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Antimicrobial activity of gallic acid against food-related *Pseudomonas* strains and its use as biocontrol tool to improve the shelf life of fresh black truffles

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ABSTRACT

Refrigeration alone or in combination with other technologies represents the main tool used in the last decades to preserve the freshness of black truffles. This is principally due to the delicateness and vulnerability of this edible hypogeous fungus, so that other invasive preservation practices cannot be adopted. However, the proliferation of some microbial species during the cold storage still represents an unsolved problem. Pseudomonads are among the main spoiler bacteria responsible for the deterioration of refrigerated black truffles. Their growth ability at low temperatures requires the use of additional hurdles to prolong the shelf-life of truffles without altering their major features. The use of natural compounds may represent an alternative system for the biocontrol of this kind of product. Specifically, gallic acid (GA) is a phenolic acid naturally present in different foods, whose effectiveness was *in vitro* demonstrated against *Pseudomonas* spp. In our study, we reported the antimicrobial activity expressed by GA not only *in vitro*, using as target bacteria *Pseudomonas putida* DSMZ 291^T, *P. fluorescens* DSMZ 50090^T, *P. fragi* DSMZ 3456^T and *Pseudomonas* spp. P30-4, previously isolated from black truffles, but also *in situ* on fresh black truffles stored at 4 °C for 28 days. Our results showed Minimum Inhibitory Concentrations (MIC) of 2.5 mg/mL GA for all tested strains, except for *P. fluorescens* DSMZ 50090^T, having a MIC corresponding to 5 mg/mL GA. The Minimum Bactericidal Concentration (MBC) was 10 mg/mL for all strains. The analysis of kinetic parameters showed that the survival declined passing from 2.5 to 10 mg/mL GA concentrations, with *P. fluorescens* confirmed to be the most resistant strain. Moreover, images obtained from Scanning Electron Microscopy revealed that *Pseudomonas* cells were strongly injured by the treatment with GA at 2.5 mg/mL concentration, displaying visible pores on the cellular surfaces, absence of flagella and lysis with loss of cytoplasmic material.

The storage test performed on fresh black truffles confirmed *in situ* the GA antimicrobial activity observed *in vitro*, with a drastic reduction not only of *Pseudomonas* spp., but also of the other assessed microbial groups, including *Enterobacteriaceae* and *Eumycetes*. Finally, sensory analysis established the absence of off-flavours and the preservation of positive features in black truffles treated with 2.5 mg/mL GA and stored for 28 d at 4 °C. The results obtained in this study suggest that GA is a potential biocontrol tool to decontaminate and preserve fresh black truffles during refrigerated storage.

1. Introduction

Due to their attributes and sensorial traits, truffles are widely used and appreciated in the gourmet culinary world (Patel et al., 2017). Both seasonal and ecological features influence their availability, and even the shelf-life of fresh products is very limited because of their high perishability. The increasing demand for this hypogeous edible fungus has led the food industry to the development and introduction in the market of a wide variety of truffle-based products, such as sauce, oil, pasta and cheese. For their production, different methods were tested with the aim to guarantee the preservation of the typical aroma in the final products. However, all the technologies applied on truffles, such as sterilization, hot air drying, dehydration, freeze-drying or lyophilisation (Ballestra et al., 2010; Campo et al., 2017), showed detrimental effects on the sensory characteristics (Ballestra et al., 2010). Other methods used in the preservation of edible fungi, including truffles, are summarised in a recent review by Xue et al. (2017), which discussed advantages and disadvantages of processing conditions on fungi. In the case of truffles, it is important to underline that they exhibit their maximum sensorial properties when fresh, and after only a few days

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from harvest they lose the typical taste and smell (Campo et al., 2017). Some authors have recently assumed that the formation of the distinctive truffle aroma might be partially derived from microbes naturally colonising the fruiting body (Vahdatzadeh et al., 2015). Conversely, as a matter of fact, the deterioration of texture, smell and colour which occurs in the postharvest stage is coupled with the proliferation of some pathogenic and/or spoilage microorganisms (Reale et al., 2009; Rivera et al., 2011; Sorrentino et al., 2013). For this reason, different postharvest preservation technologies have been proposed for the maintenance of organoleptic properties of fresh products and to extend their shelf-life. Among them, refrigeration and freezing based technologies (Pennazza et al., 2013; Saltarelli et al., 2008; Savini et al., 2017) are probably the most studied ones. Refrigeration, in particular, enables the restraint of a large part of microorganisms unable to grow at low temperatures. However, this technology, used alone, is not adequate to inhibit psychrotrophic microorganisms, such as *Pseudomonas* spp. and moulds, both having a very fast growth rate and high affinity for the oxygen, and thus able to dominate the microbiota of refrigerated foods (Casaburi et al., 2015; Tremonte et al., 2005, 2014). Taking in mind the consumer demand for a reduced use of chemical preservatives or additives in foods, in a previous study we used a selected strain of *Lactobacillus plantarum* to inhibit the growth of spoilage moulds in refrigerated black truffles allowing its shelf-life extension (Sorrentino et al., 2013). Considering that total mesophilic bacteria, including a large proportion of *Pseudomonas*, are able to grow and to overrun on truffles kept at 4 °C (Ballestra et al., 2010), in this study we evaluated the effect of gallic acid (GA) combined with refrigeration (4 °C) on the growth of some *Pseudomonas* species. The choice of this phenolic acid was primarily due to its "safe" nature, so that it is widely present in the human diet and is often used by the food industry as flavouring agent (Larsen et al., 2010). Moreover, GA is frequently occurring in a variety of food (Daglia et al., 2014; Gutiérrez-Larraínzar et al., 2012). Gallic acid (3,4,5 trihydroxybenzoic acid) is a water-soluble phenolic acid with three hydroxyl groups, thought to be related to its relative toxicity to microorganisms. Specifically, GA possesses powerful antioxidant and antibacterial activities, where bacterial cell membranes appear to be the main target, leading to irreversible changes in permeability profile, rupture and pore formation (Borges et al., 2013). Several studies reported the GA antimicrobial activity against several pathogens, such as *Salmonella typhimurium* (Nohynek et al., 2006), *Escherichia coli* (Díaz-Gómez et al., 2014), *Staphylococcus aureus* (Chanwitheesuk et al., 2007), *Listeria innocua* (Sun et al., 2014), *Helicobacter pylori* (Díaz-Gómez et al., 2013) and *Campylobacter* spp. (Sarjit et al., 2015). Studies focusing on the GA antimicrobial activity against spoilage bacteria such as *Pseudomonas* are also present in the literature (Borges et al., 2013; Gutiérrez-Larraínzar et al., 2012; Jayaraman et al., 2010), but the effect was tested only *in vitro*.

In the present work, we studied the antimicrobial activity of GA *in vitro* and *in situ* on fresh black truffles during a storage test at 4 °C for 28 days, with *Pseudomonas* spp. as target bacteria. The final goal was the fresh truffle shelf-life extension.

2. Materials and methods

2.1. Gallic acid solution

A stock solution of gallic acid (GA, Sigma-Aldrich, St. Luis, MO, USA) in dimethyl sulfoxide DMSO (Sigma-Aldrich) at a final concentration of 50 mg/mL, was sterilized by filtration (Filter Unit Red rim FP 30/0.2 CA-S, 0.22 μm pore size; Schleider & Schuell, Dassel, Germany) and stored at − 20 °C until use.

2.2. Bacterial strains and growth conditions

In this study, four different *Pseudomonas* strains were used. *Pseudomonas fragi* DSMZ 3456^T, *P. fluorescens* DSMZ 50090^T and *P. putida* DSMZ 291^T were from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Collection (DSMZ, Braunschweig, Germany). *Pseudomonas* spp. strain P30-4, previously isolated from black truffle stored at 4 °C (Catalano et al., 2015), was from the DiAAA (University of Molise) collection. All the strains were revitalised at 28 °C in Nutrient broth (Biolife, Milan, Italy) and then stored at 4 °C in Pseudomonas Agar Base (Oxoid, Milan, Italy). Before experiments, strains were revitalised in the same conditions.

2.3. Antimicrobial activity evaluated by agar well diffusion assay

The GA stock solution was diluted in dimethyl sulfoxide DMSO (Sigma-Aldrich) to obtain a concentration range of 0–10 mg/mL. The inhibitory action of the different concentrations obtained (0,625, 1.250, 2.500, 5.000 and 10.000 mg/mL) was assessed by the agar well diffusion assay as described by Tremonte et al. (2016). Briefly, 1 mL (inoculum size of 5 log CFU/mL) of each bacterial suspension was inoculated into 20 mL of soft medium (0.7% agar), gently mixed and poured into plates. Then, 3 mm diameter wells were made into each agar plate and 70 μL of each GA dilution were added into the wells. DMSO (Sigma-Aldrich) without GA was used as negative control. Streptomycin 25 μg (Oxoid, Milan, Italy) was used as reference control.

After incubation at 28 °C for 24–48 h, a Calibrated Densitometer (GS-800, BioRad, Hercules, CA, USA) was used for plate image acquisition and Adobe Photoshop CC software was used for inhibition halos measurement.

2.4. Preliminary screening for Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC assay were carried according to Casaburi et al. (2015) with some modifications. Trials were performed in Mueller Hinton broth (MH, Biolife). Tubes were inoculated with an overnight culture of each strain at a final concentration of 5 log CFU/mL. Serial two-fold dilutions of GA, containing from 10 to 0 mg/mL were used. DMSO (Sigma-Aldrich) without GA was used as control. The tubes were incubated for 24 h at 28 °C. 100 μL of the cell suspensions from the tubes showing no growth were sub-cultured on PAB plates. MIC was defined as the lowest concentration of GA at which bacteria failed to growth in liquid media, but yet viable when 100 μL of culture broth were plated on agar media. MBC was defined as the lowest concentration of GA at which bacteria failed to growth in liquid media, with a negative growth after incubation on agar media.

2.5. Time-kill assay

The antimicrobial effectiveness of GA was studied for each bacterial strain on the basis of data obtained by MIC and MBC determination. For this purpose, overnight cultures of each strain were harvested by centrifugation (8000 rpm, 5 min, 4 °C), suspended in phosphate buffered saline (PBS) pH 7 at a final concentration of about 7–8 log CFU/mL and exposed at three different concentration of GA (2.5, 5 and 10 mg/mL) for 12 h at 28 °C. For control, PBS without GA was used. One milliliter of cultures collected at 1 h intervals were serially diluted and plated on Pseudomonas Agar Base (Oxoid). The experimental data were fitted with software DMFit web edition.

2.6. Effect of GA on bacterial morphology

Scanning Electron Microscopy (SEM) was used to evaluate the morphology of cells exposed to 2.5 mg/mL of GA. For this purpose, overnight cultures (approximately 7 log CFU/mL) of each strain were washed with PBS, re-suspended in the same buffer containing GA and collected after 1 h of exposure. Untreated bacterial cells were used as control. After exposure, treated and untreated cells were harvested by centrifugation and fixed in 3% glutaraldehyde in 0.1 M sodium

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