

Effect of gaseous ozone treatment on biofilm of dairy-isolated *Pseudomonas* spp. strains

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Abstract

Microbial biofilms existing in food industries have been implicated as important contamination sources of spoilage and pathogenic microorganisms in the finished products. Among the innovative strategies proposed to contrast biofilms in food environments, ozone is recognised as an environmentally friendly technology but there are few studies about its effect against bacterial biofilms. The objective of this study was to evaluate the effect of gaseous ozone (50 ppm for 6 h) in inhibition and eradication of biofilm formed by twenty-one dairy-isolated *Pseudomonas* spp. strains. Before ozone treatments, all isolates were screened for biofilm formation according to a previously described method. Strains were then divided in four groups: weak, weak/moderate, moderate/strong, and strong biofilm producers based on the biofilm biomass value of each isolate determined using the optical density (OD - 595 nm). Inhibition treatment was effective on the strain (C1) belonging to the weak producers' group, on all strains classified as weak/moderate producers, on two strains (C8 and C12) belonging to the group of moderate/strong producers and on one strain (C13) classified as strong producer. Conversely, eradication treatments were ineffective on all strains tested, except for the strain C4 which reduced its biofilm-forming abilities after exposure to ozone gas. In conclusion, gaseous ozone may be used to enhance existing sanitation protocols in food processing environments, but its application alone not seems sufficient to contrast *Pseudomonas* spp. established biofilms.

Introduction

In recent decades, it has become evident that, in different environments, bacteria grow mainly as biofilms on surfaces rather than in a planktonic state (Frank, 2001). Within the food industry, bacterial biofilms are commonly found on surfaces contacting with, or without foods (González-Rivas et

al., 2018). Biofilms allow bacteria to better withstand adverse environmental conditions and may represent a source of foodstuff contamination by spoilage and/or pathogenic microorganisms (Rossi et al., 2016). Biofilms formed by spoilage bacteria may be a cause of repeated product contamination, resulting in important hygiene issues and economic losses (Sofos and Geornaras, 2010). One of the most common biofilm-forming genus is *Pseudomonas* spp., that includes ubiquitous spoilage organisms with negative quality impact on foods (Muhammad et al., 2020). Dairy industries commonly have biofilms composed by *Pseudomonas* spp. isolates (Rossi et al., 2018). The capacity of bacteria of this genus to produce exopolysaccharide promotes the formation of biofilms on surfaces and protect the microorganisms from the standard hygiene procedures (Irie et al., 2010). *Pseudomonas* spp., in fact, can form stable multispecies biofilms with other pathogens (Chmielewski and Frank, 2003). *Pseudomonas* spp. can contaminate milk through the inadequate water supply and poor hygiene procedures during processing in the dairy industry (Eneroth et al., 2000). The contamination supported by *Pseudomonas* spp. is dicey since these bacteria can produce thermostable enzymes, such as proteases and lipases, which affect both the stability and durability of dairy products (Teh et al., 2014). In addition, *Pseudomonas* spp. can cause anomalous pigmentation in fresh dairy products, such as mozzarella cheese (Cenci-Goga et al., 2014). Several species belonging to the *Pseudomonas* genus are able to grow during cold storage of raw milk in dairy environments (De Jonghe et al., 2011). *Pseudomonas* biofilms can also contaminate previously processed milk (Kives et al., 2006). In addition, biofilm detachment during processing can contribute to the contamination of the finished product (Cleto et al., 2012) and post-processing contamination can cause cheese spoilage and shelf life reduction (Segat et al., 2014).

Currently, there is no control strategy capable of entirely preventing and/or eradicating biofilms. Appropriate hygiene procedures are the main strategies used to control bacterial biofilms within the food industry (Carrascosa et al., 2021). Among the innovative anti-biofilm strategies, ozone is considered a promising eco-friendly technology (Botondi et al., 2021). It has a very high oxidation potential and leaves no toxic residues because it decomposes into oxygen. It has powerful antimicrobial properties (Aponte et al., 2018). The bactericidal effect of ozone on a broad range of microorganisms has been tested, including bacteria,

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spores, fungi, and viruses (Sheng et al., 2018). In particular, the half-life and diffusion capacity of gaseous ozone molecules are higher compared to molecules in an aqueous state (Kim et al., 1999). In addition, gaseous ozone has been studied as a tool to inactivate the microbial spoilage on surfaces and airborne microorganisms in food storage chambers (Bigi et al., 2021). To date, the application of ozone gas as an antimicrobial agent for the decontamination of storage sites and/or food has been approved by several countries (Brodowska et al., 2018; Segat et al., 2014). Italian

Ministry of Health (2010) the application of gaseous ozone in empty cheese ripening rooms, whereas the use on food is not allowed (Bigi *et al.*, 2021). To our knowledge, there are few studies that have investigated the effect of ozone in gaseous form against microbial biofilm. Thus, the aim of this study was to evaluate the anti-biofilm activity of ozone gas against *Pseudomonas* spp. to assess its potential use and effectiveness against biofilm produced by these critical food spoilage organisms.

Materials and Methods

Bacterial strains

A total of twenty-one *Pseudomonas* spp. strains isolated from dairy industries were obtained from the Bacterial Culture Collection of the Department of Veterinary Sciences, University of Turin (Table 1). Before the experiments, each strain was inoculated twice in 10 ml of Tryptic Soy Broth (TSB, Oxoid, Milan, Italy) and incubated at 25°C for 24 h. The grown cultures were used for inoculation into the wells of plastic microplates for subsequent quantification of biofilm production.

Screening of biofilm forming strains: micro-method assays

The methodology used to assess the biofilm-forming abilities of the isolates was based on the modified microtiter plate test, as proposed by Stepanović *et al.* (2007). Briefly, 200 µL of bacterial cultures, serially diluted to a concentration of 6 Log CFU/mL in TSB, were distributed (three wells for each strain) in 96-well microtiter plates (Sarstedt, Nümbrecht, Germany). Control wells were prepared with the uninoculated TSB. Plates were incubated at 25 °C for 24 h. Subsequently, the supernatant was discarded, and wells were washed thrice with 300 µL of sterile PBS (Phosphate buffer solution). Biofilms were heat-fixed (60°C for 60 min) and stained with 150 µL of 2% crystal violet (CV; Merck, Germany) for 20 min. After staining, wells were rinsed under running tap water. Therefore, the microplate was air dried, and the crystal violet was resolubilized with 150 µL of 95% ethanol (Honeywell, USA) per well. The optical density (OD) of wells was measured at 595 nm with a microtiter-plate reader (iMark plate reader, Bio-Rad, Sydney, NSW, Australia). The strains were classified as weak ($OD-C < OD-S \leq 2 \times OD-C$), moderate ($2 \times OD-C < OD-S \leq 4 \times OD-C$), strong ($4 \times OD-C < OD-S$) and no ($OD-S \leq OD-C$) biofilm producers following Stepanović *et al.* (2007).

Ozonization assays

The anti-biofilm effect of ozone was evaluated in 96-wells flat bottom polystyrene microtiter plates (Sarstedt) in an ozone-inert plexiglass chamber (Biofresh Group Ltd., Northumberland, UK) connected to an ozone generator (Model-LF5; Biofresh Group Ltd.). The injection of ozone gas in the chamber was regulated by an ozone analyzer (UV-100, EcoSensor, Santa Fe, USA). During each treatment, a fan was placed in the chamber to obtain a good distribution of the gas and containers with water were placed on the bottom to maintain high relative humidity ($\geq 90\%$). During the treatments, temperature and relative humidity were monitored with a data logger (Testo 174 H, Testo AG, Lenzkirchen, Germany). Experiments were performed in triplicate and at room temperature. Considering the literature data and the higher resistance to oxidative stress of cells in the sessile state compared to the planktonic forms (Bialka and Demirci, 2007; Botta *et al.*, 2020; Guzzon *et al.*, 2015; Marino *et al.*, 2018; Panebianco *et al.*, 2021), the treatments were performed at 50 ppm for 6 hours.

Biofilm Inhibition by ozone gas

This experimental phase was carried out to evaluate the ability of ozone gas to influence the biofilm forming abilities of iso-

lates. For this purpose, *Pseudomonas* spp. isolates were preliminary exposed to the ozone before the incubation of plates and subsequent analysed for biofilm formation. The potential preventive action of ozone gas to inhibit biofilm formation was assessed following the protocol previously described (micro-method assay) using polystyrene tissue culture plates (96 wells). After incubation, ODs (595nm) were quantified as described before. A new classification of the strains according to the formula proposed by Stepanović *et al.* (2007) was performed, to check if bacteria reduced their biofilm-forming capacities after the ozone exposure.

Biofilm Eradication by ozone gas

In this case, the treatments were carried out to evaluate the effect of ozone gas against established biofilms. Also in this case, we modified the previously described protocol (micro-method assay) including an additional step (the ozone gas treatment). In details, revitalized cultures were diluted, poured in polystyrene microtiter plates, and incubated at 25°C for 24 h to allow the biofilm formation. After incubation, TSB (Oxoid) was removed from each well and the cells organized in biofilm were exposed to ozone gas. After treatment, ODs (595nm) were quantified as described before. A new classification of the strains according to the

Table 1. *Pseudomonas* spp. strains used in the present study.

Strain ID	Source	Identification
C1	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C2	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C3	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C4	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C5	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C6	Preserving liquid	<i>Pseudomonas fluorescens</i>
C7	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C8	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C9	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C10	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C11	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C12	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C13	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C14	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C15	Mozzarella cheese	<i>Pseudomonas putida</i>
C16	Preserving liquid	<i>Pseudomonas fluorescens</i>
C17	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C18	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C19	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C20	Mozzarella cheese	<i>Pseudomonas fluorescens</i>
C21	Mozzarella cheese	<i>Pseudomonas fluorescens</i>

formula proposed by Stepanović *et al.* (2007) was performed, to check if bacteria reduced their biofilm-forming capacities after the ozone exposure.

Statistical analysis

The frequency distribution of strains in different OD values ranges obtained with the micro-method assay was calculated with socstatistics (<https://www.socscistatistics.com/>), while graphing was performed with GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, California, USA).

Results

Screening of biofilm forming strains

All strains were able to produce biofilm. In details, the majority of isolates (20/21; 95% of all strains) were strong biofilm producers while one strain (5% of the total) was classified as moderate biofilm producer according to the formula proposed by Stepanović *et al.* (2007). A mean OD value (595 nm) of 1.240 ± 0.350 (from 0.385 ± 0.070 for strain C1 to 1.829 ± 0.224 for strains C6) was detected. Single OD values of all strains are reported in Figure 1. To understand the action of ozone exposure in relation to the biofilm-forming capacities of the strains, a further grouping of the isolates was based on the frequency distribution of the strains in four ranges based on the OD (595 nm) values obtained after the micro-method assay. All isolates were then arbitrarily divided in four classes, as reported in Table 2. Most of the strains were included in the two intermediate classes, weak/moderate (OD range= 0.780-1.179) and moderate/strong (OD range = 1.180-1.579) producers, one strain was classified as weak producer (OD range = 0.385-0.779), and four strains were considered strong biofilm producers (OD range = 1.580-1.979).

Anti-biofilm effect of ozone gas

Considering the Stepanović *et al.* (2007) classification, inhibition treatment with gaseous ozone was effective on the only strain (C1) belonging to the weak producers' group, since it was classified as non-biofilm producer after the treatment. Ozone was unable to eradicate the pre-formed biofilm of this strain (Table 3). Considering our classification based on OD value ranges (Table 2), for the group of weak/moderate producers, inhibition treatments reduced the biofilm forming abilities of all strains, since these were classified in the Stepanović *et al.* (2007) classification as strong before ozone treatment and as moderate producers after ozone exposure. Even for this group, the eradication treatments

Table 2. Classification of *Pseudomonas* spp. strains in 4 classes based on the OD values ranges.

Class (OD range)	Strains	%
Weak (0.385-0.779)	C1	4.8
Weak/moderate (0.780-1.179)	C3, C4, C7, C11, C14, C18, C19, C20, C21	42.9
Moderate/strong (1.180-1.579)	C2, C5, C8, C10, C12, C15, C16	33.3
Strong (1.580-1.979)	C6, C9, C13, C17	19

Table 3. Classification of *Pseudomonas* spp. strains before and after ozone treatments according to Stepanović *et al.* (2007). Classes of *Pseudomonas* spp. according to the OD ranges (Table 2) are highlighted as follows: *weak, **weak/moderate, °moderate/strong, °°strong.

Strain ID	Classification	Classification after ozone treatments	
		Inhibition	Eradication
C1*	Moderate	Non producer	Moderate
C2°	Strong	Strong	Strong
C3**	Strong	Moderate	Strong
C4**	Strong	Moderate	Moderate
C5°	Strong	Strong	Strong
C6°°	Strong	Strong	Strong
C7**	Strong	Moderate	Strong
C8°	Strong	Moderate	Strong
C9°°	Strong	Strong	Strong
C10°	Strong	Strong	Strong
C11**	Strong	Moderate	Strong
C12°	Strong	Moderate	Strong
C13°°	Strong	Moderate	Strong
C14**	Strong	Moderate	Strong
C15°	Strong	Strong	Strong
C16°	Strong	Strong	Strong
C17°°	Strong	Strong	Strong
C18**	Strong	Moderate	Strong
C19**	Strong	Moderate	Strong
C20**	Strong	Moderate	Strong
C21**	Strong	Moderate	Strong

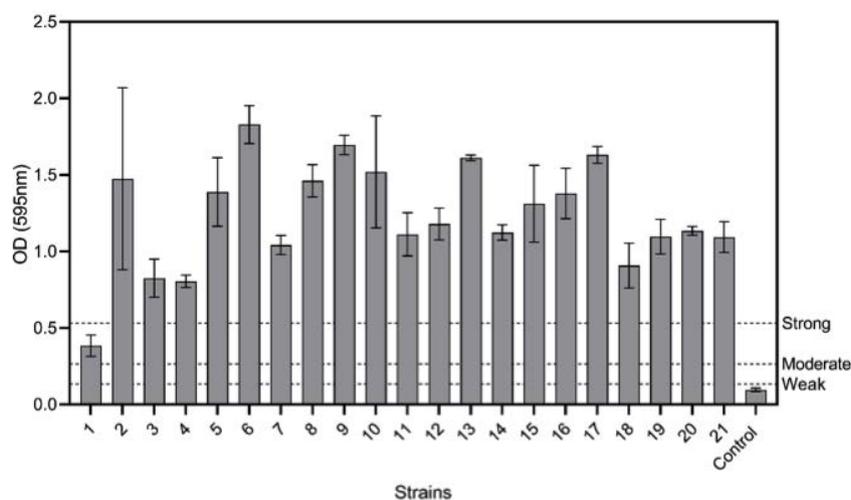


Figure 1. OD values of *Pseudomonas* spp. strains after the micro-method assay. Error bars indicate the standard deviation between three replicates.

were ineffective, except for the strain C4 which reduced its biofilm-forming abilities after exposure to ozone gas (Table 3). Regarding the group of moderate/strong producers, a reduction of biofilm forming abilities was observed only for two strains (C8 and C12) after inhibition treatments, while ozone gas was no effective on pre-formed biofilm of these strains (Table 3). Among the strains grouped in the strong biofilm producers' class, inhibition treatments led to a reduction of biofilm forming capacities of only one strain (C13) that changed from strong to moderate, while eradication treatments were never effective (Table 3).

Discussion

The presence of microbial biofilm represents a critical issue for the food industry, since it could occur also in well designed, constructed, and maintained factories. Biofilm growth on surfaces in dairy plants causes economic losses for the passage of bacteria into the final products, problems related to food deterioration and safety, difficulties during the cleaning operations in dairy farms or in processing plants (Teh *et al.*, 2014).

The ability to adhere to solid surfaces and the consecutive formation of an organised bacterial biofilm community are important steps in the establishment of *Pseudomonas* spp. in dairy manufacturing plants (Rossi *et al.*, 2016). In regard to this, *Pseudomonas* strains are common in dairy environments, they produce great quantities of EPS and are able to adhere on stainless steel surfaces forming biofilms. They can form resistant multispecies biofilms with other pathogens (Chmielewski and Frank 2003). *Pseudomonas* spp. is responsible for several changes in the appearance and aroma of food products, including milk and dairy products (Reichler *et al.*, 2018). Therefore, in food processing environments the inactivation and removal of *Pseudomonas* spp. isolates capable of forming biofilms deserve great attention. Rossi *et al.* (2016) with a study on 64 *P. fluorescens* strains originating from dairy product and dairy plant showed that 89% of the isolates were able to form biofilm at both 10 and 30°C after 48 h. Different strategies to avoid biofilm development have been used in the food industry. The main target has been on preventing bacterial contamination through physical and chemical treatments. Nevertheless, concerns have been posed about both the efficacy and safety of these methods, leading to the research and devel-

opment of new tools to counteract biofilm formation. In this context, research for new substances for the control of biofilm formation on food contact surfaces is an important area of focus. The growing negative consumer perception against artificial synthetic chemicals, however, has shifted this research effort toward the development of alternatives, environmentally friendly substances. Among alternative biocides, ozone is considered a promising eco-friendly technology (Baumann *et al.*, 2009). The use of ozone has been proved to be efficient as an antimicrobial approach in different contexts, such as medical, agricultural, marine, and food (Bigi *et al.*, 2021). Besides the well-established antimicrobial action of ozone against planktonic microorganisms, in recent years this action was also confirmed against biofilm embedded microorganisms (Marino *et al.*, 2018). It is demonstrated that gaseous ozone usually requires higher concentrations and exposure time to achieve an antimicrobial efficacy comparable with aqueous ozone (Bialka and Demirci 2007). Previous experiments, in fact, demonstrated that the antimicrobial efficacy of treatments with gaseous ozone against planktonic and sessile cells is linked to the concentration used and the exposure time. In regard to this, Guzzon *et al.* (2015) demonstrated that exposure up to 6 h to gaseous ozone reduced the microbial loads on wooden shelves used for the ripening of typical Italian cheeses. A study conducted by Botta *et al.* (2020) in slaughterhouses showed that gaseous ozone is an efficient adjunct sanitizing method if applied at high concentrations (20 and 40 ppm for 12 h). In this study, the effect of ozone gas treatment on biofilm of *Pseudomonas* spp. isolates and its potential application as an effective anti-biofilm tool was investigated. Based on literature data and considering that bacteria in biofilm state are known to express a higher resistance to oxidative stress compared to the planktonic forms (Panebianco *et al.*, 2021), ozone assays were performed with an high concentration (50 ppm) of gas for 6 hours. Firstly, all isolates were classified as biofilm producers: weak, moderate or strong producers following a previous described protocol (Stepanović *et al.*, 2007). The anti-biofilm effect of ozone was assessed by using 96-well plates to simulate the hard-to-reach areas within food processing environments, where microorganisms can easily persist as biofilm. With regard to this, the tests were carried out in TSB broth during the inhibition assay and keeping the residual TSB broth in the wells during the eradication treatments. Residual TSB broth simulated the organic matter that, in food processing environments, may persist on

surfaces or niches after routine cleaning and disinfection procedures. Based on our results, ozone gas affected the biofilm-forming abilities in strains belonging to groups classified, in our subsequent classification, as weak and weak/moderate producers. The effect after the inhibition assay may indicate a reduced capacity of cells to produce the extracellular polymeric matrix after a preliminary exposure to oxidative stress. However, the effect appeared to be less in strains included in the moderate/strong and strong producers' classes, which therefore showed a greater biofilm-forming capacity. These results suggests that the action of ozone gas is partial and strain-dependent, since it is linked to the biofilm-forming capacity of each strain. This kind of strain-dependent response to oxidative stress was recently observed also in a study conducted on *Listeria monocytogenes*, where the action of ozone gas in high concentration (50 ppm) was partial and variable among different strains (Panebianco *et al.*, 2021). Results were not satisfactory after eradication treatments, indicating that a preformed biofilm is very hard to counteract with gaseous ozone. In this case, we hypothesize that the presence of biological material derived from biofilm matrix and dead cells (biofilm biomass) might have represented the first target of the ozone activity, acting as a protective shield to oxidative damages.

This study suggests that ozone gas alone not seems sufficient to contrast *Pseudomonas* spp. established biofilms in food context. However, ozone may be applied as an additional tool to promote the optimal sanitization of surfaces within food industries. The translation of these findings in real industrial conditions is still difficult to predict, since the penetration capacity and thus the anti-biofilm activity of gaseous ozone could be countered by many factors such as the complexity of food implants (scratches and crevices), the relative humidity in food processing environments, and the presence of residual debris. Despite the unquestionable advantages of gaseous ozone as an anti-microbial agent with a low environmental impact, there are several concerns linked to the corrosion potential on materials and food equipment. In addition, we must emphasise that exposure to high levels of ozone could cause negative effects on human health. Therefore, the application of high concentrations in food industry is acceptable during the weekly closing days, in the absence of operators or by using destructive devices capable of quickly bringing ozone levels within safe limits (Botta *et al.*, 2020; Marino *et al.*, 2018; Panebianco *et al.*, 2022).

For all these reasons, further investigations aiming to confirm the anti-biofilm performances of gaseous ozone at high concentrations in real industrial and/or commercial conditions will be strongly required.

Conclusions

The present study intended to evaluate the anti-biofilm activity of ozone gas against *Pseudomonas* spp. in order to assess its potential application and effectiveness against biofilm produced by this critical food spoilage organism. Our findings showed that ozone gas (50 ppm for 6 h) alone not seems efficient on *Pseudomonas* spp. established biofilm, while its action was improved in biofilm inhibition treatments. In addition, effect of ozone gas was variable among biofilm-forming *Pseudomonas* spp. isolates. In view of this, gaseous ozone, used as an adjunct to the existing good manufacturing practice-cleaning regime, may be useful to improve the control of *Pseudomonas* spp. biofilm. Anyway, further studies are needed to evaluate the ozone gas performances in different experimental conditions and to assess the corrosion of equipment over time in food environments.

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