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# Use of hop extract as antifungal ingredient for bread making and selection of autochthonous resistant starters for sourdough fermentation

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ABSTRACT

Aiming at meeting the consumers' demand in terms of bio-preservation, the potential of the combination of the lactic acid bacteria fermentation and the addition of hop extract as natural preservative in breadmaking, was exploited. The antifungal properties of a hop (*Humulus lupulus*) extract were investigated, showing a significant inhibition of the hyphal growth of *Aspergillus parasiticus*, *Penicillium carneum*, *Penicillium polonicum*, *Penicillium paneum*, *Penicillium chermesinum*, *Aspergillus niger*, *Penicillium roqueforti*. Lactic acid bacteria belonging to species of *Enterococcus feacium*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus curvatus*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici* were isolated from hop and subjected to selection based on kinetics of growth and acidification. The sourdough (hS) enriched with hop extract (hE), started with three selected strains, had phenols concentration and antioxidant activity higher than those obtained in the same condition but without the hE. Hop-sourdough used in breadmaking delayed the fungal growth (14 days), giving a bread characterized by free aminoacids concentration, antioxidant and phytase activities higher than bread started only with baker's yeast, with or without the addition of hE. Specific volume and cell-total area of the bread containing hE improved, and its sensory profile was characterized by typical sourdough attributes, and a moderate bitter/herbaceous perception.

## 1. Introduction

Current consumer trends regarding the demand for fresh and minimally processed food products without additional chemical preservatives have promoted the research for new preservation strategy (Kramer et al., 2015). Naturally occurring compounds that show antimicrobial activity against spoilage and pathogenic microorganisms could potentially be applied for food preservation to extend the shelf-life and to improve the safety of foods and beverages at the same time (Kramer et al., 2015). Several recent studies were focused on natural preservatives from plant matrices, such as proteins and peptides or essential oils, to be used as food ingredients (Kramer et al., 2015; Rizzello et al., 2017).

The hop plant *Humulus lupulus* belongs to the family of the *Cannabinaceae* has been used for beer brewing since ancient times. Besides giving beer its typical bitter taste, hop compounds possess distinctive antimicrobial and antioxidant properties (Kramer et al., 2015).

The antimicrobial activity of bitter acids, namely α- (humulones) and β-acids (lupulones), and their isomers, was largely demonstrated mainly against Gram-positive bacteria (Kramer et al., 2015). Different fungi are also inhibited by hop acids and prenylchalcones (Mizobuchi and Sato, 1985). Recently additional antimicrobial compounds were isolated, including xanthohumol, a broad spectrum anti-infective agent working towards many bacteria, viruses, fungi and protozoa (Natarajan et al., 2008), and humulinic acids, a non-bitter derivatives of iso-α-acids (Schurr et al., 2015).

Besides the recognized potential to reduce the beer spoilage, the applicability of hop compounds as possible food preservatives has not been extensively studied, although few studies reported the inhibition of foodborne pathogens in cheeses (Larson et al., 1996) and meat products (Kramer et al., 2015).

Overall, the use of antifungal compounds from plants was proposed for extending the baked goods shelf-life (Coda et al., 2008; Rizzello

*Abbreviations:* hE, hop extract; WFH, wheat flour hydrolyzate; WFH-he, hop extract wheat flour hydrolyzate; WFH-cp, calcium propionate wheat flour hydrolyzate; WSE, water/salt-soluble extracts; TTA, Total titratable acidity; DY, dough yield; MIC, minimal inhibitory concentration; ME, methanolic extract; FQ, fermentation quotient; hS, hop sourdough; S, wheat sourdough; WB, wheat bread; $WB^{cp}$, calcium propionate wheat bread; $WB^{hE}$, hop extract wheat bread; $SWB^{hE}$, hop extract sourdough wheat bread; TFAA, Total free amino acids; $a_w$, water activity

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et al., 2015). Indeed, fungal contamination is the most common and costly spoilage for bakeries and, in many cases, it is the factor governing shelf-life (Rizzello et al., 2017).

It was already demonstrated that the water-soluble extract from *Phaseolus vulgaris* cv Pinto contained phaseolin alpha-type precursor, phaseolin erythroagglutinating and phytohemagglutinin inhibited a large spectrum of fungal species isolated from bakeries (Coda et al., 2008). Moreover, the prolonging of the shelf-life through the inhibition of the mold contamination was recently reported by using pea, lentil and faba bean flour hydrolysates as ingredients for bread making (Rizzello et al., 2015, 2017). In these cases, the antifungal activity was attributed to native proteins (nsLTP, ubiquitin, lectin alpha-1 chain, wound-induced basic protein, defensin-1, defensin-2) and mixture of peptides, which were released during hydrolysis from legume vicilins, lectins and chitinases (Rizzello et al., 2015, 2017). Also an extract of amaranth seeds was used as an ingredient for the manufacture of gluten-free and wheat flour breads, and inhibitory activity, due to agglutinin peptides, was achieved during long-term storage under pilot plant conditions (Rizzello et al., 2009). The same advantage was observed for breads enriched with sourdough-fermented wheat germ, able to delay fungal growth for at least 28 days (Rizzello et al., 2011).

Besides plant compounds, metabolites produced by sourdough lactic acid bacteria and non-*Saccharomyces* yeasts during fermentation have been screened for their antimicrobial properties (Coda et al., 2008, 2011b, 2013; Rizzello et al., 2011). In particular, lactic acid bacteria are considered as efficient bio-preservative organisms because of their acidification capacity through the lactic acid fermentation, but also for the ability to synthesize or release various antimicrobial and antifungal molecules, like bacteriocins, organic acids, bioactive peptides (Crowley et al., 2013; Gerez et al., 2009; Schnürer and Magnusson, 2005).

Despite many promising results recently collected by the scientific community, the problem of fungal contamination in the baked goods sector remains still unsolved and much awaited by consumers and manufactures, especially due to the lacking of a large activity spectrum when antifungal plant matrices and starters were used singly (Rizzello et al., 2017).

In this work, a hop extract was characterized for the antifungal activity and used as ingredient aiming to prolong the shelf-life of bread. Sourdough fermentation with lactic acid bacteria, isolated from hop, characterized and selected for pro-technological characteristics and hop resistance, was employed for bread making. First, the effects of the hop extract on the selected lactic acid bacteria performances and on the sourdough characteristics were evaluated. Then, the effects of the addition of the hop extract, together with the sourdough fermentation, on the shelf-life, as well as on rheological and sensory features of wheat bread, were also assessed.

## 2. Materials and methods

### 2.1. Hop and hop extract

Commercial Amarillo hop cones (Pinta, Disegna Group, Marostica, Italy) were used to obtain the hop extract. The proximal composition of the hop was: moisture, 11.0%; protein, 15.2%; fat, 3.4%; dietary fibers, 46.2%; total soluble carbohydrates, 2.0%; polyphenols and tannins, 4.7%; α-acids 11.1%, β-acids, 6.5%. To obtain the hop extract (hE), 1 g of milled hop was resuspended in 100 ml of distilled water, homogenized, and boiled for 1 h aiming at the isomerization of the hop acids (Jaskula et al., 2008). After cooling at room temperature, the extract was centrifuged (10,000 × *g*) for 10 min to remove eventual suspended materials and the supernatant was filtered using a 0.22 μm filter (Millipore Corporation, Bedford, MA 01730). Analysis of the total iso-α-acids in hE was carried out by HPLC, with an ÄKTA Purifier system (GE Healthcare, Buckinghmshire, UK) equipped with a Kinetex 2.6 column (Phenomenex, Torrance, CA, USA) and an UV detector operating at 270 nm. The analysis was carried out at 1 ml/min flow, 40 °C, using an eluent constituted by 85% (vol/vol) methanol, 0.05% phosphoric acid (85%) and 0.05 mM EDTA (isocratic).

### 2.2. Antifungal activity of the hop extract

Seventeen indicator microorganisms were used for antifungal assays since previously identified as the most common baked goods spoilage molds (Coda et al., 2008; Rizzello et al., 2009). *Penicillium polonicum* CBS 112490, *Penicillium chrysogenum* CBS 111214, *Penicillium paneum* CBS 101032, *Penicillium albocoremium* CBS109582, *Penicillium chermesinum* CBS117279, *Penicillium carneum* CBS112297, *Eurotium herbariorum* CBS117336, *Eurotium rubrum* CBS150.92, *Aspergillus parasiticus* CBS971.97, *Aspergillus versicolor* CBS117286, *P. bialowiezense* CBS110102 and *Penicillium brevicompactum* CBS28997 were from the Culture Collection of Centraalbureau voor Schimmelcultures (Utrecht, Holland); *Penicillium roqueforti* DPPMAF1, *Penicillium aethiopicum* DPPMAF2, *Aspergillus niger* DPPMAF3, *Aspergillus penicilloides* F1, and *Wallemia sebi* F2 were from the Culture Collection of the Department of Soil, Plant, and Food Sciences (Bari, Italy). Fungi were grown in Potato Dextrose Agar (pH 5.6) (PDA, Oxoid) at 25 °C for 24–72 h, with the exception of *W. sebi* F2, that was cultivated in Dichloran-Glycerol Agar Base (DG18, Oxoid). The inhibitory activity of hE was assayed based on hyphal radial growth rate of fungi (Coda et al., 2013). The hE was added (25%, vol/vol, final concentration) to sterilized PDA or DG18. After mixing, aliquots of 20 ml were poured into Petri plates (90 mm diameter). Control plates contained PDA or DG18 supplemented with 25% (vol/vol) of sterile water. The assay was carried out by placing a 3-mm diameter plug of growing mycelia onto the center of Petri dishes containing the culture medium. Plates were incubated aerobically at 25 °C. Three replicates were run simultaneously. The radial growth of mycelia (colony diameter, mm) in all plates was measured after 6 days of incubation. Each datum point is the mean of at least four measurements of a growing colony. The percentage of growth inhibition was calculated from mean values as follows: percentage of inhibition = [(mycelial growth under control conditions − mycelial growth in the presence of hE)/mycelial growth under control conditions] × 100.

The effect of hE on the germination of conidia was determined (Coda et al., 2008, 2013) on the indicator *P. roqueforti*. Wheat flour hydrolysate (WFH) was chosen as the substrate since representative of the chemical composition of wheat flour. WFH was obtained as previously described by Coda et al. (2013). A suspension of 20% wheat flour (wt/vol) in tap water was incubated at 30 °C for 18 h under stirring conditions (ca. 200 rpm). After incubation, the suspension was filtered onto a Whatman apparatus (Polycarp 75 SPF, Whatman International Maidstone, England) and added of yeast extract (0.3%, wt/vol), sucrose, glucose and maltose (0.25% total concentration, wt/vol). The WFH was sterilized by filtration on 0.22 μm membrane filters (Millipore Corporation, Bedford, MA01730) and stored at 4 °C before use.

After growth for 7 days on PDA plates, conidia of *P. roqueforti* DPPMAF1 were harvested in sterile water, containing 0.05% (vol/vol) Tween 80. The count of the conidia in the suspension was carried out using the Petroff-Houser Counting chamber. A fixed number of ca. $10^6$–$10^7$ conidia/ml was added to 5 ml of the mixture of WFH containing 25% (vol/vol) hE (WFH-hE). The mixtures were incubated in 60 mm Petri dishes for 24 h at 25 °C under stirring conditions. WFH alone and WFH added of 0.3% (wt/vol) calcium propionate (WFH-cp) were used as controls. To determine the percentage of germinated conidia (length/width ratio ≥ 2) at 16 and 24 h of incubation, aliquots of the suspension were examined with a Zeiss (Weimar, Germany) optical microscope (400 × magnification). Three separate replications of at least 100 conidia were used for each assay. All assays for antifungal activity were carried out at least in triplicate.