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Control of household mycoflora in fermented sausages using phenolic fractions from olive mill wastewaters

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ABSTRACT

Biopreservation using polyphenols represents an alternative to chemical molecules for improving food safety. In this work, we evaluated the antifungal activity of polyphenols extracted from olive mill wastewater (OMWWP) to reduce or eliminate the growth of undesired fungi on the surface of dry fermented sausages.
Antagonism against *Penicillium expansum* DSMZ 1282, *Penicillium verrucosum* DSMZ 12639, *Penicillium nalgiovense* MS01, *Aspergillus ochraceus* DSMZ 63304, *Cladosporium cladosporioides* MS12, and *Eurotium amstelodami* MS10 was evident at 1.25% OMWWP *in vitro*, whereas *in situ* application of 2.5% OMWWP strongly reduced undesired household fungal species such as *C. cladosporioides, Penicillium aurantiogriseum*, *Penicillium commune*, and *Eurotium amstelodami*, while a moderate antagonistic activity towards *P. nalgiovense* and *Penicillium chrysogenum* was observed at the same concentration.
OMWWP at the concentrations used in this study demonstrated species-dependent antifungal activity by inhibiting both fungal growth and spore germination. Therefore, OMWWP can be regarded as a potential alternative to synthetic antifungal compounds to preserve the product from both oxidation and undesired fungi, without changing the sensory characteristics.

1. Introduction

Olive oil production in Italy was about 480 thousand tons in 2013/2014 (Ismea, 2013), and annually approximately 1.1–1.5 times the weight of milled olives (considering the three-phase centrifugal olive oil extraction) are discarded as olive mill wastewater (OMWW). Not only is this by-product a challenge for efficient production (Garcia Garcia et al., 2000), but it also creates severe environmental problems due to both high organic load and antimicrobial activity mainly exerted by various phenolic compounds. Therefore, investigations have been carried out to generate new value-added products starting from olive oil processing discards (Cegarra et al., 1996; Soler-Rivas et al., 2006; Dermeche et al., 2013).

Control of foodborne microorganisms with natural compounds is demanded from consumers and producers, as an alternative to synthetic antimicrobial compounds. In particular, bioactive compounds extracted from agro-industrial residues have a great potential as novel fungicide sources for controlling pathogenic fungi (Osorio et al., 2010). In particular, OMWW possesses an antimicrobial potential against pathogenic bacteria and fungi, as well as molluscicidal activity (Aziz et al., 1998; Obied et al., 2007; Yangui et al., 2010; Carraro et al., 2014). Other bioactivities of OMWW include antioxidant, anti-atherogenic, and anti-inflammatory activity (Visioli et al., 1999).

Biopreservation using polyphenols represents an alternative to improve food safety, as their structure allows their diffusion through microbial membranes and their penetration into the cell, where they can interfere in the metabolic pathways, for example by hindering the synthesis of ergosterol, glucan, chitin, proteins, and glucosamine in fungi (De Souza et al., 2011).

This work is aimed to evaluate the effect of OMWW polyphenols (OMWWP) against the undesired household fungi that may grow on dry fermented sausages. It is well known that fungal growth in fermented foods can promote desirable or undesirable effects. In fermented sausages, the surface growth of moulds leads to a series of enzymatic reactions, including proteolysis, amino acid degradation, lypolysis, β-oxidation, and lactate-oxidation, which contribute positively to the flavour and other qualities of the product. Moreover, such growth also protects against spontaneous yeast and bacteria development, delays rancidity, stabilizes colour, consumes oxygen, protects against light, and can also reduce the risk of developing a dry edge, reducing water loss (Sunesen and Stanke, 2003). Nevertheless, in the absence of good quality assurance practices, some of the fungal species that grow on the surface of sausages can lead to negative consequences such as the production of off-flavours, and toxigenic and allergenic metabolites (Iacumin et al., 2009). While, in some countries, dried sausages with superficial white mycelium are considered desirable, in

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other countries consumers reject buying dried sausages covered with this kind of mould. Thus, chemical preservatives such as potassium sorbate are added to dry fermented sausages, especially on the surface, to inhibit the mould growth during the drying process (Martin Sanchez et al., 2011).

Therefore, considering the importance of obtaining safe food products preferably by means of natural compounds, the objectives of this study were: i) to evaluate the potential of the OMWWP to control undesired fungal species on the surface during ripening of Italian dry fermented sausages, and ii) to evaluate their impact on the microbiological and physical–chemical characteristics of the product.

2. Materials and methods

2.1. Phenolic compounds

OMWW phenol fraction extracts (OMWWP) were supplied by the Department of Economics and Food Science, University of Perugia, and extraction was performed as previously reported (Servili et al., 2011b). The composition of the extract used in this study was as follows: 3,4-dihydroxyphenolethanol (3,4-DHPEA) 100.23 ± 5.25 mg/g; tyrosol (p-HPEA) 21.93 ± 0.93 mg/g; verbascoside 135.20 ± 5.94 mg/g; and 2-(3,4-hydroxyphenyl) ethyl (3S, 4E)-4-formyl-3-(2-oxoethyl)hex-4-enoate (3,4-DHPEA-EDA 500.43 ± 8.15 mg/g).

2.2. In vitro evaluation of the antifungal activity of OMWWP

To evaluate the antifungal activity of OMWWP, selected strains of mycotoxigenic fungi were utilized: *Aspergillus ochraceus* DSMZ 63304, *Penicillium brevicompactum* DSMZ 3825, and *Penicillium verrucosum* DSMZ 12639 (species commonly reported as contaminants of fermented and dried meat products); *Aspergillus flavus* DSMZ 62065, *Penicillium expansum* DSMZ 1282, *Aspergillus clavatus* DSMZ 816, and *Aspergillus parasiticus* DSMZ 5771 (frequently reported as a indoor moulds); as well as natural strains isolated from Italian fermented sausages produced by the same industry in which this work was performed, and namely *Penicillium nalgiovense* MS01, *Penicillium chrysogenum* MS02, *P. chrysogenum* MS03, *Cladosporium cladosporioides* MS12, *Penicillium aurantiogriseum* MS09, *Penicillium commune* MS07, and *Eurotium amstelodami* MS10. DSMZ refers to the Deutsche Sammlung von Mikroorganismen und Zellkulturen, and MS refers to the Collection of the Faculty of Bioscience and Technology for Food, Agriculture and Environment.

All the strains were grown on potato dextrose agar (PDA, OXOID) at 28 °C for 5 to 7 days and stored at 4 °C. The spore inoculum was prepared by growing the moulds on PDA plates until the occurrence of sporulation (5–7 days). The spores were then collected with sterile water containing 0.1% Tween 80.

The agar disc diffusion method was employed for the screening of the antifungal activity of OMWWP. Polyphenol extracts were diluted in a solution of water–ethanol (4:1) at room temperature. Ethanol solution and sterile water were used as controls.

Spore suspensions of the tested fungi (about 10^8 CFU/mL) were spread on solid media plates. After keeping them at room temperature for 30 min, discs containing OMWWP stock solutions (0, 0.03, 0.62, 0.125, 2.5, 5.0, 7.5 and 10%) were placed onto the inoculated plates, which were stored at 25 °C for 5 days. Inhibition was measured as a diameter of fungal growth inhibition. Tests were done in four replicates.

2.3. Effect of OMWWP on spore germination

Spores obtained as described above were centrifuged (3000 ×*g* at 4 °C for 10 min), and re-suspended in Malt Extract Broth (MAB) (OXOID) in the presence of the same concentration of OMWWP previously used to test the mycelia, and stored at 25 °C on an orbital shaker at 350 rpm. Samples of 1 mL were taken at 6 h intervals and centrifuged at 4000 ×*g* for 3 min. The pellet was re-suspended in 100 μL glycerol:water (1:1 V/V), then the spores were analysed with an Olympus Optical microscope at 1000 magnification by using a slide haemocytometer (Bürker camera) and the percentage of germinated conidia of each suspension was calculated. The number of conidia showing germ tube formation was determined and the values were given as percentage. Spores were considered germinated if the germ tube was longer than one-half of the diameter of the conidium. The tests were performed in triplicate.

2.4. In situ evaluation of the antifungal activity of OMWWP

Antifungal activity of the OMWWP was evaluated on the surface of dry fermented sausages. The experiment was carried out in a medium size manufacturing plant in Central Italy (Salumificio Federici, Carassai, AP, Italy). Sausages were prepared with 60% lean pork, 40% pork fat, 2.5% salt, 0.05% black pepper, 0.05% white pepper, 0.05% ascorbate, 0.013% sodium nitrite, 0.014% potassium nitrate and 1% lactose. Meat was ground at 4 °C in a mincer through a 6 mm grinder plate and homogenised in a bowl mixer with a spiral dough hook with the other ingredients and 25 g/100 kg of a commercial starter culture of *Lactobacillus plantarum* (20%) and *Staphylococcus xylosus* (80%) (CHR Hansen, Germany), dissolved in 50 mL of water. The sausage mixture was stuffed into pork casings (60/63 mm diameter) to obtain sausages of 620–630 g each.

The sausages were then placed in a drying chamber under the following fermentation conditions: initially 24 °C, 80–70% of relative humidity (RH) for 12 h; successively the sausages were dried progressively up to 14/16 °C and 70/80% RH, for 96 h. Ripening was performed at 12/14 °C and 80–85% RH for 28 days. The final weight of the sausages was between 390 and 410 g.

Sausages were divided into four batches of 30 sausages each. At the 3rd day of production, when the first fungi started to appear on the sausage surface, the samples were dipped in different solutions, as follows: a) 2.5% of OMWWP, named 2.5; b) 5.0% of OMWWP, named 5.0; c) water–ethanol (4:1), solution in which polyphenol extracts were dissolved, named C; and d) sterile water, named N. The samples were dipped at 18–20 °C in the different solutions for 1 min and allowed to dry at room temperature for about 10 min. As preliminary studies evidenced that surface treatments with 7.5% and 10% extract gave a strong bitter taste, these concentrations were not used in this study.

After treatment, the samples were placed again inside the drying chamber to continue the ripening process.

For physical–chemical and microbiological analyses, samples were taken from the mix (before stuffing), at day 1 (end of fermentation), after 3 and 8 days since the treatment, and at the end of ripening (28 days). At each sampling time, three sausages from each batch were randomly chosen and two replicates were taken from each sausage to be analysed in duplicate. The experiment was repeated twice for each treatment.

While pH, water activity (a_w), moisture, and microbiological analyses were performed during maturation, protein, TBARS, volatile compounds, sensory evaluation, and visual evaluation of the mould growth were evaluated on the finished product.

2.5. Microbiological analyses

Microbiological analyses were carried out on the whole sausage after the careful removal of surface moulds. First, the pork casing was washed with ethanol, then cut with a sterile knife and removed by using sterile forceps. Samples of each sausage (10 g) were suspended in sterile 0.1% (w/v) peptone-water solution and homogenised with a Stomacher Lab-Blender 400 (PBI International Milan, Italy) at high speed and room temperature for 2 min. The following microbial groups were determined: aerobic mesophilic bacteria on Plate Count Agar (PCA) (Oxoid Ltd., Basingstoke, Hampshire, England) at 30 °C for 48 h; mesophilic lactic acid bacteria on MRS agar (Oxoid) at 30 °C for 48–