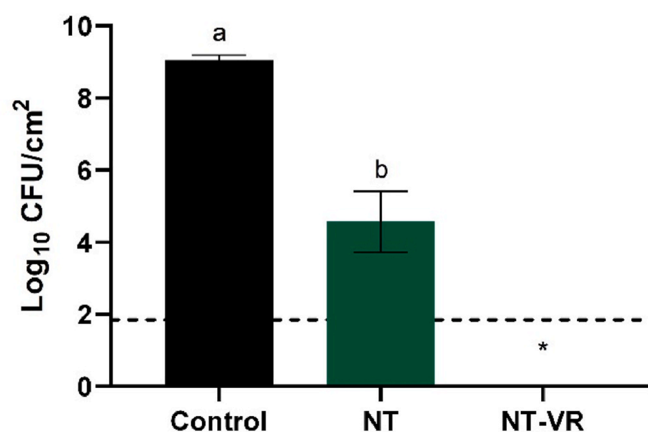

Fig. 3. Microbial counts of sessile cells in biofilms of *Pseudomonas fluorescens* formed in 316L stainless steel 24-well plates after 96 h of incubation (control), after a one-step procedure (NT), and after two-step procedures (NT-VR and NT-AM).

NT – 3% OX-NETAL 4 at 22 °C for 20 min.

NT-VR – 3% OX-NETAL 4 at 22 °C for 20 min followed by 1% OX-VIRIN at 22 °C for 20 min.

Bars represent the mean and standard deviation of at least three independent assays. Horizontal dotted line represents the limit of detection. Asterisk means that the C&D procedure reduces sessile cells counts below the limit of detection. Different letters over the bars represent statistically significant differences ($p < 0.05$) among the means of each group as determined by one-way ANOVA followed by Tukey's multiple pairwise comparisons post hoc test.

Frank (2005), where the tested alkaline detergent effectively removed *Pseudomonas putida* EPS after just 1 min of treatment. In addition, it is noteworthy that the successful removal of *P. fluorescens* biofilm after the combined application of the alkaline detergent and the peroxyacetic disinfectant (NT-VR) was achieved by applying the manufacturer's recommended concentrations. This outcome contrasted with the findings obtained by Fagerlund et al. (2020), where various C&D procedures, involving the application of alkaline detergents and peracetic acid disinfectants at manufacturer's recommended concentrations, only led to a modest 1.8 Log reduction in counts of *L. monocytogenes* sessile cells; an outcome that necessitated the authors to increase concentrations, exposure times, application temperatures, and the number of subsequent treatments to achieve a more substantial reduction.

Although C&D procedures with the alkaline detergent (NT) demonstrated high efficacy against the *P. fluorescens* biofilm (Fig. 3), the current growing general interest in enzymatic cleaning motivated us to assess the efficacy of other detergents based on enzymatic formulations, alone or in combination with the peroxyacetic disinfectant (VR).

Fig. 4 shows the counts of viable sessile cells before and after the application of two enzymatic detergents, foaming (EN) and non-foaming (EN.EC), with varying concentrations of an enzymatic cocktail (P) (0.2% and 20%) alone or followed by the application of the peroxyacetic disinfectant (VR). Specifically, Fig. 4A presents the results achieved by the non-foaming enzymatic detergent (EN.EC), whereas Fig. 4B illustrates the results achieved by the foaming enzymatic detergent (EN).

Similar to what had been observed with the alkaline detergent (NT) (Fig. 3), the sole application of the enzymatic detergents already achieved a significant reduction ($p \leq 0.05$) in sessile cell counts ranging from 1.32 to 5.17 Log cycles depending on the type of detergent and the concentration of enzymes; specifically, the foaming enzymatic detergent (EN) showed a higher efficacy against the *P. fluorescens* biofilm than the non-foaming enzymatic detergent (EN.EC) ($p \leq 0.05$). This result was consistent with a study conducted by Dallagi et al. (2022), where the foam-producing solution was more efficient in detaching the biofilm than the related non-foaming solution. In any case, the enzymatic cleaning step alone did not achieve complete eradication of the biofilm. However, the addition of a secondary step with the peroxyacetic disinfectant (VR) did result in the reduction of sessile cell counts below the detection limit ($p \leq 0.05$), even when the lowest concentration of the enzymatic cocktail (0.2%) was employed. This result confirmed the enzymes' role in disrupting the structure of the EPS, thereby increasing the sessile cells' vulnerability to the subsequent application of the

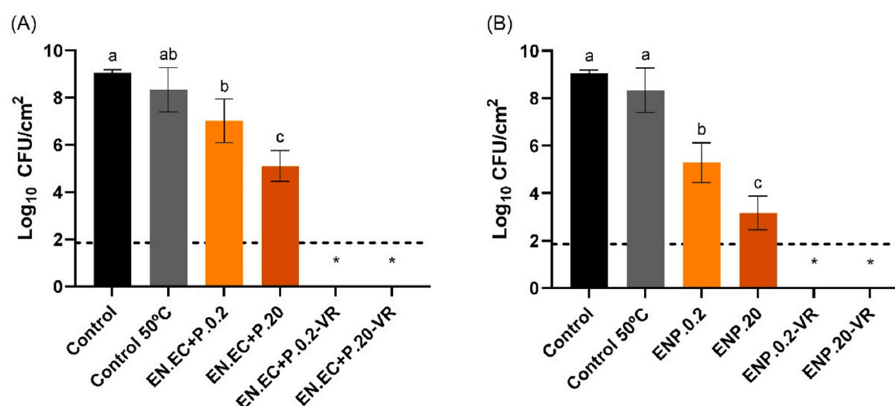

Fig. 4. Microbial counts of sessile cells in biofilms of *Pseudomonas fluorescens* formed in 316L stainless steel 24-well plates after 96 h of incubation (control), after 50 °C-procedure with SDW (50 °C-control), after one step-procedure with EN.EC0.2 and EN.EC20 (A) or EN0.2 and EN20 (B); and after two step-procedures (EN.EC0.2-VR and EN.EC20-VR (A) or EN0.2-VR and EN20-VR (B)).

EN.EC + P.0.2 – 4% ENZYOX EC along with 0.2% ENZYOX PLUS at 50 °C for 20 min.

EN.EC + P.20 – 4% ENZYOX EC along with 20% ENZYOX PLUS at 50 °C for 20 min.

EN.EC + P.0.2-VR – 4% ENZYOX EC along with 0.2% ENZYOX PLUS at 50 °C for 20 min followed by 1% OX-VIRIN at 22 °C for 20 min.

EN.EC + P.20-VR – 4% ENZYOX EC along with 20% ENZYOX PLUS at 50 °C for 20 min followed by 1% OX-VIRIN at 22 °C for 20 min.

EN + P.0.2 – 4% ENZYOX along with 0.2% ENZYOX PLUS at 50 °C for 20 min.

EN + P.20 – 4% ENZYOX along with 20% ENZYOX PLUS at 50 °C for 20 min.

EN + P.0.2-VR – 4% ENZYOX along with 0.2% ENZYOX PLUS at 50 °C for 20 min followed by 1% OX-VIRIN at 22 °C for 20 min.

EN + P.20-VR – 4% ENZYOX along with 20% ENZYOX PLUS at 50 °C for 20 min followed by 1% OX-VIRIN at 22 °C for 20 min.

Bars represent the mean and standard deviation of at least three independent assays. Horizontal dotted line represents the limit of detection. Asterisk means that the C&D procedure reduces sessile cells counts below the limit of detection. Different letters over the bars represent statistically significant differences ($p < 0.05$) among the means of each group as determined by one-way ANOVA followed by Tukey's multiple pairwise comparisons post hoc test.

disinfectant. Tsiaprazi-Stamou et al. (2019) also demonstrated that introducing a disinfection step after the enzymatic cleaning step led to a greater reduction in the percentage of viable sessile cells and more extensive removal of biomass. However, the efficacy of the C&D procedures employed in that study was much lower than observed in the present study.

The conditions for applying these enzymatic detergents can be costly since elevated temperatures are required for optimal activity. Still, certain commercial enzymatic detergents can nevertheless be applied at room temperature according to manufacturer's instructions. To explore this aspect, we tested the efficacy of the enzymatic detergents employed in our study and other commercial enzymatic detergents at both 22 °C and at 50 °C against the *P. fluorescens* biofilm (Fig. S2). As can be observed, none of the enzymatic detergents showed efficacy at 22 °C against the *P. fluorescens* biofilm, whereas, at 50 °C, they were highly successful, resulting in the complete eradication of viable sessile cells. This proves that enzymatic detergents used here must be applied at 50 °C to be effective, at least against a *P. fluorescens* biofilm and under this study's experimental conditions.

Overall, C&D procedures involving the separate, successive application of cleaning and disinfection agents proved effective against the *P. fluorescens* biofilm, regardless of whether the cleaning step involved an alkaline or an enzymatic detergent. However, for subsequent experiments, given the higher cost associated with enzymatic detergents, we opted for the alkaline detergent (NT) followed by the peroxyacetic disinfectant (VR).

Once we had selected the following C&D procedure, we assessed and compared the efficacy of the peroxyacetic disinfectant (VR) applied by aerosolization with its efficacy applied by immersion.

3.3. Comparison of the efficacy of C&D agents applied by immersion and by aerosolization against surface-dried cells and biofilms of P. fluorescens

As the ISO Standard 17272:2020 examines the disinfectant activity of specific procedures against surface-dried cells, we started by testing the efficacy of the peroxyacetic disinfectant (VR) applied by either immersion or aerosolization against *P. fluorescens* surface-dried cells, then against *P. fluorescens* biofilms.

The sole application of the peroxyacetic disinfectant (VR), either by immersion or by aerosolization, successfully reduced the quantity of *P. fluorescens* surface-dried cells below the detection limit ($p \leq 0.05$) (Fig. S3). This finding differed from other studies, which had found several biocides to be ineffective against bacterial cells attached to stainless steel surfaces (Møretrø et al., 2009, 2019). For instance, Møretrø et al. (2009) had observed that the application of a disinfectant based on peracetic acid and hydrogen peroxide by immersion only led to a reduction of approximately 1.5 Log cycles in the counts of *Salmonella* sp. surface-dried cells. Similarly, Møretrø et al. (2019) had observed that the aerosolization of hydrogen peroxide achieved a maximum reduction of only 1.5 Log in the number of viable surface-dried cells of *L. monocytogenes*.

The remarkable efficacy of the peroxyacetic disinfectant (VR) displayed in the present study against *P. fluorescens* surface-dried cells is promising, as cell attachment is the initial step in the process of biofilm formation. The application of the peroxyacetic disinfectant (VR) using one of the two application techniques (immersion or aerosolization) was not only effective in inactivating *P. fluorescens* surface-dried cells but also in prevention of biofilm development: this is indeed the measure that is most effective in controlling biofilms (X. Liu, Yao, et al., 2023).

After having noted the high efficacy of the peroxyacetic disinfectant (VR) against surface-dried cells through immersion and aerosolization techniques, subsequent tests were conducted targeting sessile cells

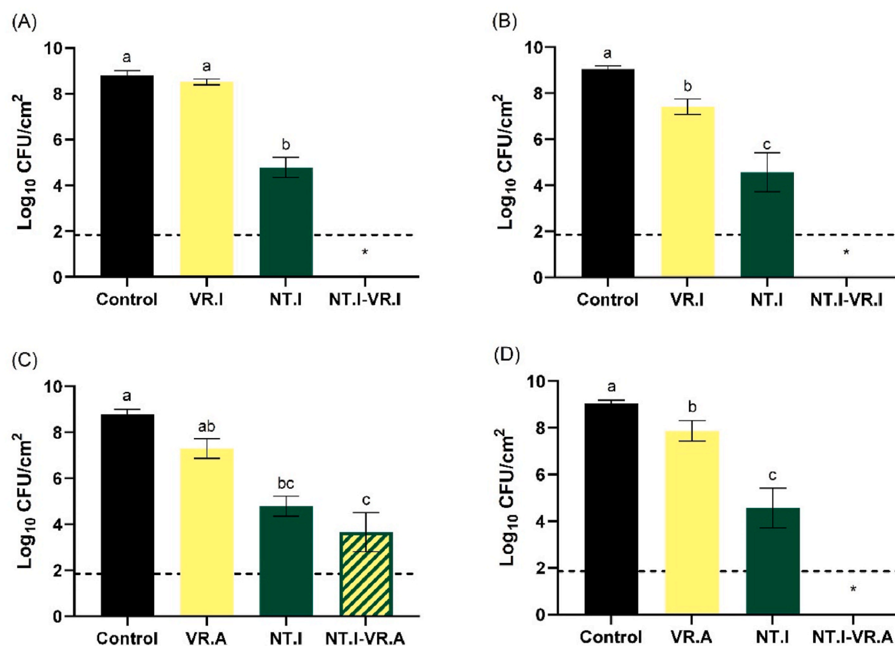

Fig. 5. Microbial counts of sessile cells in biofilms of *Pseudomonas fluorescens* formed on 316L stainless steel discs (A, C) or in 316L stainless steel 24-well plates (B, D) after 96 h of incubation (control) and after one step-procedures (VR and NT), and two step-procedure (NT-VR) applying the disinfection step by immersion (A, B) or by aerosolization (C, D).
VR – 1% OX-VIRIN at 22 °C for 20 min.
NT – 3% OX-NETAL 4 at 22 °C for 20 min.
NT-VR – 3% OX-NETAL 4 at 22 °C for 20 min followed by 1% OX-VIRIN at 22 °C for 20 min.
Bars represent the mean and standard deviation of at least three independent assays. Horizontal dotted line represents the limit of detection. Asterisk means that the C&D procedure reduces sessile cells counts below the limit of detection. Different letters over the bars represent statistically significant differences ($p < 0.05$) among the means of each group as determined by one-way ANOVA followed by Tukey's multiple pairwise comparisons post hoc test.

within biofilms (Fig. 5).

Fig. 5 shows viable sessile cells counts before and after the sole application of either the alkaline detergent (NT) or the peroxyacetic disinfectant (VR) by immersion or aerosolization, and also the combined application of OX-NETAL and the peroxyacetic disinfectant (VR) applying the disinfection step by immersion or aerosolization.

In this case, the sole application of the peroxyacetic disinfectant (VR) by either immersion or aerosolization did not achieve a reduction of sessile cell counts below the detection limit. This discrepancy between this disinfectant's efficacy against surface-dried cells and sessile cells (Fig. 5) can be attributed to the influence of the cell phase on susceptibility to biocides, as surface-dried cells are more susceptible to C&D agents than the stress-hardened cells residing within an already established biofilm (Otter et al., 2015).

As mentioned, the sole application of the peroxyacetic disinfectant (VR) by aerosolization (Fig. 5C and D) did not achieve a pronounced inactivation of *P. fluorescens* sessile cells (only 1.1 and 1.51 Log cycles of inactivation in biofilms formed in 24-well plates and on discs, respectively). This finding is consistent with Kim et al. (2023), who found that the aerosolization of peroxide hydrogen (2%) and peracetic acid (0.01%) for a period of 20 min did not achieve a reduction of more than 1.5 Log cycles in sessile cell counts of *Escherichia coli*, *L. monocytogenes*, and *Salmonella* Typhimurium. Conversely, however, Park et al. (2012) observed that the sole application of 100 ppm (0.01%) of peracetic acid by aerosolization for a period of 50 min effectively reduced the sessile cell counts of the same pathogenic microorganisms to levels below the detection limit, probably due to a longer exposure time.

In any case, the inefficacy of the peroxyacetic disinfectant (VR) against the *P. fluorescens* biofilm incited us to include a cleaning step with the alkaline detergent (NT) prior to the disinfection step with the peroxyacetic disinfectant (VR). The two-step procedure in which we applied the peroxyacetic disinfectant (VR) by immersion did manage to achieve a reduction of *P. fluorescens* sessile cells below the detection limit ($p \leq 0.05$), regardless of whether the biofilm was formed in 24-well plates or on discs (Fig. 5A and B). Conversely, the two-step procedure in which we applied the peroxyacetic disinfectant (VR) by aerosolization achieved a reduction in *P. fluorescens* sessile cells below the detection limit when the biofilm had formed in 24-well plates (Fig. 5D). However, this was not the case when the biofilm was formed on discs: in the latter case, the reduction achieved was 5.14 Log cycles (Fig. 5C). This result cannot be attributed to a higher cell concentration as there were no statistical differences ($p > 0.05$) between the sessile cell counts of the biofilms formed in 24-well plates and those formed on discs. Instead, it might be due to a more pronounced production of biomass on discs as compared to 24-well plates. It has been demonstrated that EPS matrix acts as a barrier delaying or preventing antimicrobials from reaching cells within the biofilm by limiting antimicrobial diffusion and/or by chemically interacting with the extracellular proteins and polysaccharides (Mah & O'Toole, 2001; Simões et al., 2009). Hence, in the current case, a more dense EPS matrix might be responsible for the lower efficacy of the aerosolized peroxyacetic disinfectant (VR) against the biofilm formed on discs.

Overall, although immersion treatments exhibited a greater efficacy than aerosolization against the biofilms of *P. fluorescens* formed on discs, the application of the disinfectant by aerosolization also provided good results in terms of inactivation of both surface-dried and sessile cells. In addition, previous studies have shown that some processing areas or equipment may be difficult to reach when applying a liquid disinfectant (Møretrø et al., 2019) (referred to in this study as immersion technique); thus, aerosolization could serve as a useful alternative in the application of disinfectants for hard-to-clean places in food processing environments. In fact, the low standard deviation observed in viable cell counts after aerosolization procedures points to the robust repeatability of this application technique. This observation aligns with the results reported by Oh et al. (2005), who found that the efficacy of an aerosolization technique remains unaffected by the biofilm's location.

4. Conclusions

The current study demonstrated the strong biofilm-forming ability of a *P. fluorescens* strain isolated from the poultry meat plant. Regarding C&D procedures, the sole application of detergents, disinfectants or detergent-disinfectants failed to completely eradicate the biofilm of *P. fluorescens*, regardless of the active compound in each C&D agent. On the contrary, two-step C&D procedures involving the separate application of detergents and disinfectants at manufacturer's recommended concentrations managed to successfully eradicate *P. fluorescens* biofilms, regardless of whether the detergent employed was alkaline or enzymatic at 50 °C. Hence, despite the robust nature of *P. fluorescens* biofilm, the successfully tested C&D agents achieved its removal without having to increase concentration, exposure time, or application temperature. Furthermore, the notable efficacy of C&D procedures involving the application of a disinfectant agent by aerosolization against surface-dried as well as sessile cells of *P. fluorescens* suggested that this combined application procedure could serve as a viable alternative to the commonly utilized immersion technique.

CRediT authorship contribution statement

Natalia Merino: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Carlota García-Castillo:** Writing – original draft, Methodology, Investigation, Data curation. **Daniel Berdejo:** Writing – review & editing, Methodology, Investigation, Data curation. **Elisa Pagán:** Methodology, Investigation. **Diego García-Gonzalo:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Rafael Pagán:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgements

This research was supported by Grant PID 2021-123404NB-I00 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe" and by the Government of Aragón (grant Grupo AESA). Department of Science, Technology, and University Education of the Government of Aragón (Spain) provided N. Merino with grant to carry out this research. Ministry of Science, Innovation, and Universities provided E. Pagán with grant (FPU17/02441) to carry out this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110614.

References

Antoniou, K., & Frank, J. F. (2005). Removal of *Pseudomonas putida* biofilm and associated extracellular polymeric substances from stainless steel by alkali cleaning. *Journal of Food Protection, 68*, 277–281. https://doi.org/10.4315/0362-028X-68.2.277

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology, 19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics, 30*, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Borges, A., Meireles, A., Mergulhão, F., Melo, L., & Simões, M. (2020). Biofilm control with enzymes. In M. Simoes, A. Borges, & L. Chaves Simoes (Eds.), *Recent trends in biofilm science and Technology* (pp. 249–271). https://doi.org/10.1016/B978-0-12-819497-3.00011-8

Caraballo, A., González, M. I., Cuesta-Astroz, Y., & Torres, G. (2020). Metagenomic characterization of bacterial biofilm in four food processing plants in Colombia. *Brazilian Journal of Microbiology, 51*, 1259–1267. https://doi.org/10.1007/s42770-020-00260-x

Carrascosa, C., Raheem, D., Ramos, F., Saraiva, A., & Raposo, A. (2021). Microbial biofilms in the food industry—a comprehensive review. *International Journal of Environmental Research and Public Health, 18*, 2014. https://www.mdpi.com/1660-4601/18/4/2014.

Chen, L., Yang, J., Yu, J., Yao, Z., Sun, L., Shen, Y., & Jin, Q. (2005). Vfdb: A reference database for bacterial virulence factors. *Nucleic Acids Research, 33*, 325–328. https://doi.org/10.1093/nar/gki008

Cousin, M., Jay, J., & Vasavada, P. (2001). Psychrotrophic microorganisms. In *Compendium of methods for the microbiological examination of foods*. https://doi.org/10.2105/9780875531755ch13

Dallagi, H., Faille, C., Gruescu, C., Aloui, F., & Benezech, T. (2022). Foam flow cleaning, an effective and environmentally friendly method for controlling the hygiene of closed surfaces contaminated with biofilms. *Food and Bioproducts Processing, 136*, 236–248. https://doi.org/10.1016/j.fbp.2022.09.014

Fagerlund, A., Heir, E., Møretrø, T., & Langsrud, S. (2020). *Listeria Monocytogenes* biofilm removal using different commercial cleaning agents. *Molecules, 25*. https://doi.org/10.3390/molecules25040792

Gram, L., Bagge-Ravn, D., Ng, Y. Y., Gymoese, P., & Vogel, B. F. (2007). Influence of food soiling matrix on cleaning and disinfection efficiency on surface attached *Listeria monocytogenes*. *Food Control, 18*, 1165–1171. https://doi.org/10.1016/j.foodcont.2006.06.014

Jolley, K. A., Bray, J. E., & Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res, 3*, 124. https://doi.org/10.12688/wellcomeopenres.14826.1

Kim, S.-Y., Kim, S.-H., & Park, S.-H. (2023). Inactivation of foodborne pathogen biofilm cells using a combination treatment with gaseous chlorine dioxide and aerosolized sanitizers. *Journal of Food Protection, 86*, Article 100105. https://doi.org/10.1016/j.jfp.2023.100105

Kocot, A. M., & Olszewska, M. A. (2020). Interaction of *Pseudomonas aeruginosa* and *Staphylococcus aureus* with *Listeria innocua* in dual species biofilms and inactivation following disinfectant treatments. *Lebensmittel-Wissenschaft und -Technologie, 118*, Article 108736. https://doi.org/10.1016/j.lwt.2019.108736

Lindsay, D., & von Holy, A. (2006). What food safety professionals should know about bacterial biofilms. *British Food Journal, 108*, 27–37. https://doi.org/10.1108/00070700610637616

Liu, J., Wu, S., Feng, L., Wu, Y., & Zhu, J. (2023). Extracellular matrix affects mature biofilm and stress resistance of psychrotrophic spoilage *Pseudomonas* at cold temperature. *Food Microbiology, 112*, Article 104214. https://doi.org/10.1016/j.fm.2023.104214

Liu, X., Yao, H., Zhao, X., & Ge, C. (2023). Biofilm formation and control of foodborne pathogenic bacteria. *Molecules, 28*. https://doi.org/10.3390/molecules28062432

López, D., Vlamakis, H., & Kolter, R. (2010). Biofilms. *Cold Spring Harbour Protocols, 2*, a000398. https://doi.org/10.1101/cshperspect.a000398

Maggio, F., Rossi, C., Chaves-López, C., Serio, A., Valbonetti, L., Pomilio, F., Chiavaroli, A. P., & Paparella, A. (2021). Interactions between *L. monocytogenes* and *P. fluorescens* in dual-species biofilms under simulated dairy processing conditions. *Foods, 10*, 176. https://doi.org/10.3390/foods10010176

Mah, T. F., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology, 9*, 34–39. https://doi.org/10.1016/s0966-842x(00)01913-2

Martín-Espada, M. C., D'Ors, A., Bartolomé, M. C., Pereira, M., & Sánchez-Fortún, S. (2014). Peracetic acid disinfectant efficacy against *Pseudomonas aeruginosa* biofilms on polystyrene surfaces and comparison between methods to measure it. *Lebensmittel-Wissenschaft und -Technologie, 56*, 58–61. https://doi.org/10.1016/j.lwt.2013.11.013

Masotti, F., Cattaneo, S., Stuknytė, M., & De Noni, I. (2019). Airborne contamination in the food industry: An update on monitoring and disinfection techniques of air. *Trends in Food Science & Technology, 90*, 147–156. https://doi.org/10.1016/j.tifs.2019.06.006

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., Bhullar, K., Canova, M. J., De Pascale, G., Ejim, L., Kalan, L., King, A. M., Koteva, K., Morar, M., Mulvey, M. R., O'Brien, J. S., Pawlowski, A. C., Piddock, L. J., Spanogiannopoulos, P., … Wright, G. D. (2013). The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy, 57*, 3348–3357. https://doi.org/10.1128/aac.00419-13

Merino, N., Pagán, E., Berdejo, D., Pagán, R., & García-Gonzalo, D. (2021). Combining culturing technique and metabarcoding to study microbiota in the meat industry. In M. Magnani (Ed.), *Detection and enumeration of bacteria, yeast, viruses, and Protozoan in foods and freshwater* (pp. 37–51). Springer US. https://doi.org/10.1007/978-1-0716-1932-2_5.

Møretrø, T., Fanebust, H., Fagerlund, A., & Langsrud, S. (2019). Whole room disinfection with hydrogen peroxide mist to control *Listeria monocytogenes* in food industry related environments. *International Journal of Food Microbiology, 292*, 118–125. https://doi.org/10.1016/j.ijfoodmicro.2018.12.015

Møretrø, T., & Langsrud, S. (2017). Residential bacteria on surfaces in the food industry and their implications for food safety and quality. *Comprehensive Reviews in Food Science and Food Safety, 16*, 1022–1041. https://doi.org/10.1111/1541-4337.12283

Møretrø, T., Vestby, L. K., Nesse, L. L., Storheim, S. E., Kotlarz, K., & Langsrud, S. (2009). Evaluation of efficacy of disinfectants against *Salmonella* from the feed industry. *Journal of Applied Microbiology, 106*, 1005–1012. https://doi.org/10.1111/j.1365-2672.2008.04067.x

Nahar, S., Mizan, M., Ha, A., & Ha, S.-D. (2018). Advances and future prospects of enzyme-based biofilm prevention approaches in the food industry. *Comprehensive Reviews in Food Science and Food Safety, 17*, 1484–1502. https://doi.org/10.1111/1541-4337.12382

Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C., & Koutsoumanis, K. P. (2008). Meat spoilage during distribution. *Meat Science, 78*, 77–89. https://doi.org/10.1016/j.meatsci.2007.06.020

Odeyemi, O. A., Alegbeleye, O. O., Strateva, M., & Stratev, D. (2020). Understanding spoilage microbial community and spoilage mechanisms in foods of animal origin. *Comprehensive Reviews in Food Science and Food Safety, 19*, 311–331. https://doi.org/10.1111/1541-4337.12526

Oh, S. W., Gray, P. M., Dougherty, R. H., & Kang, D. H. (2005). Aerosolization as novel sanitizer delivery system to reduce food-borne pathogens. *Letters in Applied Microbiology, 41*, 56–60. https://doi.org/10.1111/j.1472-765X.2005.01711.x

Otter, J. A., Vickery, K., Walker, J. T., deLancey Pulcini, E., Stoodley, P., Goldenberg, S. D., Salkeld, J. A. G., Chewins, J., Yezli, S., & Edgeworth, J. D. (2015). Surface-attached cells, biofilms and biocide susceptibility: Implications for hospital cleaning and disinfection. *Journal of Hospital Infection, 89*, 16–27. https://doi.org/10.1016/j.jhin.2014.09.008

Park, S.-H., Cheon, H.-L., Park, K.-H., Chung, M.-S., Choi, S. H., Ryu, S., & Kang, D.-H. (2012). Inactivation of biofilm cells of foodborne pathogen by aerosolized sanitizers. *International Journal of Food Microbiology, 154*, 130–134. https://doi.org/10.1016/j.ijfoodmicro.2011.12.018

Puga, C. H., Dahdouh, E., SanJose, C., & Orgaz, B. (2018). *Listeria monocytogenes* colonizes *Pseudomonas fluorescens* biofilms and induces matrix over-production. *Frontiers in Microbiology, 9*, 1706. https://doi.org/10.3389/fmicb.2018.01706

Quijada, N. M., Rodríguez-Lázaro, D., Eiros, J. M., & Hernández, M. (2019). Tormes: An automated pipeline for whole bacterial genome analysis. *Bioinformatics, 35*, 4207–4212. https://doi.org/10.1093/bioinformatics/btz220

Quintieri, L., Caputo, L., Brasca, M., & Fanelli, F. (2021). Recent advances in the mechanisms and regulation of QS in dairy spoilage by *Pseudomonas* spp. *Foods, 10*, 3088. https://doi.org/10.3390/foods10123088

Rossi, C., Serio, A., Chaves-López, C., Anniballi, F., Auricchio, B., Goffredo, E., Cenci-Goga, B. T., Lista, F., Fillo, S., & Paparella, A. (2018). Biofilm formation, pigment production and motility in *Pseudomonas* spp. isolated from the dairy industry. *Food Control, 86*, 241–248. https://doi.org/10.1016/j.foodcont.2017.11.018

Santos Rosado Castro, M., da Silva Fernandes, M., Kabuki, D. Y., & Kuaye, A. Y. (2021). Modelling *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* biofilm formation on stainless steel surfaces and controlling through sanitisers. *International Dairy Journal, 114*, Article 104945. https://doi.org/10.1016/j.idairyj.2020.104945

Simões, M., Bennett, R. N., & Rosa, E. A. (2009). Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Natural Product Reports, 26*, 746–757. https://doi.org/10.1039/b821648g

Sofos, J. N., & Geornaras, I. (2010). Overview of current meat hygiene and safety risks and summary of recent studies on biofilms, and control of *Escherichia coli* O157:H7 in nonintact, and *Listeria monocytogenes* in ready-to-eat, meat products. *Meat Science, 86*, 2–14. https://doi.org/10.1016/j.meatsci.2010.04.015

Srey, S., Jahid, I. K., & Ha, S.-D. (2013). Biofilm formation in food industries: A food safety concern. *Food Control, 31*, 572–585. https://doi.org/10.1016/j.foodcont.2012.12.001

Trejo, M., Douarche, C., Bailleux, V., Poulard, C., Mariot, S., Regeard, C., & Raspaud, E. (2013). Elasticity and wrinkled morphology of *Bacillus subtilis* pellicles. *Applied Physical Science, 110*, 2011–2016. https://doi.org/10.1073/pnas.1217178110

Troller, J. A. (1993). Chapter 4 - cleaning. In J. A. Troller (Ed.), *Sanitation in food processing* (2nd ed., pp. 30–51). Academic Press. https://doi.org/10.1016/B978-0-12-700655-0.50009-2.

Tsiaprazi-Stamou, A., Monfort, I. Y., Romani, A. M., Bakalis, S., & Gkatzionis, K. (2019). The synergistic effect of enzymatic detergents on biofilm cleaning from different surfaces. *Biofouling, 35*, 883–899. https://doi.org/10.1080/08927014.2019.1666108

Wang, H., Cai, L., Li, Y., Xu, X., & Zhou, G. (2018). Biofilm formation by meat-borne *Pseudomonas fluorescens* on stainless steel and its resistance to disinfectants. *Food Control, 91*, 397–403. https://doi.org/10.1016/j.foodcont.2018.04.035

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology, 20*, 257. https://doi.org/10.1186/s13059-019-1891-0

Yuan, L., Sadiq, F. A., Wang, N., Yang, Z., & He, G. (2021). Recent advances in understanding the control of disinfectant-resistant biofilms by hurdle technology in the food industry. *Critical Reviews in Food Science and Nutrition, 61*, 3876–3891. https://doi.org/10.1080/10408398.2020.1809345