

Antimicrobial activity of olive mill wastewater extract against *Pseudomonas fluorescens* isolated from mozzarella cheese

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Abstract

Olive mill wastewater polyphenol extract was tested for antimicrobial activity against 64 strains of *Pseudomonas fluorescens* responsible for mozzarella discoloration. The extract showed a minimum inhibitory concentration (MIC)₅₀ value of 5 mg/mL and a MIC₉₀ value of 7 mg/mL. The MBC₅₀ and MBC₉₀ values corresponded to 6 and 8 mg/mL, respectively. The MIC concentration (7 mg/mL) was demonstrated to have a bacteriostatic effect while maintaining the bacterial concentration on the levels of the inoculum for 48 hours. The 3/2 MIC concentration was responsible for four logs CFU/mL depletion in colony count after 24 h. As the extract concentration decreased from MIC value, no inhibitory effects were recorded.

Introduction

Microbial spoilage is a major concern in the food industry, as microbial contamination and growth can result in undesirable changes in appearance, texture, flavour and odour that reduce food quality or cause food loss (Sperber and Doyle, 2009). The *Pseudomonas* spp. group is strictly connected to food spoilage, especially for animal-derived foods like meat, poultry, milk and fish, and its importance is related to the food trade and hygienic standards (Liao, 2006). Because of the psychrotrophic and mesophilic characteristics of these bacteria, they can replicate at refrigeration temperatures, and long periods of shelf-life can easily increase the *Pseudomonas* concentration in food (Andreani *et al.*, 2014; Marchand *et al.*, 2009; Munsch-Alatossava *et al.*, 2005). Specifically, *Pseudomonas fluorescens* represents part of the main microflora of raw milk

and dairy products that can be contaminated via defiled water and soil, inadequately sanitised milking surfaces, storage and transporting equipment (Munsch-Alatossava *et al.*, 2005; Chiesa *et al.*, 2014). Heat-stable lipases and proteases produced by *Pseudomonas fluorescens* and other similar pseudomonads are a cause of milk spoilage and are responsible of casein breakdown, bitterness and ropiness due to the production of slime and coagulation of proteins (Jay, 2000).

Interest in the aforementioned bacteria in dairy products increased after the case of *blue mozzarella* that occurred in 2010 when some European consumers noted a blue discoloration on some mozzarella products (Martin *et al.*, 2011; Andreani *et al.*, 2014). This colour change was due to the production by *P. fluorescens*: under certain conditions, a blue, non-diffusible pigment as well as the soluble pigment pyoverdine fluoresces under UV light. Despite all the efforts made by mozzarella manufacturers to avoid or minimise contamination, it is difficult to eradicate *P. fluorescens* even with extraordinary sanitation processes due to its remarkable adaptability and ability to produce biofilm (Sperber and Doyle, 2009). Hence, there is a strong need for alternative methods that are able to limit bacterial growth during the product's shelf-life.

Currently, there is a worldwide effort to minimise the use of chemical preservatives because of consumer preferences towards more natural and healthier products. Consequently, we are seeing an increasing scientific interest in the search for natural antimicrobial compounds like biopreservatives (Serra *et al.*, 2008). In recent decades, research has shown the potential exploitation of plant products as a bioactive compound source of industrial interest (Prado Martin *et al.*, 2013). The application of natural antimicrobial compounds in food could be useful to prevent the growth of spoilage microorganisms such as *Pseudomonas* spp. (Gammariello *et al.*, 2008). Different by-products derived from olive oil extraction process are rich in phenolic compounds (PC), which have demonstrated possible application in feed and food industry (Branciarì *et al.*, 2015). Among natural products, olive-mill wastewater (OMW), a by-product of the olive oil extraction process, could be an alternative source for biologically active phenols compounds that can be used in the food industry. The phenol substances present in OMW are aglycon derivatives: the dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA or p-HPEA (3,4-DHPEA-EDA or p-HPEA-EDA), the isomer of oleuropein aglycon (3,4-DHPEA-EA), the lygstroside aglycon (p-HPEA-EA) and the hydroxycinnamic acid derivative verbascoside (Servili *et al.*, 2011). These substances are proven to inhibit or delay the rate of growth of a range of bacte-

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ria, so that they may be used as alternative food additives (Pereira *et al.*, 2006). The aim of the study is to evaluate antimicrobial activity of PC extracted from OMW against different *P. fluorescens* strains responsible of mozzarella discoloration.

Materials and Methods

Purification and evaluation of phenolic compound from olive mill wastewater

In order to obtain OMW rich in phenols to be used for the production of polyphenolic extract (PE), Italian *Olea europaea* cultivars with a high content of PC were selected (*Moraiolo* cultivar). The OMW PE was produced according to Servili *et al.* (2011). The PC evaluation was performed with high-performance liquid chromatography (HPLC) analysis using an Agilent Technologies Model 1100 consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, a diode array detector (DAD) set at 278 nm (Esposto *et al.*, 2015).

Bacterial strains, culture media

P. fluorescens ATCC 13525 and 64 strains of *P. fluorescens* stored in the Institute for

Experimental Veterinary Medicine of Umbria and Marche collection (IZSUM-PF no. 259-299, 310-334) were tested to evaluate the antimicrobial effect of the PE; the 64 strains derived from fresh *pasta filata* cheeses that exhibited the blue pigment defect. The stored strains were revitalised on Brain Heart Infusion Broth (BHI; cod. 4012302; Biolife Italiana s.r.l., Milan, Italy), incubated at $25\pm 1^\circ\text{C}$ for 24 hours under aerobic conditions.

Dilution of polyphenolic extract

The PE, stored at -20°C was characterised by a creamy consistency and had a colour between yellow and orange. The physical characteristics of the extract meant that it could not be used directly. For this reason, in order to increase the solubility of all its components, it was necessary to dilute it with an ethanol/water 60/40 (v/v) solution (mother solution, maintained at a temperature of $+4^\circ\text{C}$). At the time of use, the mother solution was diluted again to obtain the concentration of polyphenols and ethanol required by the protocol for the evaluation of the antimicrobial effect. The ethanol used was purchased from Carlo Erba Reagents s.r.l. (cod. 414605; Milan, Italy).

In vitro evaluation of antimicrobial effects of polyphenolic extract

In order to test the antibacterial effect of PE, minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined. The tests were conducted using a standard microdilution technique according to Clinical Laboratory Standards Institute guidelines adapting the protocol as suggested by Othman *et al.* (2011).

The bacterial suspension to be used for the assay was prepared as previously reported, adjusting the number of bacteria to approximately 1×10^5 CFU/mL with fresh BHI. The phenolic extract concentrations tested were 8, 7, 6, 5, 4 and 3 mg/mL of total PC. Moreover, for each bacterial strain four controls were set up. These included the antibacterial effect of ethanol (using the same concentrations

already present in the extract), antibiotic control (with ciprofloxacin), organism control (wells containing BHI and the bacterial suspension), negative control (wells containing BHI and the solution of polyphenols at the same concentration tested).

At time 0, the microplates were read in optical density at 620 nm using a spectrophotometer (Multiskan FC Thermo Scientific, Vantaa, Finland) and immediately incubated for 18 to 20 hours at $25\pm 1^\circ\text{C}$ in aerobic conditions. After the incubation time the second reading at 620 nm was performed. MIC was defined as the lowest concentration of extract that produced no bacterial growth when compared to time 0 wells. MIC₅₀ and MIC₉₀ were defined as the extract concentration that inhibits 50 and 90% of the isolates, respectively. Moreover, in order to define the MBC, 100 μL of the well's content was plated on Penicillin Piramicin Agar (PPA; cod. 4019602; Biolife Italiana S.r.l.) dishes then incubated for 24 hours at $25\pm 1^\circ\text{C}$ in aerobic conditions. The MBC was represented as the smallest amount of PE that was capable of killing the bacterial inoculum, demonstrated by the total absence of growth. MBC₅₀ and MBC₉₀ were defined as the extract concentration, which kills 50 and 90% of the isolates, respectively.

In vitro evaluation of death kinetics of *Pseudomonas fluorescens* with different concentrations of polyphenolic extract

The bacterial survival curve was studied over a range of multiples MICs, 1/10 MIC, 1/5 MIC, 1/2 MIC, MIC, 3/2 MIC on four randomly selected strains of *P. fluorescens* already tested for the evaluation of antimicrobial effect of PE. The assay was carried out based on the method reported by Bubonja-Sonje *et al.* (2011) with some modifications. The stored strains were revitalised in BHI incubated at $25\pm 1^\circ\text{C}$ for 24-48 h under aerobic conditions. The bacterial suspensions were separately enumerated and pooled together before the inoculum. The starting inoculum was prepared adjusting it to

approximately 1×10^5 CFU/mL with fresh BHI broth. Sterile 96-well microtiter plates were added with 80 μL of inoculum and 80 μL of extract to produce final concentration equivalent to 1/10 MIC, 1/5 MIC, 1/2 MIC, MIC, 3/2 MIC; negative (wells containing BHI) and positive (wells containing BHI and bacterial suspension) controls were also included.

The plates were incubated at $25\pm 1^\circ\text{C}$ and, at predetermined time points (0, 3, 9, 12, 24, 48 h), a 100 μL sample was removed from each well with test suspension, 10-fold serially diluted in sterile peptone water (cod. A1702HA; Diagnostic International Distribution s.p.a., Milan, Italy) and plated on PPA for colony count determination. The experiment was performed in duplicate and replicated three times, and the data obtained were averaged and plotted as log CFU/mL vs time. Growth curves of *P. fluorescens* pooled strains, under different extract concentrations, were built using DMFit version 2015 (ComBase online freeware) by fitting data to the Baranyi and Robertes's model (Baranyi and Roberts, 1994). From the obtained curves, the duration of lag phase (λ), maximum growth rate (μ_{max}) and final value were calculated. Data were then compared by one-way ANOVA followed by post hoc Tukey tests. The significance were set at $P < 0.05$. When bacteria were not detected at the level of 1.0 log CFU/mL, a value of -0.5 log CFU/mL was assigned (Ranucci *et al.*, 2014).

Results and Discussion

Polyphenolic extract composition

The content of each PC of the extract was characterised by 12.3 ± 0.4 mg/g of p-HPEA (tyrosol), 56.5 ± 1.1 mg/g of 3,4 DHPEA (hydroxytyrosol), 80.0 ± 4.1 mg/g of verbascoside and 532.5 ± 9.8 of 3,4 DHPEA-EDA. The total amount of PC corresponded to 681.3 ± 10.7 mg/g (Figure 1 and Table 1).

In the extract used, the most represented molecule was 3,4-DHPEA-EDA. Furthermore, the variability of phenols content in OMW is reported in literature (Dermeche *et al.*, 2013).

Table 1. Phenolic compounds detected on the olive mill wastewater polyphenol extract.

Compounds	m/z	Molecular formula	References
3,4-DHPEA hydroxytyrosol	153	$\text{C}_8\text{H}_{10}\text{O}_3$	Di Maio <i>et al.</i> (2011); De Marco <i>et al.</i> (2007)
p-HPEA Tyrosol	137	$\text{C}_8\text{H}_{10}\text{O}_2$	Di Maio <i>et al.</i> (2011); De Marco <i>et al.</i> (2007)
3,4-DHPEA-EDA	377	$\text{C}_{19}\text{H}_{22}\text{O}_8$	Di Maio <i>et al.</i> (2013); De Marco <i>et al.</i> (2007)
Verbascoside	623	$\text{C}_{29}\text{H}_{36}\text{O}_{15}$	De Marco <i>et al.</i> (2007); Obied <i>et al.</i> (2007); Dermeche <i>et al.</i> (2013)

The differences in the extract composition may vary greatly depending on factors such as cultivar, geographical origin, the ripening of the fruit, the milling technique and the extraction method (El-Abbassi *et al.*, 2012; Pereira *et al.*, 2006; Vinha *et al.*, 2005; Dermeche *et al.*, 2013).

In vitro evaluation of antimicrobial effects of polyphenolic extract

The antimicrobial effect of the PE was examined by broth microdilution susceptibility assay using different concentrations on 65 strains of *P. fluorescens*. The inhibitory effect of each of the six dilutions of PE tested (3, 4, 5, 6, 7, 8 mg/mL) is reported in Table 2. As shown, the MIC₅₀ value was 5 mg/mL, MIC₉₀ was 7 mg/mL, and MBC₅₀ and MBC₉₀ values corresponded to 6 and 8 mg/mL, respectively.

Differences in the antimicrobial effect among strains were registered, as shown in Table 2. Five mg/mL of the extract killed the 15% of the strains tested, whereas the concentration able to kill 100% of the bacteria tested was 8 mg/mL. Fasolato *et al.* (2015) found that bactericidal effect of PE was exerted when 6

mg/mL was used, but only two *P. fluorescens* strains were tested. The composition of the extract could also influence the bacterial dynamics and resistance mainly for the difference in the compounds and their structure. Bisignano *et al.* (1999) reported higher MIC values for oleuropein than hydroxytyrosol tested on several ATCC bacteria, and Obied *et al.* (2007) found that the antibacterial effect against Gram+, Gram- bacteria and yeasts was exerted more by hydroxytyrosol than by oleuropein. Although both compounds have an o-diphenol system, which is responsible of antibacterial activity, these compounds have lower inhibitory effects than their products of hydrolysis (Medina *et al.*, 2013). The antibacterial activity is mainly related to the dialdehydic form of decarboxymethyl elenolic acid, free or linked to tyrosol or hydroxytyrosol (Medina *et al.*, 2009). The PE tested was characterised by a relative high amount of 3,4 DHPEA-EDA, in turn characterised by the elenolic acid structure linked to hydroxytyrosol, which has a strong antimicrobial activity similar to that of commercial biocide (Medina *et al.*, 2013).

The results obtained confirm that *P. fluo-*

rescens exerts a certain resistance to both synthetic (Langsrud *et al.*, 2003) and natural biocides (Fasolato *et al.*, 2015; Medina *et al.*, 2009), but they also show that not all the strains have the same resistance. Furthermore, Gutierrez-Larrainzar *et al.* (2013) tested synthetic PC (thymol, carvacrol, eugenol, hydroquinone, *p*-hydroxybenzoic acid and gallic acid) on *P. fluorescens*, and found a significant intraspecific variability for some of the molecules tested.

In vitro evaluation of death kinetics of *Pseudomonas fluorescens* with different concentrations of polyphenolic extract

The results of the death kinetic data and their estimation are reported in Figure 2 and Table 3.

The use of different extract concentrations resulted in different levels of *P. fluorescens* inhibition.

The addition of the PE extract at 1/10 MIC showed no difference when compared to PC for all the parameters considered. A longer than

Table 2. Minimum bactericidal concentration and minimum inhibitory concentration of the polyphenolic extract for the sixty-five *Pseudomonas fluorescens* strains tested (number of strains and overall percentage).

Concentration (mg/mL)	MIC	MBC		
	Number of strains inhibited	Percentage of strains inhibited	Number of strains killed	Percentage of strains killed
8	65	100	65	100
7	65	100	53	82
6	53	82	36	55
5	41	63	10	15
4	11	17	0	0
3	0	0	0	0

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

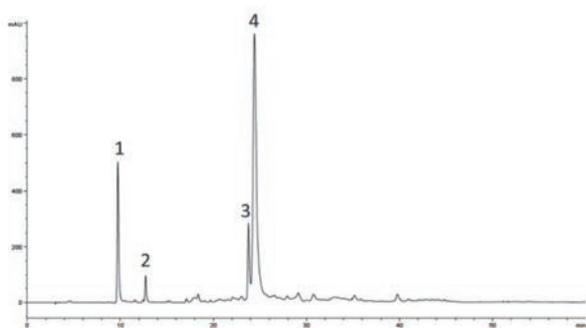


Figure 1. High-performance liquid chromatogram of the extract from olive mill wastewaters polyphenol recorded with diode array detector at 278 nm. Peak numbers: 1, hydroxytyrosol (3,4-DHPEA); 2, tyrosol (*p*-HPEA); 3, verbascoside; 4, dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA).

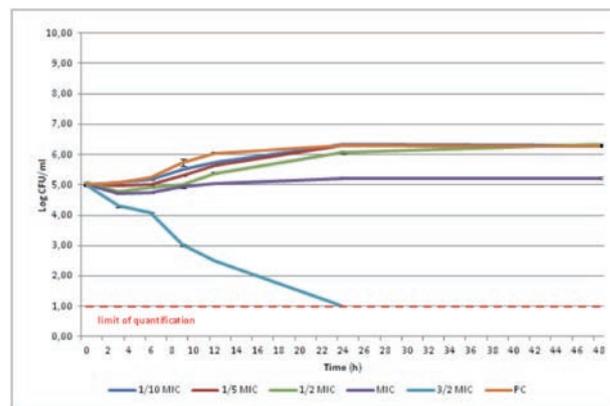


Figure 2. Growth of the *Pseudomonas fluorescens* strains with different concentrations of polyphenolic extract (average values for four pooled strains tested in triplicate \pm standard deviation). MIC, minimum inhibitory concentration; PC, phenolic compound.

Table 3. Estimation of the death kinetic parameters using the Baranyi-Roberts model.

	PC	1/10 MIC	1/5 MIC	1/2 MIC	MIC	3/2 MIC	SEM	P
Initial value (log CFU/mL)	5.01	5.01	5.00	4.91	4.82	4.95	0.023	0.167
λ (h)	4.17 ^b	4.33 ^b	7.02 ^c	7.66 ^c	7.72 ^c	0.94 ^a	1.089	<0.001
μ_{max} (log CFU/mL/h)	0.14 ^c	0.10 ^{bc}	0.13 ^c	0.07 ^b	0.06 ^b	-0.22 ^a	0.055	<0.001
Final value (Log CFU/mL)	6.29 ^c	6.31 ^c	6.28 ^c	6.32 ^c	5.22 ^b	-0.05 ^a	1.037	<0.001
R ² value	0.993	0.995	0.995	0.929	0.52 ^d	0.942		
SE of fit	0.047	0.039	0.040	0.159	0.143	0.240		

PC, positive control; MIC, minimum inhibitory concentration; λ , lag phase; μ_{max} , maximum growth rate; SEM, standard error of mean; CFU, colony forming unit; SE, standard error. ^{a-c}Different letters in the same row denote difference between the means (P<0.05).

the PC was observed from 1/5 MIC onwards. Nevertheless, the μ_{max} decreased only when the concentration used was higher than 1/2 MIC. Despite these differences, the final values were similar among PC, 1/10 MIC, 1/5 MIC and 1/2 MIC, as confirmed in Figure 2 after 24 hours. As the extract concentration decreased from MIC value no inhibitory effects was recorded.

The MIC concentration resulted in extended λ and reduced μ_{max} compared to PC. Furthermore, the inoculum *P. fluorescens* concentration was similar to the final value (initial value=4.82 and final value=5.22). The 3/2 MIC concentration had a noticeable effect on *P. fluorescens* death kinetics and was responsible for four logs CFU/mL depletion in colony count after 24 h, reaching the limit of quantification of the method (10 CFU/mL). At this concentration the μ_{max} was negative and significantly different from the other concentration used. Furthermore, the value was the lowest recorded because this PE concentration has a lethal effect on the strains tested already after 3 hours. No study on bacterial death kinetics of PE on *P. fluorescens* is reported in the literature. Serra *et al.* (2008) described the study of bacterial growth kinetics in the presence of different concentrations of olive extract, showing no inhibition of growth for *E. coli* and poor inhibition for *Salmonella pomona*, while for *Bacillus cereus* the extract exerts a dose dependent inhibition. Servili *et al.* (2004) did not find a significant reduction on lactic bacteria growth of yogurt added with 0.1-0.2 mg/mL PE. The dose-dependent effect was observed also by Bubonja-Sonje *et al.* (2011), who tested total olive oil polyphenols on *Listeria monocytogenes* growth.

Conclusions

The OMW extract shows a dose-dependent antimicrobial effect against *P. fluorescens*, even though remarkable differences among the tested strains were observed. The results of the death kinetic study on *P. fluorescens* could be of utmost interest in the use of such

compounds in limiting the growth of these spoilage bacteria. The use of this extract needs further investigation to better define the real potential of this promising natural preservative and its most suitable applications in food industry.

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