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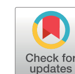
Clean-in-place disinfection of dual-species biofilm (*Listeria* and P*seudomonas*) by a green antibacterial product made from citrus extract



Andrea C. Medina-Rodríguez[a], Alejandro Ávila-Sierra[a], Juan J. Ariza[b], Enrique Guillamón[b], Alberto Baños-Arjona[b], José M. Vicaria[a,*], Encarnación Jurado[a]

[a] *Chemical Engineering Department, Faculty of Sciences, University of Granada, Avda. Fuentenueva, s/n, 18071, Granada, Spain*
[b] *DMC Research Center, Camino de Jayena, 82, 18620, Alhendín, Granada, Spain*

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ABSTRACT

Food industries, which must ensure safety and quality of manufactured products, require effective and regular cleaning and disinfection processes. One of the most difficult issues to overcome is to ensure the elimination of biofilms that can generate contamination during food processing. Despite chemical disinfection is the most used strategy, it also requires to be optimised, reducing energy costs, time, and the environmental impact of these operations. In this work, the effectiveness of an environmental-friendly commercial disinfectant (Mico E-PRO®) made from food-grade citrus extracts has been addressed by two methods (i) *in-vitro* tests for evaluation of antimicrobial efficacy and (ii) a lab-simulated CIP system, where the efficacy was compared with NaClO 1%. In both cases, Mico E-PRO® shows that it is an effective product for the killing of *E. coli* (MBC = 625 ppm), *L. monocytogenes* (MBC = 625 ppm), *S. enterica* (MBC = 625 ppm) and *P. aeruginosa* (MBC = 156.25 ppm), being *P. aeruginosa* the most sensible to the disinfectant. The product also shows good bactericidal effect against a mature biofilm formed by *L. innocua* and *P. putida* – similar efficacy to that obtained with NaClO 1%. Overall, results demonstrated that Mico E-PRO® is a good option as a natural sanitiser for CIP systems, running as an efficient and safe alternative to the traditional chemicals-based disinfectants.

1. Introduction

Biofilms, which may resist to antimicrobial and cleaning products, have become a severe problem in food industries (Srey et al., 2013). Biofilm formation has evolved as an adaptive strategy of microorganisms to survive to aggressive environments that, inside of processing lines, may generate sources of recurrent contamination, as well as other undesirable issues such as reduction of both flow rate and efficiency, increased cleaning costs, and even surface corrosion (Parkar et al., 2004). Despite the most of bacterial species have the ability to form biofilm, its resistance to both removal and disinfection depends on several factors such as surface characteristics and biofilm composition (Papaioannou et al., 2018), being necessary to understand inhibitory mechanisms under extreme conditions (Plakunov et al., 2017). Some species are more resistant to biocides than others (e.g. *L. monocytogenes* develops higher tolerance to cleaning and disinfection over time for classic disinfectants as peracetic acid or quaternary ammonium compounds disinfectants (Fagerlund et al., 2017)). Therefore, cleaning and disinfection of processing lines are complex processes that require special attention for the elaboration of well-designed sanitation programmes.

For sanitation protocols, the combination of chemical action, mechanical forces, temperature, and time is necessary to achieve the required cleaning and disinfection levels. In industry, Cleaning-In-Place (CIP) systems are used to ensure high and recurrent cleanliness of processing lines. Detergents, which are generally formulated to remove soils (e.g. proteinaceous, fatty, carbohydrate or mineral soils), have a limited effect on biofilm removal (Wirtanen et al., 1996). For instance, acidic and alkaline formulations produce a significant reduction in the viability of cells (Furukawa et al., 2010), being the alkaline one more effective on biofilms detaching (Kumari & Sarkar, 2014). Therefore, the cleaning phase is responsible for an initial biofilm removal estimated between 1 and 3 log orders of microorganisms (Bremer et al., 2006).

In spite of achieving an effective cleaning phase, significant numbers of microorganisms could remain on surfaces or spread through the installation, requiring a disinfection stage for total inhibition. Within the methods to inactivate or remove biofilms from food processing facilities are included physical (e.g. electric fields and ultrasonics) and chemical methods, being the chemical ones the most commonly used for controlling pathogenic and spoilage of microorganisms.

* Corresponding author.
E-mail address: vicaria@ugr.es (J.M. Vicaria).

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Table 1
References and uses of the strains used.

Strain	Reference	Use	Isolation
Listeria monocytogenes	CECT 4032	In vitro test	Clinical isolate of meningitis associated with food intake
Escherichia coli	CECT 405	In vitro test	Clinical isolate
Salmonella enterica	CECT 7160	In vitro test	Clinical isolate of infection associated with food intake
Pseudomonas aeruginosa	CECT 116	In vitro test	Animal room water bottle
Listeria innocua	CECT 910	In vitro test and biofilm formation	Enviromental sample
Pseudomonas putida	DSM 12264	In vitro test and biofilm formation	Sludge of wastewater treatment system

Unfortunately, some species can survive to repeated processes (e.g. *Listeria monocytogenes* (Colagiorgi et al., 2017)), in where dual-species biofilms with foodborne pathogens are even more resistant to disinfectants than their single species. In fact, biofilm maturity, the presence of food waste, as well as other environmental factors might play an important role in their response to chemicals treatments (Kocot & Olszewska, 2020). Therefore, special attention should be paid to the biofilm formation in order to design sanitation protocols.

Previous works demonstrated the importance of undertaking laboratory disinfectant tests under appropriate in-use conditions – only some of the commercial disinfectants achieved the effectiveness reported (Taylor et al., 1999). If disinfection is not effective, microorganisms will remain at concentrations that may affect product integrity. The regular use of chemical disinfectants could also lead to several disadvantages such as surface damage, high water consume, as well as high environmental impact. Therefore, it is required to develop new environmental-friendly disinfectant which can inactivate cells and improve biofilm removal without deteriorating equipment surfaces or food attributes.

Nowadays, compounds extracted from plants such as essential oils, phenolic compounds, flavonoids and other related substances, which have a strong antimicrobial activity, are being investigated (Fancello et al., 2020; Wang et al., 2020). These products can damage both the cell wall and the membrane, inducing cell lysis and facilitating the leakage of cellular content - organic acids can pass the cell membrane and acidify the cell cytoplasm causing lethal cell damage (Harich et al., 2017). Previous works highlighted the effect of essential oils and citrus extracts against typical methods of disinfection. For instance, Vetas et al. (2017) studied the disinfection efficiency of sage and spearmint essential oils against planktonic and biofilm of *Staphylococcus aureus* cells in comparison with sodium hypochlorite. Vázquez-Sánchez et al. (2018) also found that *Lippia sidoides*, *Thymus vulgaris* and *Pimenta pseudochariophyllus* oils were highly effective against both planktonic cells and biofilm of *L. monocytogenes*. Furthermore, the application of oils in binary combinations decreased the required doses to kill 99.99% of biofilm cells. Thus, disinfection products as Mico E-PRO® (DOMCA SA, Spain), that contains bioactive compounds synthesised naturally by plants, are used for sanititation of food (e.g. vegetables, fruits and IG range food) and food-contact surfaces. On the other hand, García-Heredia, Orue, Heredia, and García (2013) studied six commercial antimicrobial formulations based on citrus extracts against *Salmonella* biofilm, achieving to inhibit 75% of *Salmonella* formation. Castillo et al. (2015) reported that the treatment of biofilms of *Campylobacter jejuni* with both polyphenolic compounds and a disinfectant based on citrus substances reduced the activity of the bacteria from 60% to 99%, with respect to the control sample. The results mentioned above are a stimulus for future studies of essential oils and citrus extracts for controlling biofilm formation, avoiding poisoning and formation of carcinogens compounds such as trihalomethanes (Bachelli et al., 2013), preserving equipment (Tang et al., 2010), and reducing the environmental impact of sanitation processes.

This work highlights the efficiency as CIP disinfectant of a commercial product (Mico E-PRO®) made from citrus extracts against both six different strains (*Listeria monocytogenes*, *Listeria innocua*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa* and *Pseudomonas putida*) and a mature dual-species biofilm formed by *Listeria innocua* and *Pseudomonas putida*. Mico E-PRO® is a food-grade product that does not need rising after application. This study was addressed by using two approaches: *in-vitro* tests for evaluation of the antimicrobial efficacy, and a lab-simulated CIP system for analysing the disinfectant power of the product on both the remaining attached biofilm and the migrated cells to the disinfection water.

2. Materials and methods

2.1. Bacterial strains and used media

The used microorganisms were obtained from CECT (Spanish Type Culture Collection) and DSMZ (Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures) (Table 1). Target strains are originally from food and stored at −70 °C. Different culture media were supplied by Biokar Diagnostics: Cefalotina, Fusidic acid, Cetrimida (CFC) used for the selective isolation of *Pseudomonas* spp; MacConkey Agar used for the growth of enterobacteria and the chromogenic medium Compass Listeria for the isolation and selective growth of *Listeria* spp. and the Tryptic Soy Broth (TSB) was used for biofilm formation.

2.2. Disinfectants tested

Mico E-PRO®, a food-grade product consisting of an extract from bitter orange (*Citrus aurantium*), containing antibacterial flavonoids as naringenin and stabilisers such as glycerin, lactic acid and citric acid (provided by DOMCA SA, Spain) has been used as disinfectant of interest. 1% v/v NaClO aqueous solution (VWR, Pensilvania, USA) has also been used.

2.3. In-vitro tests for the evaluation of the antimicrobial activity

In-vitro models (agar diffusion method, minimun bactericidal concentration, and time-kill curves) were used to determine the effectiveness of Mico E-PRO® against microorganisms.

For agar difussion method (Calvo & Asensio, 1999), all bacterial cultures were grown on selective mediums Cefalotina, Fusidic acid, Cetrimida (CFC) agar, chromogenic medium Compass Listeria and MacConkey agar plates (Biokar Diagnostics) for *Pseudomonas* spp, *Listeria* spp, and Enterobacteriaceae (*E. coli* and *S. enterica*), respectively, at 37 °C for 24 h. Steril cellulose disc (Whatman® Antibiotic Assay Discs 6 mm diameter) impregnated with the tested product (15 μl Mico E-PRO® pure/disc) was placed on the plates centre of culture media. The inhibition zone of bacterial growth is proportional to the degree of inhibition produced. All assays were made in duplicate.

The MBC (Minimum Bactericidal Concentration) is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by a pre-determined reduction such as 99.9%. The broth microdilution method was used in this study as recommended by the National Committee for Clinical Laboratory Standards (CLSI, 2012); decreasing concentrations (10000; 5000; 2500; 1250; 625; 315.5; 156.25; 78.125; 39.06; 19.53 and 9.76 ppm) of the antimicrobial agent, generally in dilutions 1:2 (in

peptone water) and inoculated with the different bacterial strains at final concentration of 5 log CFU/mL (approx.). A well without antimicrobial product was inoculated with bacteria and used as positive control. An another well neither antimicrobial nor bacteria was used as negative control. Each well in which there was no cell growth (measured by absorbance at 620 nm) was tested by sub-culturing to selective agar plates and incubated at 37 °C for 24 h to determine the MBC. All assays were made in duplicate.

Time-kill curves were done following the procedure described by Guerillot et al. (1993). Different tests were carried out on buffered peptone water (Biokar Diagnostics), starting from an initial inoculum of bacteria adjusted to 6–8 log CFU/mL (depending on strain). A collection of tubes with different Mico E-PRO® concentrations (78.125; 156.25; 312.5; 625 and 1000 ppm) was used. A tube without the product is used as a control. At different time intervals, samples were collected (5, 15, 30, 60, 120 min, and 5 and 24 h) and then serially diluted, plated in selective agar and incubated at 37 °C for 24–48 h. Kill curves are represented by expressing the results of log CFU/ml against time. The limit of detection of methods of count used is 0.3 log CFU/mL. All assays were made in duplicate.

2.4. Substrate and biofilm formation for CIP system

The substrate, which is the surface where the bacteria are retained, was made by stainless steel fibres AISI 410 (diameter 2.0–2.1 cm, weight 0.80–0.81g, fibre width 0.51 mm) (Vicaria et al., 2018). Porous spheres were selected due to the complexity encountered to remove the adhered soil. The soiling agent used is a mature biofilm formed by *Listeria innocua* CECT 910 and *Pseudomona putida* DSM 12264 (these strains were used for safety reasons in order to avoid contamination of the facilities where the device was placed).

The scheme of the method for substrate sterilization and incubation of microorganisms is shown in Fig. 1. For biofilm formation, stainless steel spheres were previously cleaned with an aqueous solution of 1% Triton X-100 at room temperature. The residual detergent was removed with sterile distilled water. Then, spheres were soaked into a solution of 70% ethanol for 10 min and rinsed again with sterile water, and finally sterilised in an autoclave at 121 °C for 15 min. Once sterilised, they were placed in groups of 8 units inside of sterile bottles with 80 mL of 0.89% NaCl aqueous solution with *L. inocua* and *P. putida* (approximate concentration of 8 log CFU/ml). The bottles were kept static and incubated at 4 °C for 24 h to facilitate the bacteria fixation (Tan et al., 2017). After incubation, the remaining suspension was slowly removed by a sterile Pasteur pipette. Spheres were immersed and gently shaken in 80 mL of sterile distilled water for 5 s. The washed spheres were transferred to 200 mL flasks, containing 80 mL of TSB and maintained at 28 °C for 10 days under static conditions to develop the biofilm.

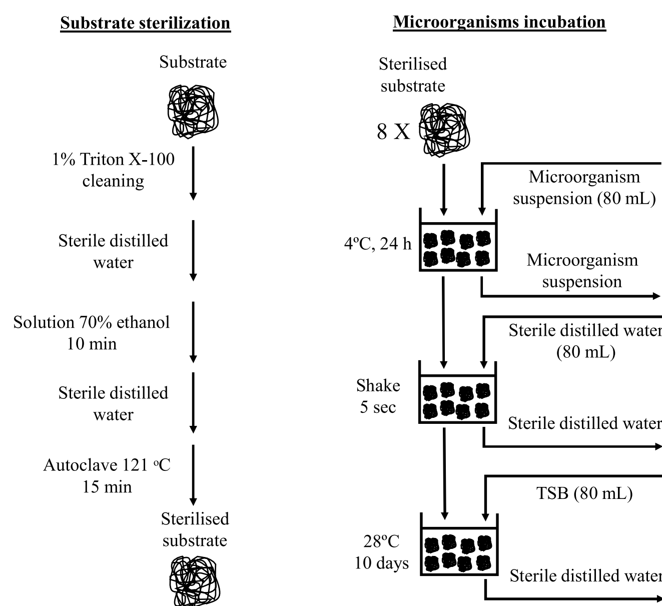

Fig. 1. Substrate sterilization and microorganism incubation on substrate.

2.5. Enumeration of biofilm cells

The spheres with the formed biofilm were gently washed with sterile saline solution to remove unattached cells. Then, each sphere was placed into 3 mL of sterile saline solution where by shaking vigorously for 30 s, adhered cells were removed from the surface. To quantify the number of viable cells on the biofilm, serial decimals dilutions of the cell suspension were made and plated on different culture media. *L. innocua* (COMPASS Listeria) was incubated at 37 °C and *P. putida* (CFC Agar) at 30 °C. Finally, plates were incubated for 48 h before colony counting: initial concentration of inoculum is shown in Table 2. When it was not possible to quantify the bacteria below the detection limit (< 0.3 log CFU/mL), an investigation was carried out by pre-enrichment in TSB (Biokar Diagnostics) expressing the results as presence or absence.

2.6. Biofilm removal method

The biofilm removal process was studied in a lab-simulated CIP system denominated Bath-Substrate-Flow (BSF) (Jurado-Alameda et al., 2015). This device allows modification of the main factors in both cleaning and disinfection processes (e.g. temperature, chemical agent, soiling agent, flow rate, and substrate). A scheme of BSF is illustrated in Fig. 2. The device is formed by a jacketed tank (1) containing the cleaning/disinfection solution (volume 1L); a pump (model 5006, Heidolph) (2) which supplies a recirculation flow of 60L/h; and a column (3) with a capacity of 50 mL (diameter = 2.5 cm, height = 8.5 cm) where the substrate is placed and thermostatically controlled by a water bath (4) (model Ultraterm, P-Selecta) (Avila-Sierra et al., 2020). The disinfection solution (1.2 L) used, 2.5% Mico E-PRO® or 1% NaClO aqueous solutions, was added to the BSF and pumped through the circuit (thermally controlled at 40 °C). Eight spheres with the formed biofilm are placed into the column to begin the experiment. Samples are taken from the tank at 10, 20 and 30 min to measure the bacterial concentration as a function of time. Once the disinfection process is finished, the device is stopped and spheres collected to determine the number of viable biofilm cells. Furthermore, a study of the disinfection water at different processing times (10, 20, and 30 min) was done to quantify the number of viable bacteria. At the end of each cleaning test, disinfection of the BSF is carried out by recirculating a 1% NaClO aqueous solution for 10 min, following by a three-cycle cleaning with water at 50 °C for 5 min. A control test was made using the same conditions that the disinfection test, substituting the Mico E-PRO® solution by distilled water.

3. Results and discussion

The formation of biofilms is a common problem in the food industry, constituting a potential risk of cross-contamination since they can favour the growth of pathogenic and spoilage microorganisms (Brooks & Flint, 2008). Techniques such as industrial CIP and disinfection are regularly used to reduce the number of harmful bacteria. In order to evaluate the effectiveness of Mico E-PRO® upon those processes, this work analyses (i) the disinfectant power of the product against different food-related microorganisms, and (ii) the biofilm disinfection achieved on both the remaining attached biofilm and the migrated cells to the disinfection water.

Table 2
Data show the reduction of each strain before and after treatment (40 °C, 30 min). Experimental results are expressed as log (CFU/mL). Experiments with preenrichment of culture medium were performed in cases where the detection limit was less than 0.3 log CFU/mL, the results being expressed as presence or absence.

Strain	Initial inoculum	Control test	NaClO 1%	Mico E-PRO® 2.5%	NaClO 1% (*)	Mico E-PRO® 2.5% (*)
L. innocua	7.5 ± 0.9	5.9 ± 0.3	< 0.3	< 0.3	Absence (0%)	Absence (0%)
P. putida	7.9 ± 1.0	6.5 ± 0.9	< 0.3	< 0.3	Absence (0%)	Presence (18%)

[*] survival percentage formula: Survival percentage = (number of spheres with bacterial presence) · 100/(number of total spheres).

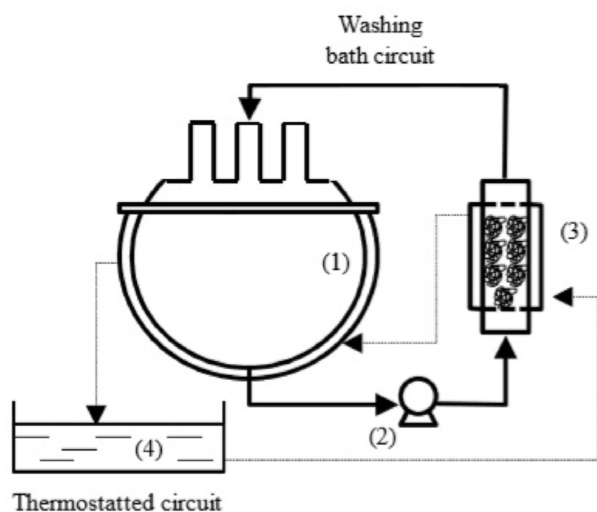

Fig. 2. Scheme of BSF device including a jacketed tank (1), pump (2), substrate column (3), and a thermostatically controlled bath (4).

3.1. In-vitro tests for the evaluation of the antimicrobial activity

The effectiveness of Mico E-PRO® as a disinfectant has been tested against the collection of strains detailed in Table 1. *L. monocytogenes*, which is implicated in most of the frequent contamination routes through cross-contamination from food-contact surfaces (Porsby et al., 2008), as well as other sources of potential risk like Gram-negative bacteria (e.g. *Pseudomonas aeruginosa* and *Escherichia coli*) have been selected. For the agar diffusion test, different concentrations of Mico E-PRO® (pure, 5%, and 2%) were tested against *L. monocytogenes*, *L. innocua*, *E. coli, S. enterica*, *P. putida* and *P. aeruginosa*. Table 3 shows the obtained results as diameter of the inhibition zone. As expected, the inhibition zone is greater when the pure product is used - *Pseudomonas* spp. (*P. aeruginosa* and *P. putida*) are the most sensitive microorganism with an inhibition zone of 40 mm. Reduced inhibition areas were observed by decreasing the product concentration, where *Pseudomonas* spp. remain as the most sensitive microorgamism with inhibitions areas of 17 and 13 mm for Mico E-PRO® concentrations of 5 and 2%, respectively. The bacteria that showed the highest resistance to the product was *L. monocytogenes,* with a 9 mm inhibition zone at 2% dose. Table 3 also details the results obtained in the MBC tests. Mico E-PRO® reduces microbial counts below the limit of detection (0.3 log CFU/mL) of *E. coli, S. enterica, L. innocua* and *L. monocytogenes,* at a concentration of 625 ppm for 24 h. On the other hand, *Pseudomonas* spp were more sensitive with a total elimination at 156.25 ppm.

Time-kill curves give additional information about the biocidal action and the relationship between the concentration of the antimicrobial agent and its biocidal activity - they were plotted as log CFU/mL as a function of time. All the bacteria strains tested are susceptible to Mico E-PRO®. Fig. 3 shows a decrease of the initial cells concentration as a function of time at different product concentrations (312.5, 625.0 and 1000 ppm). Fig. 3A and B shows how the initial concentration of both *E. coli* inoculum (5.8 log CFU/mL) and *S. enterica* (6.8 log CFU/ml) decreased up to < 0.3 log CFU/mL at 24 h for all the concentrations tested. Mico E-PRO® reduces the *E. coli* concentration between 30 and 60 min at 625 ppm, while at 1000 ppm, total disinfection is reached below 30 min. For *S. enterica*, disinfection time is reduced considerably (5–10 min at 625 ppm, and below 5 min at 1000 ppm). On the other hand, Fig. 3C shows how the initial inoculum concentration of *L. monocytogenes* decreases with time (7.9 log CFU/mL) up to < 0.3 log CFU/mL at 24 h and 1000 ppm. At 625 ppm and 312.5 ppm of product, there is still 0.6 log CFU/mL and 2 log CFU/mL of the microorganism (referred to 24 h), respectively. *P. aeruginosa* presented the highest reduction during the first 5 min, requiring times between 30 and 60 min for achieving a complete disinfection at 156.25 ppm. The initial inoculum concentration of *P. aeruginosa* (Fig. 3D) decreased (7.5 log CFU/mL) up to < 0.3 log CFU/mL, at 24 h, for all the tested concentrations (78.125, 156.25 and 312.5 ppm). *L. monocytogenes* was the most resistant cell of the four-group tested, requiring both longer exposure time and higher disinfectant concentration than other type of cells to complete inactivation.

To achieve good reduction levels, the reduction of microorganisms should be at least 5-log cycles (EN1040, 2006; EN1276, 2000). The good performance of Mico E-PRO® highlighted above remarks its high disinfection effectiveness comparing with some of the common chemical-based disinfectants. For example, for planktonic cells of *P. aeruginosa*, it was observed that orthophthalaldehyde (OPA), peracetic acid (PAA), and NaClO were effective at the first minute (reduction of 100%, 99.98%, and 99.99%, respectively), while H_2O_2 was effective at the 5th minute (100%) (Kose & Yapar, 2017). The concentrations used were 0.55% OPA, 0.3% PAA, 5% H_2O_2 and 1% NaClO. Despite this work uses much lower product concentrations (< 0.1%), similar disinfection levels are reached, verifying its good effectiveness against the strains tested.

Table 3
Result of the antibiosis tests on solid medium and minimum bactericidal concentration (MBC) of Mico E-PRO® against reference strains.

Strain	Reference	MBC (ppm)	Inhibition zone (mm)		
			Mico E-PRO® Pure	Mico E-PRO® 5%	Mico E-PRO® 2%
Listeria monocytogenes	CECT 4032	625	30 ± 1	12 ± 1	9 ± 1
Listeria innocua	CECT 910	625	30 ± 1	12 ± 1	10 ± 1
Escherichia coli	CECT 405	625	36 ± 0	15 ± 0	12 ± 0
Salmonella enterica	CECT 7159	625	34 ± 2	14 ± 0	11 ± 1
Pseudomonas aeruginosa	CECT 116	156.25	40 ± 1	17 ± 1	13 ± 1
Pseudomonas putida	DSM 12264	156.25	40 ± 2	17 ± 1	13 ± 1

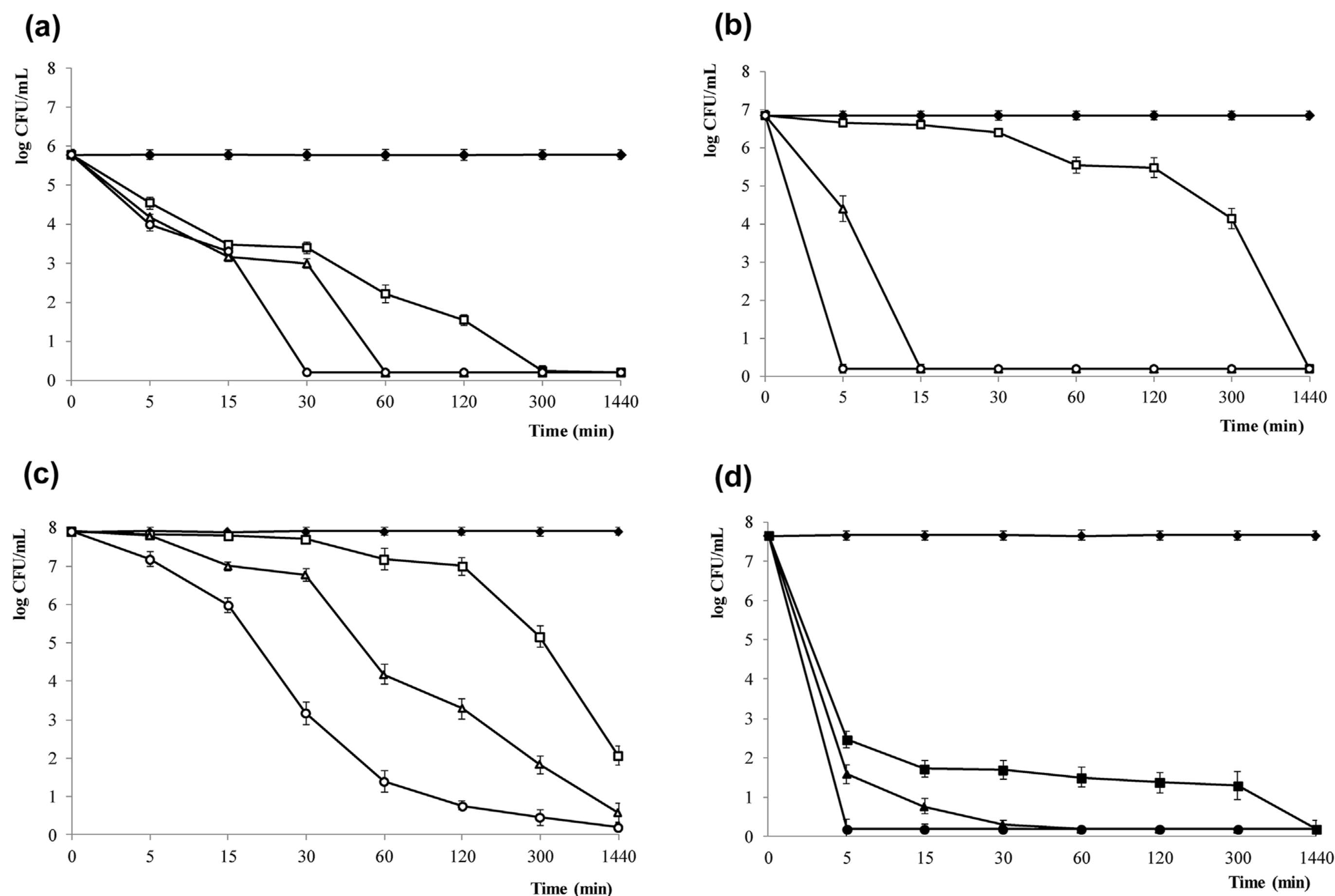

Fig. 3. Viability evolution. 3A) *E. coli*; 3B) *S. enterica* CECT 7160; 3C) *L. monocytogenes* CECT 4032; and 3D) *P. aeruginosa* CECT 116 in presence of Mico E-PRO®. Concentrations tested: ◆ 0 ppm (of each strain); □ 312.5 ppm; Δ 625 ppm; and ○ 1000 ppm of Mico E-PRO®. For *P. aeruginosa*, the concentrations Mico E-PRO® tested are ■ 78.13 ppm; ▲ 156.25 ppm; and ● 312.5 ppm.

3.2. Biofilm disinfection in a lab-simulated CIP

Microorganisms adhered to food-contact surfaces are more resistant to disinfection than planktonic cells (Królasik et al., 2010), requiring the use of higher concencetration of disinfectant to produce good cell inactivation (Robbins, Fisher, Molts & Martin, 2005). Among the disinfectants mentioned above (OPA, PAA, NaClO, and H_2O_2), NaClO was the most effective disinfectant against *P. aeruginosa* biofilm (99.8% decrease of cells at 60 min Kose & Yapar, 2017)) – all the disinfectant showed values over 92% of reduction at 60 min. Toté, Horemans, Berghe, Maes, and Cos (2010) also reported that H_2O_2 and NaClO were effective against both *P. aeruginosa* biofilm matrix and the live microbial cells in the biofilm layers. However, PAA was the least effective disinfectant against live cells (Tote et al., 2010). Other works showed that H_2O_2 efficacy could be enhanced by combination with a strong acid (peracetic acid), ethanol (Perumal et al., 2014), or followed by ozone (Tachikawa & Yamanaka, 2014). Against *Listeria monocytogenes* biofilm, Cabeça et al. (2012) reported that NaClO and PAA were the most effective disinfectants, while biguanide, iodine, and quaternary ammonium compounds disinfectants were the least effective. Taylor et al. (1999) also reported that the efficacy of some products might be compromised by certain conditions such as low temperatures and the presence of organic residues - temperature has a marked effect on biocidal efficacy (Gelinas et al., 1984).

The disinfectant effect of Mico E-PRO® on a mature biofilm (described in section 2.4) has been verified by simulating CIP industrial conditions (flow, time and temperature) in a BSF device, and comparing the results with NaClO 1%, used as a control. To avoid the risks involved from working with cells such as *L. monocytogenes*, and similar to that done in other works (Perni et al., 2006; Vaz-Velho et al., 2001), *Listeria innocua* and *Pseudomonas putida* were selected for biofilm formation. The conditions used for biofilm formation (saline solution and temperature) were selected to ensure a good cell adhesion to the stainless steel fibres.

To evaluate how the BSF flow (60 L/h) can affect the biofilm dettachment due to the migration of microorganisms from the biofilm to the washing water, a control test using distilled water was carried out at 40 ºC and 30 min (Table 2). There was a bacterial run-off to the washing water, decreasing the number of viable *L.innocua* and *P.putida* cells on the stainless steel spheres from 7.5 to 7.9 to 5.9 and 6.5 log CFU/ml, respectivately. The reduction is within the range reported in literature after the cleaning phase (between 1 and 3 log orders of microorganisms from surfaces) (Bremer et al., 2006). No inactivation was noticed after water treatment.

Table 2 shows the results obtained in the CIP disinfection tests. Tests were performed in duplicate, using in each assay 8 spheres with biofilm to know the initial microorganism concentration, and other 8 spheres for each disinfection test. The initial inoculum concentration for *Listeria innocua* and *Pseudomonas putida* were 7.5 log CFU/mL and 7.9 log CFU/mL, respectively. The disinfection results showed a complete initial reduction (< 0.3 log CFU/mL) for *L. innocua* and *P. putida* when both NaClO solution and Mico E-PRO® 2.5% are used in parallel. According to the recommended by FDA (U.S. Food and Drug Administration), 1% NaClO is a successful disinfectant for short contact times. In fact, disinfection with NaClO 1% aqueous solution, at 40 °C and 30 min, produces total disinfection of the microorganisms involved in the biofilm

Table 4
Evolution of the viability of *L. innocua* CECT 910 and *P. putida* DSM 12264 in the washing water after disinfection at different times (40 °C). Results expressed as log (CFU/mL).

Test	10 min		20 min		30 min	
Strain	*L. innocua*	*P. putida*	*L. innocua*	*P. putida*	*L. innocua*	*P. putida*
Control	5.58	2.12	5.82	4.23	5.82	5.08
Mico E-PRO® 2.5%	Absence	Absence	Absence	Absence	Absence	Absence
NaClO 1%	Absence	Absence	Absence	Absence	Absence	Absence

formation (shown as absence in Table 2). On the other hand, despite Mico E-PRO® (Mico E-PRO® 2.5% aqueous solution, at 40 °C and 30 min) achieved total absence of *L. innocua* in 100% of the spheres, and *P. putida* was recovered in 3 out of the 16 spheres after the confirmation test.

Finally, a study of the disinfection water at different processing times (10, 20, and 30 min) was done to quantify the level of biofilm disinfection. In Table 4 can be seen both the microorganisms concentration and the disinfectant level achieved. The cells of *P. putida* inside of biofilm matrix are more resistant then the *L. innocua* ones. Disinfection of L. *innocua* biofilm is not significantly affected by increasing of the disinfection time. Comparing the achieved results with the performance of other chemicals-based formulations ready-to-use for food industry, Mico E-PRO® shows a good performance. For example, Królasik et al. (2010) studied a comercial product (useable concentration 0.5–1%) formed by hydrogen peroxide (25–30%) and peroxyacetic (2–5%) acid for inhibition of planktonic cells. For 0.5% and 10 min, a reduction levels of viable cells of *L. innocua* of nearly 6 log CFU/mL, and *P. putida* of more than 7 log CFU/mL, were observed. However, they reported low effectiveness of this disinfectant for biofilm inhibition. There was a reduction of 1–2 and 3–4 log CFU/cm^2 at 0.5% and 10 min and 30 min, respectively. Mico E-PRO® 2.5% showed both effective biofilm disinfection of the cells adhered to stainless steel, and total killing of the cells into the disinfection water. Therefore, this product could be a promising alternative to common chemical-based disinfectants for CIP purposes, achieving a better grade of industrial sustainability.

4. Conclusions

Biofilm formation into food-processing lines might be a source of potential contamination, compromising both quality and safety of processed products. As an environmental-friendly alternative to the use of typical disinfectants, the disinfection effectiveness of a food-grade product composed by citrus extract rich in flavonoids (Mico E-PRO®) has been tested. The product demonstrated a good bactericidal activity of broad-spectrum, being effective in the elimination of strains such as *E. coli, L. monocytogenes* and *S. enterica*. *Pseudomonas* spp. was the most sensitive bacteria to this product with a MBC value of 156.25 ppm. Moreover, a continuous disinfection process has also been performed in a simulated CIP system to compare the disinfectant effect of Mico E-PRO® against a traditional method (NaClO). The product showed good bactericidal effect against *L. innocua* and *P. putida* - part of a mature biofilm developed on stainless steel fibres - obtaining similar results to that of the achieved by sodium hypochlorite. Overall, results demonstrated that Mico E-PRO® is a good option as a natural sanitiser for CIP systems, running as an efficient and safe alternative to the traditional chemical-based disinfectants.

CRediT authorship contribution statement

Andrea C. Medina-Rodríguez: Validation, Formal analysis, Investigation, Resources, Writing - original draft, Visualization. **Alejandro Ávila-Sierra:** Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization. **Juan J. Ariza:** Methodology, Validation, Formal analysis, Visualization, Supervision. **Enrique Guillamón:** Conceptualization, Supervision. **Alberto Baños-Arjona:** Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing, Supervision. **José M. Vicaria:** Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Encarnación Jurado:** Conceptualization, Project administration, Funding acquisition.

Declaration of competing interest

None.

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