



Short communication

Antimicrobial efficacy of a polyphenolic extract from olive oil by-product against “Fior di latte” cheese spoilage bacteria

Rossana Roila^a, Andrea Valiani^b, David Ranucci^{a,*}, Roberta Orteni^b, Maurizio Servili^c, Gianluca Veneziani^c, Raffaella Branciarì^a

^a Dipartimento di Medicina Veterinaria, Università degli Studi di Perugia, Via San Costanzo 4, 06126 Perugia (PG), Italy

^b Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, “Togo Rosati”, Via Salvemini 1, 06126 Perugia (PG), Italy

^c Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Borgo XX Giugno 74, 06121 Perugia (PG), Italy

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ABSTRACT

This study aimed to evaluate a polyphenol extract from olive oil by-product, to improve the preservation of “Fior di latte” cheese during storage. Nine batches of “Fior di latte” cheese were manufactured in a local dairy production plant: three batches treated with 250 µg/mL of phenols from olive oil by-products in the governing liquid; three batches treated with 500 µg/mL of phenols in the governing liquid, and three untreated batches as the control group. The phenols effectively retarded the growth of *Pseudomonas fluorescens* and *Enterobacteriaceae*, acting mainly on the lag phase of the microorganisms, resulting in the prolongation of the time needed to reach the acceptable microbial limit. The delay in the growth of the spoilage bacteria was considered to indirectly influence the sensorial acceptability threshold, recorded through survival analysis. The combination of the two above-mentioned factors promotes the extension of “Fior di latte” cheese shelf life. by > 2 and 4 days, for PA and PB respectively.

1. Introduction

Olive oil extraction produce a variety of solid and liquid wastes and in varying amounts, depending on the production techniques. The liquid fraction is characterised by diverse compounds that cannot be treated by conventional wastewater treatment systems, so safe disposal is of serious environmental concern (Branciarì et al., 2015, 2016). Nevertheless, an important aspect of this waste material is its high content of phenolic compounds with antioxidant and antibacterial activity, such as 3,4-dihydroxyphenylethanol (3,4-DHPEA or hydroxytyrosol), *p*-hydroxyphenylethanol (*p*-HPEA or tyrosol) and secoiridoids derivatives, in particular, the dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA or *p*-HPEA (3,4-DHPEA-EDA or *p*-HPEA-EDA, respectively). The use of appropriate extracts of these by-products could represent an alternative source of chemical preservatives commonly used in the food industry (Branciarì et al., 2016, 2017). The use of natural antimicrobial compounds in foodstuff could prove useful to limit the growth of spoilage bacteria, especially in fresh dairy products, among which “Fior di latte” cheese represents a typical Mediterranean “pasta filata” product, largely consumed throughout Europe, especially in Italy. “Fior di latte” is produced in various shapes, usually stored submerged in governing liquid (GL,

typically conditioning brine and whey), and packaged in thermo-sealed plastic bags. Numerous studies have underlined that “Fior di latte” cheese is often spoiled by *Pseudomonas* spp. growing on the cheese surface and it is recognised that the major vehicle of contamination is represented by water used during manufacture (Lucera et al., 2014). “Fior di latte” cheese is characterised by a short shelf life and continuous efforts are made to prolong it, by means of process innovation and quality improvement of the raw materials (Gammariello et al., 2008; Lucera et al., 2014).

The current work aimed to investigate the potential use of phenols from olive oil by-products, to control the growth of spoilage bacteria in “Fior di latte” cheese. The specific effect of the addition of phenols into the cheese-making procedures, on the inhibition of *P. fluorescens* growth, was considered.

2. Materials and methods

2.1. Purification and evaluation of phenolic compounds

Extract of oil mill wastewater rich in phenols was obtained from olives of Moraiolo cultivar and was obtained according to Servili et al. (2011).

* Corresponding author.

E-mail address: david.ranucci@unipg.it (D. Ranucci).

Solid phase extraction (SPE) was applied to extract the phenolic compounds from the GL. One millilitre of the GL was loaded into SPE Bond Elut Jr-C18, 1000 mg cartridges (Agilent Technologies), and 50 mL methanol applied to elute the phenolic compounds. The methanolic extract was completely evaporated and then dissolved with 1 mL of methanol filtered through polyvinylidene fluoride (PVDF) syringe filters (Whatman, Clifton, NJ, USA).

For the extraction of phenolic compounds from “Fior di Latte” cheese, 20 g of sample was added with 100 mL of methanol + water (80:20% v/v) + butylated hydroxytoluene (20 mg/L) + trichloroacetic acid (1 M, with final proportion in the solution of 0.2%) and homogenised (Ultra-Turrax T50 homogeniser) for 1 min. After centrifugation of the mixture at 5000 rpm for 5 min, the supernatant was recovered and loaded into SPE HF Mega Bond Elut-C18, 5 g cartridges (Agilent Technologies).

The phenolic compounds were determined by high-performance liquid chromatography (HPLC), using an Agilent Technologies system Model 1100 (Agilent Technologies, Santa Clara, CA, USA), consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment and a diode array detector set at 278 nm (Roila et al., 2016). The results were expressed as milligrams of total polyphenols per gram of extract.

2.2. “Fior di latte” cheesemaking

The “Fior di Latte” cheese was manufactured in an industrial plant from cow milk that was pasteurized at 72 °C for 15 s. Acidification was carried out with natural starter culture and stretching of the curd was performed over 10 min at 86 °C. The obtained products were transferred to the cooling vat where water at 8–10 °C, allowed the chilling and firming of “Fior di Latte” cheese before the mechanised packaging step was performed. The cheese was packaged in GL. The 100 g rounded shaped “Fior di latte” cheese, singularly packaged in heat-sealed plastic bags with 100 mL of GL, were considered.

2.3. Extract addition

The polyphenolic extract used contained 565.9 mg/g total phenolic compounds, comprising 15.3% 3,4-DHPEA, 3.5% *p*-HPEA, 13.2% verbascoside and 68% 3,4-DHPEA-EDA.

Nine batches of 15 “Fior di latte” cheeses each, were considered: three batches were added with the polyphenolic extract to a final concentration of 250 µg total polyphenols/mL GL (PA); three batches were combined with the polyphenolic extract to a final concentration of 500 µg total polyphenols/mL GL (PB), and three batches with no addition performed, to serve as control (CTR). Batches were randomly selected from the commercially produced cheese by the dairy plant, considering that historical records highlight the same *P. fluorescens* load in these products (data not shown). The extract was prepared and diluted to obtain the above-stated final concentrations of PA and PB, respectively. The extract was added to GL by injecting it into the package, on the same day of production, with sterile disposable syringes through specific adhesive patches that guaranteed the closure of the hole and, therefore, the maintenance of the conditions inside the package.

2.4. Microbiological analyses

Ten grams of “Fior di latte” cheese was added with 90 mL of sterile peptone water (Oxoid, Basingstoke, UK) in a stomacher bag and mixed for 1 min with a stomacher (model 4153-50; International PBI, Milan, Italy). Serial decimal dilutions of cheese homogenates were performed, and all samples tested for *P. fluorescens* (ISO/TS 11059, 2009 – IDF/RM 225:2009) and *Enterobacteriaceae* (AFNOR AES 10/07–01/08). According to the sampling scheme, analyses were performed at 12 h (T1), and 7, 14, 21 and 24 days of storage (T2, T3, T4 and T5, respectively)

and the analytical determinations were conducted, for each batch (9), on three different cheese forms in duplicate.

2.5. Evaluation of *Pseudomonas fluorescens* and *Enterobacteriaceae* growth dynamics

Growth curves of *P. fluorescens* and *Enterobacteriaceae*, naturally present in “Fior di Latte” cheese, in PA, PB and CTR groups, were defined using DMFit version 2018 (ComBase online freeware) by fitting the experimental data to the model of Baranyi and Roberts (1994). From the resulted curves, the initial values, duration of lag phase (λ), maximum growth rate (μ_{max}) and final values were calculated.

2.6. Sensory analyses

To assess the persistence of product's characteristics during storage (sensory shelf life), a set of sensory tests were carried out using a 12-trained member panel. The tests were performed on three samples for each PA, PB and CTR batch, at the various storage times (0, 7, 14, 21 and 24 days). For the sensory evaluation “Fior di latte” cheese was maintained packaged in GL at 12 ± 2 °C for 2 h before testing, and then 25 g of sample was placed in a plastic cup, labelled with a randomly-generated three-digit code and served to the assessors. The assessors defined the acceptance or rejection of the product at the different storage times, by answering to the question: “Do you find it acceptable? (yes/no)”.

A preference test on the cheese of the three groups considered was also performed at 5 days of storage, in accordance with ISO 8587:2006. The samples were ranked from 1 (“the least preferred”) to 3 (“the most preferred”). Additionally, the 12 assessors were asked to justify their choices.

2.7. Statistical analyses

Data of total phenolic content in the GL and cheese, and the resulted microbial growth parameters were statistically analysed by the generalised linear model procedure of SAS (SAS Institute, Inc., Cary, NC, USA, 2001) by an analysis of variance (ANOVA) model. The mixed model adopted, considered the treatments performed (PA, PB and CTR) and the time (T1, T2, T3, T4, T5) as fixed effects, including replicate (batch) as a random factor nested within the treatment and the time. The batch effect was not significant for all the parameters tested ($p > 0.05$) and was not reported in the results.

The sensory shelf life of the product during storage was analysed by survival statistic analysis, using XLStat2015 software (Addinsoft, NY, USA, 2015). The cut-off point was set by Weibull distribution, considering a 50% rejection probability by the assessors.

Data on preference test were analysed using Friedman's test and the significance of difference was calculated by least significant difference (ISO 8587, 2006).

3. Results and discussion

Fig. 1 displays the evolution of the added polyphenols in the GL and “Fior di latte” cheese, during storage. The PA had twice the amount of polyphenols as the PB samples, and the content was proportional to the quantity added. Fig. 1A reveals a significant decrease in GL polyphenols contents during storage. A significant proportional increase in the cheese samples was observed, until 14 days of storage (Fig. 1B), followed by a slight decrease throughout the end of storage. The recovery of polyphenols in the cheese is a consequence of the complexation between phenolics compounds and proteins, especially caseins (Pripp et al., 2005). The decrease in phenolic compounds of “Fior di latte” cheese during storage could be due to catabolism and changes in protein structure that may release the bound phenolic compounds, which, in turn, can undergo oxidation and hydrolysis in an acid environment

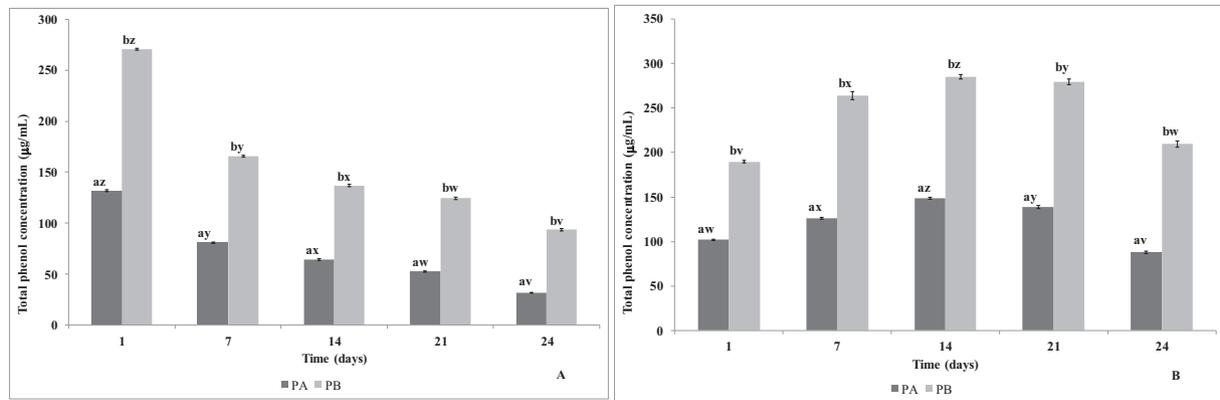


Fig. 1. Total polyphenols content in the governing liquid (A) and “Fior di latte” cheeses (B) treated with two different concentrations of olive oil polyphenols extract (250 µg/mL) PA and (500 µg/mL) PB.

Table 1

Estimated growth dynamic parameters using the Baranyi and Roberts model for *Pseudomonas fluorescens* and *Enterobacteriaceae* growth in “Fior di latte” cheese.

Microorganism and parameters	CTR	PA	PB	SEM	p-Value
<i>P. fluorescens</i>					
Initial value (log CFU/g)	0.957	0.971	1.064	0.035	0.087
λ (h)	47.83 ^a	33.75 ^a	103.86 ^b	14.733	0.008
μ_{max} (log CFU/mL/h)	0.0271 ^b	0.0191 ^a	0.0197 ^a	0.001	< 0.001
Final value (log CFU/g)	7.235	7.325	7.605	0.142	0.189
<i>Enterobacteriaceae</i>					
Initial value (log CFU/g)	1.047	0.949	0.956	0.062	0.128
λ (h)	0.00 ^a	120.86 ^b	125.81 ^b	19.48	< 0.001
μ_{max} (log CFU/mL/h)	0.0175	0.0224	0.0167	0.003	0.245
Final value (log CFU/g)	7.103	6.116	7.075	0.430	0.209

CTR: “Fior di latte” cheese maintained in untreated governing liquid (GL) PA: “Fior di latte” cheese maintained in GL added with polyphenolic extract derived from olive oil by-product to a final concentration of 250 µg total polyphenols/mL; PB: “Fior di latte” cheese maintained in GL added with polyphenolic extract derived from olive oil by-product to a final concentration of 500 µg total polyphenols/mL; λ : lag phase; μ_{max} : maximum growth rate; CFU: colony-forming units; a, b: Different superscript letters in the same row denote difference between the means ($p < 0.05$).

(Servili et al., 2011).

Table 1 and Fig. 2 report the evolution of *P. fluorescens* and *Enterobacteriaceae* in “Fior di latte” cheese during storage. The curves represent the best fitting for *P. fluorescens* (Fig. 2A) and *Enterobacteriaceae* (Fig. 2B) growth experimental data. Concerning *P. fluorescens*, the addition of polyphenols in GL resulted in extended lag phase (λ) in PB samples, in comparison with CTR and PA, and in a reduction of the maximum growth rate (μ_{max}) in PA and PB samples

compared to CTR samples. These results confirm the finding of an *in vitro* trial that reported the use of a polyphenol extract obtained from olive oil by-products lead to an extension in λ value and a reduction of μ_{max} of the growth of *P. fluorescens* strains isolated from “Fior di latte” cheese (Roila et al., 2016). Even *in vitro*, a dose-dependent effect was highlighted for polyphenol concentrations in the range 0.7–10.5 mg/mL, which could explain the differences in the duration of λ recovered between treatments. The dose-dependency was also observed by Bubonja-Sonje et al. (2011), who tested total olive oil polyphenols on *Listeria monocytogenes* growth.

For the *Enterobacteriaceae*, the addition of polyphenols resulted in extended λ in PA and PB samples in comparison to CTR. For this microbial class, the treatment did not affect the μ_{max} and the final value. The efficacy of polyphenols against microorganisms belonging to the *Enterobacteriaceae* family is still debated. Serra et al. (2008) documented no *Escherichia coli* growth inhibition and poor *Salmonella pomona* inhibition in the presence of various concentrations of olive polyphenol extract *in vitro*. Gupta et al. (2010) described that maximum specific growth rate was reduced and the lag phase increased *in vitro* upon addition of crucifer vegetables extract to *Salmonella enterica* subsp. *enterica* serotype Abony. Other effects of herbal phenol compounds on bacteria belonging to the *Enterobacteriaceae* family were noted in fresh cheeses (Khorshidian et al., 2018) but Gammariello et al. (2008) highlighted a limited effect of several different essential oils containing polyphenols against total coliforms in “Fior di latte” cheese. Wahba et al. (2010) registered a reduction in the growth and final value of coliforms in Egyptian Kareish cheese added with different plant extracts.

Pseudomonas spp. and *Enterobacteriaceae* are considered to be the bacterial groups responsible for the most important spoilage phenomena (i.e., surface spoilage, proteolysis) of Mozzarella (Altieri et al.,

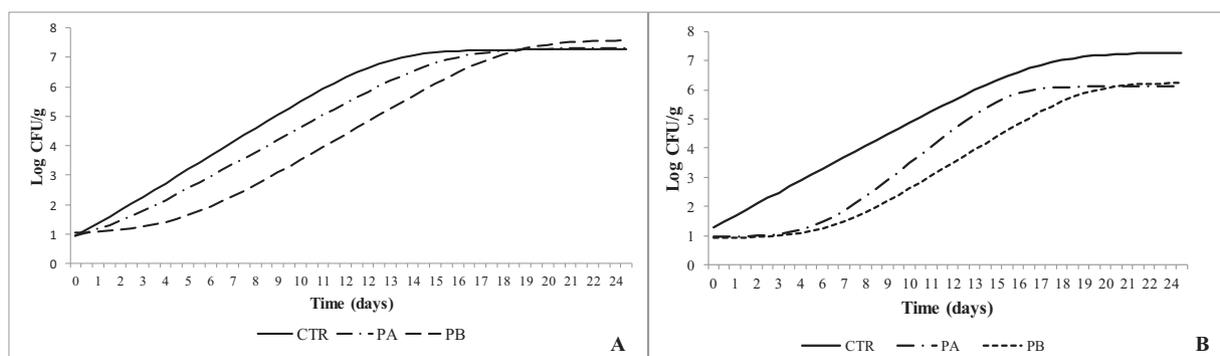


Fig. 2. Estimated growth curves of *Pseudomonas fluorescens* (A) and of *Enterobacteriaceae* (B) using the Baranyi and Roberts model, in “Fior di Latte” cheeses treated with two different concentrations of olive oil polyphenols extract (250 µg/mL) PA and (500 µg/mL) PB and CTR samples.

Table 2
Estimated sensory shelf life analyses of the “Fior di latte” cheese by Weibull distribution.

Percentage	CTR days	PA days	PB days
1%	2.129	3.630	5.307
5%	4.025	5.996	8.084
10%	5.332	7.484	9.734
1st quartile 25%	7.895	10.197	12.616
Median 50%	11.132	13.368	15.831
3rd quartile 75%	14.595	16.549	18.932
90%	17.795	19.348	21.582
95%	19.722	20.981	23.098
99%	23.331	23.951	25.810

CTR: “Fior di latte” cheese maintained in untreated governing liquid; PA: “Fior di latte” cheese maintained in GL added with polyphenolic extract derived from olive oil by-product to a final concentration of 250 µg total polyphenols/mL; PB: “Fior di latte” cheese maintained in GL added with polyphenolic extract derived from olive oil by-product to a final concentration of 500 µg total polyphenols/mL.

2005) that affect the quality and consequently provoke consumer unacceptability (Lucera et al., 2014). The previous literature stated that a microbial load equal to 10^6 CFU/g for *P. fluorescens* and 10^5 CFU/ml for *Enterobacteriaceae* (Altieri et al., 2005; Gammariello et al., 2008) might represent the contamination level at which the alterations of the product start to appear (Lucera et al., 2014). In the current study, the above-mentioned threshold was reached for *P. fluorescens* at 265 h (11.04 days) for CTR, at 311 h (12.96 days) for PA, and at 369 h (15.39 days) for PB cheeses, respectively. For *Enterobacteriaceae*, the threshold limit was reached at 255 h (10.63 days) for CTR, 323 h (13.46 days) for PA, and at 369 h (16.25 days) for PB cheese, respectively. As above reported, based on the *P. fluorescens* data, samples with PA and PB showed a significant shelf life increase ($p < 0.05$) relative to the untreated polyphenol-free “Fior di latte” cheese (CTR). This result appears to be related to the influence exerted by the experimental addition of olive mill wastewater-derived polyphenols on extending the lag phase (λ) for both microbial classes and on reducing μ_{max} for *P. fluorescens*, rather than to a reduction of the maximum cell load reached in stationary phase, as mentioned by some authors for other preservation methods (Altieri et al., 2005; Gorrasi et al., 2016). Data regarding lactic acid bacteria are not mentioned here because they were not affected by the addition of polyphenols. The trend of this microbial group was similar in all the samples (data not shown), confirming the limited effect of such molecules on this microbial group. These results corroborate the findings of Servili et al. (2011), who verified that the inhibitory effect on lactic bacteria was very limited in a fermented milk beverage fortified with these substances.

The estimated shelf life of the products, set by the Weibull distribution of the sensory data (Table 2), revealed that 50% of the assessors found the products unacceptable at 11.136, 13.366 and 15.831 days for CTR, PA and PB, respectively. The degradation processes were most evident in the CTR group, causing defects mainly in the texture (reduction in elasticity) but also in the flavour and appearance, as reported in the assessors' comments (data not shown). These processes could be the consequence of the growth of *P. fluorescens*, as it is the most frequently represented protease-producing psychotropic bacteria to negatively influence the rheological properties of Mozzarella cheese (Baruzzi et al., 2012).

The rank sums of the preference test carried out at 5 days of storage were: CTR (74), PA (66) and PB (40). Friedman's test indicated PB was least preferred ($p < 0.05$) while there was no difference between CTR and PA ($p > 0.05$), thereby corroborating the hypothesis that the lower concentrations of the polyphenols did not negatively impact on the sensorial characteristics of the cheese. The lack of effects in the sensory attributes of the low dose of polyphenols could be mainly explained by the fact that olive oil phenol compounds bound to the

proteins are not perceived by bitterness receptors on the tongue. Bitterness is related to the concentration of free (unbound) phenolics in the aqueous phase rather than to the overall concentration of phenolics in the food (Pripp et al., 2005).

4. Conclusions

This paper reported an innovative and valuable use of olive oil mill by-product extract as a natural preservative, applicable for use in the food industry. The kinetic data on *P. fluorescens* and *Enterobacteriaceae* in the presence of polyphenols added to a fresh dairy product, highlight the possible role of the extract in limiting the growth of the spoilage microorganisms considered, which extended the median shelf life by > 2 and 4 days, for PA and PB respectively. Nonetheless, a better appreciation of the product was recorded in CTR and PA samples than PB. 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 the food industry.

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