LWT - Food Science and Technology 189 (2023) 115467

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

# Combined use of *Trametes versicolor* extract and sourdough fermentation to extend the microbiological shelf-life of baked goods

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ARTICLE INFO

*Keywords:*
Biopreservation
Bread
Antifungal activity
Antimicrobial peptides
Lactic acid bacteria

ABSTRACT

Fungal spoilage is the main responsible for the short shelf-life of baked goods. Indeed, many chemical preservatives (e.g., calcium propionate and ethanol) are often included in their formulation leading to consumer dissatisfaction. Here, an *in-vitro* and *in-situ* integrated approach was used to investigate the potential antifungal activity of the extract obtained from *Trametes versicolor* as biological preservative. An intense inhibition towards most of the *Aspergillus* and *Penicillium* species and a broad spectrum of activity on typical spoilage fungi of the bakery products, as well as high thermal stability and intense activity at pH 4.00 characterized the *Trametes* extract. The antifungal potential of the extract has been exploited in sour bread made with organic acids (chemical) or type II-sourdough (biological). Compared to baker's yeast bread produced as control (pH 5.6), the acidified samples were characterized by a longer mold-free shelf-life, with indicator mycelia that became visible after 6–9 days of storage at room temperature. Sourdough was effective to counteract the negative effect of the extract supplementation on the leavening performances, textural and sensory features of the bread samples.

## 1. Introduction

For centuries, wild mushrooms have attained remarkable interest in the fields of medicine and food processing due to their proficient nutritional and therapeutic properties (Glavinic et al., 2021; Reis, Anabela, Isabel, Vasconcelos, & Patricia, 2017; Stevanovic et al., 2018). Globally, about 14,000 species of mushrooms are known and, among them, about 2000 are considered edible (Garofalo et al., 2017). Among wild mushrooms, *Trametes versicolor* gained popularity that led to a broad-purposes utilization in the food and pharmaceutical industries (Reis, Martins, Vasconcelos, Morales, & Ferreira, 2017). *T. versicolor* is a saprotrophic mushroom species that grows on woods in the temperate zones of Asia, Europe, and North America, representing one of the most common "shelf mushrooms" (Ma, Chen, Dong, & Lu, 2013; Nagy et al., 2017). Due to its polypore nature and woody taste, it is not widely employed directly as food; nevertheless, its dried powder, aqueous homogenate or extracts are largely added as ingredients in different foods and beverages (Prasad, Rathore, Sharma, & Yadav, 2015). Moreover, *T. versicolor* derived products are traditionally used in Asia as alternative medicine in the therapy of many diseases, including cancers and certain infections (Bains et al., 2021). Indeed, besides their recognized nutritional importance, mushrooms are well-known for their bioactive compounds (lectins, polysaccharides, polysaccharide-peptides and polysaccharide-protein complexes, lanostanoids, other terpenoids, alkaloids, sterols, and phenolic structured compounds), which are responsible for different biological and therapeutic activities, including antimicrobial, antioxidant, anti-inflammatory, antidiabetic, anticancer properties, antiviral, and anti-immunomodulatory activities (Bains et al., 2021).

Many bioactive compounds have been identified in *T. versicolor* extracts, that are already employed in the preparations of commercial nutraceuticals in the form of capsules, tablets, syrups, tea, and food additives (Oyedepo & Morakinyo, 2020). Recently, the capability of the crude *T. versicolor* extract to strongly inhibit phytopathogenic fungi (and their mycotoxins synthesis) such as *Alternaria solani* (Ashraf et al., 2023), *Aspergillus parasiticus* (Zjalic et al., 2006), *Aspergillus flavus* (Scarpari et al., 2014, 2016), *Aspergillus carbonarius* (Loncar et al., 2023), and *Fusarium langsethiae* (Parroni et al., 2019) suggested a

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https://doi.org/10.1016/j.lwt.2023.115467
Received 10 July 2023; Received in revised form 23 October 2023; Accepted 25 October 2023
Available online 4 November 2023

promising use as a biopesticide in agriculture.

Thanks to its long safety history (Bains et al., 2021; Cerig, 2021) and the relevant antimicrobial activity, the use of *T. versicolor* extract as biopreservative agent in bakery products, whose shelf-life is mainly compromised by fungal contamination, was exploited in this work.

Mold contamination is the main cause of bread wastage, since it determines large economic losses for both the bakery industry and consumers (Verni et al., 2023). The application of preventive measures and, especially, the use of antifungal ingredients (i.e., preservatives), are the most common strategies adopted by the bakery industry. Overall, the use of chemical preservatives such as weak acids and their salts, such as salts of propionic and sorbic acids, is very common in breadmaking. Their use in bakery products is regulated within EU and, depending on the type of product, they can be added up to 0.1–0.3% (g:g). However, the use of chemical preservatives in the food industry has been going under a deep revision, also due to the growing consumer appreciation of more natural foods and concomitant negative perception of artificial additives (Asioli et al., 2017). The research for natural substitutes of the commonly used organic acids preservatives has intensified recently (Nionelli et al., 2020). To fulfill the growing requests for more natural food products, the antifungal effects has been sought in compounds of natural origin, such as those deriving from plants or obtained via bio-processing with microorganisms and/or enzymes. These methods, defining the concept of biopreservation (Stiles, 1996), have become an increasingly important field of research (Nionelli et al., 2020).

First, the antifungal activity of *T. versicolor* extract was investigated *in-vitro*, aiming at determining its spectrum of activity, thermal resistance, and pH-dependence; then, it was used as ingredient in breadmaking to assess the *in-situ* effects on dough leavening, technological and sensory features of bread and capability to extend the microbial shelf-life of baked goods under high-inoculum conditions.

## 2. Materials and methods

### 2.1. Mycelia-purified culture filtrate from Trametes versicolor

*Trametes versicolor* strain C used in this study was registered at CABI biosciences (UK) and deposited in the culture collection of Department of Environmental Biology of Sapienza University of Rome as ITEM 117. The isolate was cultivated in Potato Dextrose Agar (*PDA, Oxoid* Ltd., Basingstoke, UK) at 25 °C and the cultures were sub-cultured every 30 days. (Scarpari et al., 2017).

To produce the mycelia-purified culture filtrate used for the experiments (Trametes extract, TE), *T. versicolor* was grown for 5 days in Potato Dextrose Broth (*PDB, Oxoid* Ltd.) and incubated at 25 °C under stirring conditions (100×*g*). The liquid culture was homogenized, in sterile condition using Waring blender 8012. After homogenization, an aliquot (5% mL:mL) of the fungal culture was inoculated in 500 mL of PDB in 1 L-Erlenmeyer flasks and incubated for 14 days at 25 °C under stirring conditions (100×*g*). The mycelia were then separated from the culture filtrates (TE) by paper filters (Whatmann). Then the TE was concentrated 10 times with Rotavapor (Rotavapor ® R-300, Buchi, Essen city, Germany), and it was freeze dried and used for the antifungal multiwell assay.

### 2.2. Antifungal activity

#### 2.2.1. Microorganisms, culture media, growth conditions, and identification

*Forty-six fungal strains, previously isolated from baked goods or bakery environments were used as indicators to assess the TE spectrum of activity (Table 1).* Most of the fungal strains were routinely cultured in *PDB (Oxoid* Ltd.*) in the dark under stirring conditions at 25 °C.* Aspergillus niger *DSM 737 was cultivated at 30 °C.* Aspergillus glaucus *DSM 16660 and* Eurotium rubrum *DSM 62631 were cultivated in M40Y broth (Malt extract, 20.*0 g/L*; Yeast extract, 5.*0 g/L*; Sucrose, 400.*0 g/L). Hyphopichia burtonii *DSM 3505 was cultivated in Malt Extract Broth (ME, Oxoid).*

**Table 1**
List of the fungal strains used for the assessment of the TE spectrum of activity.

| Species | strain ID | Collection |
|---|---|---|
| *Aspergillus flavus* | **PIC1** | Department of Soil, Plant, and Food Science, University of Bari, Italy |
| *Aspergillus clavatus* | **PIC2** | |
| *Penicillium echinulatum* | **PIC3** | |
| *Paecilomyces variotii* | **PIC4** | |
| *Penicillium palitans* | **PIC5** | |
| *Penicillium brevicompactum* | **FER1** | |
| *Penicillium brevicompactum* | **FER2** | |
| *Penicillium brevicompactum* | **FER3** | |
| *Penicillium rubens* | **OLD1** | |
| *Penicillium carneum* | **OLD2** | |
| *Cordyceps farinosa* | **OLD3** | |
| *Aspergillus niger* | **OLD4** | |
| *Cordyceps farinosa* | **OLD5** | |
| *Penicillium bialowiezense* | **OLD6** | |
| *Penicillium rubens* | **OLD9** | |
| *Penicillium bialowiezense* | **OLD6** | |
| *Penicillium lanosocoeruleum* | **OLD8** | |
| *Penicillium carneum* | **OLD12** | |
| *Penicillium albocoremium* | **OLD13** | |
| *Penicillium paneum* | **OLD15** | |
| *Penicillium paneum* | **OLD17** | |
| *Penicillium roqueforti* | **DPPMAF1** | |
| *Aspergillus pseudoglaucus* | **CBS 123.28** | CBS-KNAW Fungal Biodiversity Centre (Utrecth, Netherlands) |
| *Penicillium polonicum* | **CBS 222.28** | |
| *Penicillium bialowiezense* | **CBS 227.28** | |
| *Penicillium crustosum* | **CBS 340.59** | |
| *Penicillium lanosocoeruleum* | **CBS 484.84** | |
| *Penicillium paneum* | **CBS 101032** | |
| *Penicillium albocoremium* | **CBS109582** | |
| *Penicillium carneum* | **CBS 112297** | |
| *Penicillium chermesinum* | **CBS 117279** | |
| *Aspergillus niger* | **DSM 737** | Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Brunswick, Germany |
| *Aspergillus amoenus* | **DSM 1943** | |
| *Aspergillus parasiticus* | **DSM 1300** | |
| *Aspergillus glaucus* | **DSM 16660** | |
| *Eurotium rubrum* | **DSM 62631** | |
| *Penicillium aurantiogriseum* | **DSM 1250** | |
| *Penicillium brevicompactum* | **DSM 2215** | |
| *Penicillium chrysogenum* | **DSM 895** | |
| *Penicillium citrinum* | **DSM 1179** | |
| *Penicillium corylophylum* | **DSM 62832** | |
| *Penicillium griseofulvum* | **DSM 896** | |
| *Penicillium expansum* | **DSM 1282** | |
| *Penicillium roqueforti* | **DSM 1079** | |
| *Penicillium viridicatum* | **DSM 62878** | |
| *Hyphopichia burtonii* | **DSM 3505** | |
| *Moniliella suaveolens* | **DSM 2400** | |

#### 2.2.2. Antifungal multiwell assay

Aiming at determining the antifungal activity of the TE, an assay based on the use of 96-multiwell plates (Greiner CELLSTAR®, Sigma-Aldrich St. Louis, Missouri, USA) was set-up. Each well contained: 10 μL of a $10^5$ conidia/mL suspension (*circa* 1000 conidia) of each strain; 190 μL of PDB (HiMedia) or PDB mixed with 0.5 or 1.0% (g:mL) of the freeze-dried TE. Multiwell plates were incubated at 25 or 30 °C depending on the indicator tested and fungal growth monitored at 24, 48 and 72 h through a Multiskan FC spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at wavelength 620 nm. The development of fungal mycelia after 72 h of incubation (data not showed) made absorbance measurement no longer possible.

Conidia were harvested from the Petri dishes containing fungal strains in active growth phase (5–7 days) by using sterilized water containing 0.01% Triton X-100 (Sigma-Aldrich St. Louis, Missouri, USA). Suspension was filtered through a sterile Miracloth Millipore filter (Merk, Darmstadt, Germany) and centrifuged at 5000 rpm for 15 min. Conidia were then resuspended in 1 mL of Triton containing 0.01% Triton X-100 (Sigma-Aldrich St. Louis, Missouri, USA) and enumerated by using Fast-Read 102® (Biosigma, Italy) counting chambers. The fungal growth is reported as absorbance difference (ΔAbs).

$$\Delta Abs = Abs\ inoculated\ well - Abs\ control\ well$$

The inoculated wells contain the spores and the TE, the control wells contain the TE at different concentrations. Results were evaluated through a T-test at 3 significance levels (*p*-values 0.05, 0.01 and 0.001).

#### 2.2.3. Thermal stability and effect of the pH

The thermal stability and effect of the pH of the TE was determined using the *Penicillium roqueforti* DPPMAF1. The strain was used as indicator microorganism for the assays since it corresponds to one of the most common bread spoilage molds, presenting high resistance to the preservatives commonly added to baked goods (Rizzello, Lavecchia, Gramaglia, & Gobbetti, 2015).

The heat stability of the TE was assessed after heating it for 5 min at 100 °C (tTE, thermal treated TE). Aiming at investigating the effect of the pH on the antifungal activity, a PDB (Oxoid) containing 10% (mL:mL) TE (TE-PDB) or tTE (tTE-PDB) was corrected at pH 4.0, 5.6, and 6.6 with HCl/NaOH 1 M, and employed in the test for the determination of the mycelial dry weight as described by Gourama (1997), with some modifications (Coda et al., 2008).

In details, the indicator was inoculated ($5 \times 10^4$ conidia/mL) in PDB, TE-PDB and tTE-PDB after the pH correction and incubated for 48 h at 25 °C. Then, the mycelial biomasses were collected by filtration through a Whatman apparatus (0.22-μm membrane filter). After the filters were dried at room temperature, the dry weight was measured until it became constant (24–48 h).

### 2.3. Baking test

Aiming at verifying the *in-situ* antifungal activity of the TE, it was used as ingredient in breadmaking.

#### 2.3.1. Sourdough making and characterization

A type-II sourdough was produced by using type 0 wheat flour (Coop, Casalecchio di Reno, Italia) having the following proximal composition: (moisture, 14.5 % (g:g); proteins, 11.75% (g:g) on dry matter/d.m.; carbohydrates, 83.4% (g:g) on d.m.; dietary fibers 2.9% (g:g) on d.m.; fat, 1.2% (g:g) on d.m.; ash, 0.7% (g:g) on d.m.

Sourdough, having a dough yield (DY, dough weight × 100/flour weight) of 160, was produced with the selected lactic acid bacteria (LAB) strains *Lactiplantibacillus plantarum* LB1 and *Furfurilactobacillus rossiae* LB5 (Rizzello, Nionelli, Coda, De Angelis, & Gobbetti, 2010).

LAB strains were singly cultivated in De Man, Rogosa and Sharpe (MRS, Oxoid) at 30 °C until the late exponential phase of growth was reached (*circa* 10 h), then cells were harvested by centrifugation (10,000×*g*, 10 min, 4 °C); washed twice in 50 mM phosphate buffer, pH 7.0, and re-suspended in tap water before use. Each strain was inoculated at the final cell density of 7 Log cfu/g. Fermentation was carried out at 30 °C for 24 h.

The pH and total titratable acidity (TTA) of the experimental doughs were determined before (t0) and after (t24) fermentation. The pH was determined by a FiveEasy Plus pHmeter (Mettler-Toledo, Columbus, Ohio, USA) with a food penetration probe. TTA was determined as the amount of 0.1 M NaOH required to adjust the end pH of 10 g dough in sterile water to 8.3. A water/salt-soluble extract (WSE) of the sourdough was prepared and used to determine the content of total free amino acids (TFAA) by a Biochrom $30^+$ series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England) with a Li-cation-exchange column (4.6 × 200 mm internal diameter), as described by Verni, Dingeo, Rizzello, and Pontonio (2021). The WSE was also used to analyze lactic and acetic acids, respectively with K-DLATE and K-ACET kits (Megazyme International Ireland Limited, Bray, Ireland). The quotient of fermentation (QF) was determined as the molar ratio between lactic and acetic acids.

#### 2.3.2. Breadmaking

Six experimental bread samples were manufactured by using the bread machine Ariete 132 Panexpress 750 (De Longhi Appliances Srl, Campi Bisenzio, Italy) and the type 0 flour above described. Experimental bread samples were as follow: cYB, a control bread, leavened with 2% (g:g) baker's yeast; acYB, a chemically acidified control bread, in which a solution 1 mol/L of lactic acid was used to correct the pH of the dough at 4.00 before leavening with 2% (g:g) baker's yeast; cSB, a control sourdough bread, containing 25% (g:g) of the type-II sourdough previously described, leavened with 2% (g:g) baker's yeast; tYB, a bread containing 10% (g:g) of the TE and leavened with 2% (g:g) baker's yeast; taYB, a chemically acidified bread (pH was corrected at 4.00 with 1 M lactic acid solution) containing 10% (g:g) of the TE and leavened with 2% (g:g) baker's yeast; tSB, a bread containing 10% (g:g) TE and 25% (g:g) sourdough, leavened with 2% (g:g) baker's yeast.

All bread samples were obtained from doughs with DY 160 corresponding to a flour/water ratio of 62.5/37.5% (g:g) and all were added with commercial baker's yeast (AB Mauri Italy S.p.a., Casteggio, Italia). Proofing was performed at 28 °C for 1.5 h and baking at 180 °C for 50 min. Recipes are reported in Supplementary Table 1.

#### 2.3.3. Bread characterization

*2.3.3.1. Chemical characterization.* The analysis of pH, TTA, organic acids, and TFAA of the dough after proofing were carried out as reported above.

*2.3.3.2. Technological characterization*

*2.3.3.2.1. Evaluation of dough leavening performance.* The dough leavening performance of the different samples was evaluated determining the volume increase of a 15 mL-dough placed in a graduated cylinder top, covered with a piece of Parafilm® and allowed to ferment at 28 °C (Torreggiani et al., 2023). Results were expressed as difference between the initial and the final volume (ΔV, mL).

*2.3.3.2.2. Texture profile analysis (TPA).* Texture profile analysis was performed by using an FRTS-100 N Texture Analyzer (Imada, Toyohashi, Japan) equipped with a 3 cm cylinder probe FR-HA-30 J on boule-shaped loaves (200 g) stored for 2 h at room temperature after baking (Perri, Greco Miani, Amendolagine, Pontonio, & Rizzello, 2022). The instrument settings were test speed 1 mm/s, 30% deformation of the sample, and two compression cycles, and the parameters evaluated were hardness, cohesiveness, springiness, and chewiness.

*2.3.3.2.3. Color measurement.* The chromaticity coordinates of the crust and crumb of the bread samples were obtained by a CS-10 colorimeter (CHN Spec Technology, Hangzhou, China) and reported as color

difference, $\Delta E^*_{ab}$, calculated by the following equation

$$\Delta E*ab = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

where ΔL, Δa and Δb are the differences for L*, a* and b* values between sample and reference (a white ceramic plate having L = 92.2, a* = 0.15 and b* = 0.85). Hue (H*), chroma (C*), and browning index (BI) were respectively calculated using the following equations as reported by Abd El-Baset and Almoselhy (2023):

$$C* = \sqrt{a*^2 + b*^2}$$

$$H* = \tan^{-1}[b* / a*]$$

$$BI = \frac{100}{0.17}\left(\frac{a* + 1.75L*}{5.645L* + a* - 0.012b*} - 0.31\right)$$

*2.3.3.3. Sensory characterization.* Sensory analysis of bread samples was performed by a trained panel group composed of ten assessors (5 male and 5 females, mean age: 30 years, range: 25–54 years) with proven skills and previous experience in sensory evaluation of bread, pasta, and other cereal-based products. According to the ethical guidelines of the sensory laboratory, enrolled panellists did not suffer from any food intolerances or allergies, received information on the objectives of the study, and provided written informed consent. The sensory attributes, scored with a scale from 0 to 10 (with 10 the highest score), were discussed with the assessors during the introductory 2 h-training session (Montemurro et al., 2023). The training of panelists was conducted according to the method described by Elía (2011). Sensory attributes included: visual and tactile perception (crust and crumb color, elasticity, friability); taste (sweetness, bitterness, astringency, salty taste, herbaceous taste, acidic taste); and scent perception (acidic odor). Bread slices (1.5 cm thick) were coded and served in a randomized order 4 h after baking. A glass of water was drunk by the panellists after each sample tasting.

### 2.3.4. Shelf-life monitoring

For each experimental bread, 4 slices were cut after 1 h-cooling. Slices, 12 cm high and 1.5 cm wide, were inoculated by nebulization with a suspension of $10^2$ conidia/mL of *P. roqueforti* DPPMAF1 and then packed in polyethylene bags to maintain constant moisture and kept at room temperature. A bread sample produced with the same protocol of the cYB but containing 0.1% (g:g) calcium propionate (cYBcp), was added to the experimental breads set. Mold growth was estimated by visual observation for a period of 15 days after baking (3-days intervals) and approximately quantified as the percentage of the surface covered by the visible mycelia of the indicator mold (Rizzello, Verni, Bordignon, Gramaglia, & Gobbetti, 2017).

## 2.4. Statistical analysis

All the data were reported as the means of the data collected in three independent analyses. Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $p < 0.05$, using the statistical software Statistica 12.5 (StatSoft Inc., Tulsa, USA).

# 3. Results

## 3.1. Antifungal activity of the T. versicolor extract

The TE resulted active towards 40 out of the 46 tested strains. All the *Aspergillus* spp. strains, except for *A. parasiticus* DSM 1300, resulted significantly inhibited by both the TE concentrations tested, especially during the first 48 h of incubation. For some of the *Aspergillus* strains, inhibition was still evident after 48 h (*A. clavatus* PIC2, *A. niger* OLD4, and *A. niger* DSM 737) (Table 2).

Regarding the *Penicillium* spp. strains, the inhibitory effect of the TE was also more evident in the first 48 h of incubation, and in few cases, it became significant during the third day (*P. carneum* OLD12, *P. polonicum* CBS 222.28, *P. roqueforti* DSM 1079, and *P. viridicatum* DSM 62878) (Table 2). The strongest inhibition was observed towards *P. rubens* OLD1, *P. rubens* OLD9, *P. roqueforti* DPPMAF1, *P. chermesinum* CBS 117279, and *P. citrinum* DSM 1179, that resulted inhibited during the entire period monitored. The growth of *P. echinulatum* PIC3, *P. lanosocoeruleum* OLD8, *P. albocoremium* OLD13, *P. aurantiogriseum* DSM 1250, and *P. griseofulvum* DSM 896 was not affected by the TE (Table 2).

Among the other species included in the assay, TE resulted active towards *C. farinosa* OLD3 and *H. burtonii* DSM 3505 (especially during the first 24 h of incubation), while a weak inhibition during the second day was observed on *Paec. variotii* PIC4. A strong inhibition was observed for *C. farinosa* OLD5 and *M. suaveolens* DSM 2400, for which the effect was significant during all three days of the test.

## 3.2. Thermal stability and pH-dependence of the activity

The growth of *P. roquefortii* DPPMAF1 in PDB not supplemented with TE was comparable ($p > 0.05$) at pH 4.0 and 5.6, while a significant ($p < 0.05$) decrease of the recovered biomass was observed when the substrate was corrected at pH 6.6.

The addition of the TE to the substrate caused a huge decrease ($p < 0.05$) of the recovered biomass at pH 4.0 (−70% compared to PDB) while a slight, although significant, decrease was observed at pH 5.6 (only −16% compared to PDB). The antifungal activity seemed disappeared at higher pH level (Fig. 1).

Thermal treatment did not affect ($p > 0.05$) the antifungal activity of the TE, since no significant differences in the dry mycelial weight was observed in any of the conditions tested (Fig. 1).

## 3.3. Bread characterization

### 3.3.1. Sourdough characterization

A semi-solid type-II sourdough (De Vuyst, Comasio, & Kerrebroeck, 2023) was produced to be used as acidifying ingredient in breadmaking. Before sourdough fermentation, pH of the dough was 6.42 ± 0.03 (TTA of 2.30 ± 0.15 mL), while during fermentation it decreased to 3.76 ± 0.04 (TTA was 13.1 ± 0.18 mL). Lactic and acetic acids in sourdough corresponded to 93.88 ± 0.12 and 17.3 ± 0.22 mmol/kg respectively (QF of 5.4). TFAA, corresponding to 580 ± 12 mg/kg at t0, were found at the concentration of 1206 ± 21 mg/kg at the end of the 24 h of fermentation.

### 3.3.2. Dough characterization and leavening performances

After leavening, the pH of the cYB dough was 5.86 ± 0.22 (Table 3) and, as expected, the addition of sourdough caused the decrease of the pH to 4.36 ± 0.20 in cSB, a value that did not significantly ($p > 0.05$) differ from the pH value of the chemically acidified dough (Table 3).

Regardless the addition of the TE, the two chemically acidified doughs presented the highest TTA value. The addition of the TE did not cause ($p > 0.05$) modification of the TTA, compared to the corresponding control, in any of the types of doughs (Table 3).

Sourdough bread doughs cSB and tSB were characterized by the presence of 33.48–34.10 mmol/kg of lactic acid and 6.22–6.55 mmol/kg of acetic acid, as the consequence of the lactic acid fermentation of the starters, without significant ($p > 0.05$) differences between the samples. The QF ranged from 5.2 to 5.4.

Volume increase of the dough made without the addition of sourdough and TE (cYB) corresponded, under the experimental conditions, to 17.82 mL. A significantly ($p < 0.05$) higher value was found in cSB, while the chemical acidification led to a significantly ($p < 0.05$) lower value in acYB. The addition of the TE caused significant ($p < 0.05$) lowering of the volume increase in all doughs (tYB, taYB, and tSB).

**Table 2**
Antifungal activity of the freeze-dried TE added to substrate at 0, 0.5 and 1% (g:mL) as determined by a multiwell assay. The growth of the indicator strains was monitored at 24, 48, and 72 h by measuring absorbance at 620 nm. Data represent the ΔAbs (Inoculated well-Control well) mean of three independent experiments. The T-test was performed at *p*-values 0.05, 0.01 and 0.001.

| Indicator | TE supplementation | Absorbance ($\lambda$ 620 nm) | | | *p*-value: 0.05*; 0.01**; 0.001*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | g:mL | 24 h | 48 h | 72 h | 24 h | | 48 h | | 72 h | |
| *Aspergillus flavus* **PIC1** | 0 | 0.035 | 0.349 | 0.667 | | | | | | |
| | 0.5 | 0.025 | 0.462 | 0.810 | 0.210 | | **0.021** | * | 0.315 | |
| | 1 | 0.019 | 0.330 | 0.809 | 0.168 | | 0.632 | | 0.340 | |
| *Aspergillus clavatus* **PIC2** | 0 | 0.068 | 0.250 | 2.007 | | | | | | |
| | 0.5 | 0.017 | 0.226 | 0.332 | **0.009** | ** | 0.470 | | **0.003** | ** |
| | 1 | 0.014 | 0.429 | 0.823 | **0.006** | ** | 0.254 | | 0.115 | |
| *Penicillium echinulatum* **PIC3** | 0 | 0.014 | 0.213 | 1.018 | | | | | | |
| | 0.5 | 0.006 | 0.179 | 0.365 | 0.333 | | 0.333 | | 0.320 | |
| | 1 | 0.013 | 0.165 | 0.324 | 0.886 | | 0.288 | | 0.298 | |
| *Paecilomyces variotii* **PIC4** | 0 | 0.002 | 0.100 | 0.387 | | | | | | |
| | 0.5 | 0.001 | 0.001 | 0.231 | 0.305 | | **0.033** | * | 0.344 | |
| | 1 | 0.001 | 0.116 | 0.286 | 0.087 | | 0.880 | | 0.590 | |
| *Penicillium palitans* **PIC5** | 0 | 0.003 | 0.003 | 0.003 | | | | | | |
| | 0.5 | 0.024 | 0.061 | 0.080 | **0.000** | *** | **0.013** | * | 0.090 | |
| | 1 | 0.070 | 0.440 | 0.789 | **0.003** | ** | 0.275 | | 0.315 | |
| *Penicillium brevicompactum* **FER1** | 0 | 0.003 | 0.137 | 0.223 | | | | | | |
| | 0.5 | 0.013 | 0.126 | 0.199 | **0.004** | ** | 0.767 | | 0.586 | |
| | 1 | 0.029 | 0.147 | 0.227 | **0.007** | ** | 0.766 | | 0.897 | |
| *Penicillium brevicompactum* **FER2** | 0 | 0.006 | 0.138 | 1.375 | | | | | | |
| | 0.5 | 0.003 | 0.076 | 0.175 | 0.358 | | **0.036** | * | 0.184 | |
| | 1 | 0.002 | 0.068 | 0.191 | 0.315 | | **0.039** | * | 0.188 | |
| *Penicillium brevicompactum* **FER3** | 0 | 0.003 | 0.111 | 0.775 | | | | | | |
| | 0.5 | 0.007 | 0.094 | 0.843 | 0.109 | | 0.299 | | 0.940 | |
| | 1 | 0.029 | 0.088 | 0.355 | **0.042** | * | 0.149 | | 0.510 | |
| *Penicillium rubens* **OLD1** | 0 | 0.007 | 0.127 | 0.450 | | | | | | |
| | 0.5 | 0.001 | 0.067 | 0.111 | **0.032** | * | **0.029** | * | **0.037** | * |
| | 1 | 0.002 | 0.058 | 0.076 | **0.029** | * | **0.030** | * | **0.046** | * |
| *Penicillium carneum* **OLD2** | 0 | 0.001 | 0.058 | 0.115 | | | | | | |
| | 0.5 | 0.152 | 0.169 | 0.168 | **0.005** | ** | 0.057 | | 0.577 | |
| | 1 | 0.182 | 0.520 | 0.839 | **0.001** | *** | 0.095 | | 0.135 | |
| *Cordyceps farinosa* **OLD3** | 0 | 0.001 | 0.013 | 0.005 | | | | | | |
| | 0.5 | 0.015 | 0.036 | 0.427 | **0.018** | * | 0.365 | | 0.070 | |
| | 1 | 0.052 | 0.049 | 0.425 | **0.002** | ** | **0.017** | * | 0.322 | |
| *Aspergillus niger* **OLD4** | 0 | 0.008 | 0.176 | 2.038 | | | | | | |
| | 0.5 | 0.006 | 0.164 | 0.419 | 0.596 | | 0.578 | | **0.012** | * |
| | 1 | 0.012 | 0.131 | 0.318 | 0.477 | | 0.260 | | **0.009** | ** |
| *Cordyceps farinosa* **OLD5** | 0 | 0.001 | 0.003 | 0.003 | | | | | | |
| | 0.5 | 0.034 | 0.125 | 0.198 | **0.000** | *** | 0.086 | | 0.088 | |
| | 1 | 0.093 | 0.079 | 0.045 | **0.007** | ** | **0.000** | *** | **0.005** | ** |
| *Penicillium rubens* **OLD9** | 0 | 0.024 | 0.154 | 0.554 | | | | | | |
| | 0.5 | 0.010 | 0.082 | 0.121 | 0.070 | | **0.010** | ** | **0.001** | *** |
| | 1 | 0.005 | 0.060 | 0.096 | **0.035** | * | **0.006** | ** | **0.002** | ** |
| *Penicillium bialowiezense* **OLD6** | 0 | 0.000 | 0.180 | 0.359 | | | | | | |
| | 0.5 | 0.127 | 0.157 | 0.170 | **0.002** | ** | 0.699 | | 0.196 | |
| | 1 | 0.188 | 0.462 | 0.717 | **0.000** | *** | 0.401 | | 0.571 | |
| *Penicillium lanosocoeruleum* **OLD8** | 0 | 0.026 | 0.198 | 0.520 | | | | | | |
| | 0.5 | 0.004 | 0.142 | 1.117 | 0.121 | | 0.122 | | 0.514 | |
| | 1 | 0.014 | 0.149 | 0.249 | 0.291 | | 0.161 | | 0.102 | |
| *Penicillium carneum* **OLD12** | 0 | 0.024 | 0.202 | 0.335 | | | | | | |
| | 0.5 | 0.026 | 0.286 | 0.396 | 0.862 | | 0.077 | | 0.291 | |
| | 1 | 0.041 | 0.366 | 0.718 | 0.296 | | **0.011** | * | **0.004** | ** |
| *Penicillium albocoremium* **OLD13** | 0 | 0.004 | 0.143 | 1.063 | | | | | | |
| | 0.5 | −0.001 | 0.140 | 0.343 | 0.233 | | 0.864 | | 0.270 | |
| | 1 | 0.003 | 0.101 | 0.253 | 0.785 | | 0.069 | | 0.231 | |
| *Penicillium paneum* **OLD15** | 0 | 0.000 | 0.132 | 0.521 | | | | | | |
| | 0.5 | 0.001 | 0.173 | 0.313 | 0.136 | | **0.023** | * | 0.399 | |
| | 1 | 0.001 | 0.172 | 0.425 | 0.366 | | 0.386 | | 0.728 | |
| *Penicillium paneum* **OLD17** | 0 | 0.001 | 0.011 | 0.250 | | | | | | |
| | 0.5 | 0.002 | 0.094 | 0.294 | **0.000** | *** | 0.467 | | 0.788 | |

(*continued on next page*)

**Table 2** (*continued*)

| Indicator | TE supplementation | Absorbance ($\lambda$ 620 nm) | | | *p*-value: 0.05*; 0.01**; 0.001*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | g:mL | 24 h | 48 h | 72 h | 24 h | | 48 h | | 72 h | |
| | 1 | 0.001 | 0.043 | 0.209 | **0.005** | ** | 0.739 | | 0.733 | |
| *Penicillium roqueforti* **DPPMAF1** | 0 | 0.234 | 0.267 | 0.648 | | | | | | |
| | 0.5 | 0.001 | 0.016 | 0.045 | **0.000** | *** | **0.003** | ** | **0.035** | * |
| | 1 | 0.001 | 0.021 | 0.036 | **0.000** | *** | **0.006** | ** | **0.039** | * |
| *Aspergillus pseudoglaucus* **CBS 123.28** | 0 | 0.004 | 0.007 | 0.010 | | | | | | |
| | 0.5 | 0.020 | 0.151 | 0.264 | **0.030** | * | 0.070 | | 0.067 | |
| | 1 | 0.065 | 0.173 | 0.262 | **0.014** | * | **0.047** | * | 0.065 | |
| *Penicillium polonicum* **CBS 222.28** | 0 | 0.089 | 0.252 | 0.753 | | | | | | |
| | 0.5 | 0.002 | 0.122 | 0.479 | 0.380 | | 0.065 | | 0.133 | |
| | 1 | 0.004 | 0.106 | 0.248 | 0.419 | | **0.050** | * | **0.000** | *** |
| *Penicillium bialowiezense* **CBS 227.28** | 0 | 0.004 | 0.144 | 0.422 | | | | | | |
| | 0.5 | 0.010 | 0.123 | 0.637 | 0.127 | | 0.466 | | 0.380 | |
| | 1 | 0.037 | 0.136 | 0.371 | **0.006** | ** | 0.728 | | 0.250 | |
| *Penicillium crustosum* **CBS 340.59** | 0 | 0.040 | 0.304 | 1.304 | | | | | | |
| | 0.5 | 0.033 | 0.252 | 0.622 | 0.410 | | **0.049** | * | 0.085 | |
| | 1 | 0.026 | 0.198 | 1.526 | 0.145 | | **0.011** | * | 0.523 | |
| *Penicillium lanosocoeruleum* **CBS 484.84** | 0 | 0.037 | 0.179 | 1.009 | | | | | | |
| | 0.5 | 0.021 | 0.134 | 0.611 | 0.158 | | 0.077 | | 0.517 | |
| | 1 | 0.024 | 0.122 | 0.266 | **0.015** | * | **0.004** | ** | 0.258 | |
| *Penicillium paneum* **CBS 101032** | 0 | 0.000 | 0.116 | 0.232 | | | | | | |
| | 0.5 | 0.146 | 0.142 | 0.121 | **0.000** | *** | 0.358 | | 0.199 | |
| | 1 | 0.195 | 0.264 | 0.242 | **0.000** | *** | **0.002** | ** | 0.713 | |
| *Penicillium albocoremium* **CBS109582** | 0 | 0.001 | 0.026 | 0.177 | | | | | | |
| | 0.5 | 0.018 | 0.071 | 0.319 | **0.018** | * | 0.107 | | 0.415 | |
| | 1 | 0.046 | 0.050 | 0.828 | **0.014** | * | 0.308 | | 0.081 | |
| *Penicillium carneum* **CBS 112297** | 0 | 0.007 | 0.088 | 0.340 | | | | | | |
| | 0.5 | 0.002 | 0.074 | 0.214 | 0.243 | | 0.326 | | 0.296 | |
| | 1 | 0.037 | 0.081 | 0.184 | **0.023** | * | 0.613 | | 0.221 | |
| *Penicillium chermesinum* **CBS 117279** | 0 | 0.005 | 0.204 | 0.452 | | | | | | |
| | 0.5 | 0.001 | 0.119 | 0.337 | **0.006** | ** | **0.012** | ** | **0.024** | * |
| | 1 | 0.002 | 0.080 | 0.292 | 0.406 | | **0.003** | ** | 0.210 | |
| *Aspergillus niger* **DSM 737** | 0 | 0.043 | 0.175 | 1.631 | | | | | | |
| | 0.5 | 0.036 | 0.196 | 1.034 | 0.700 | | 0.543 | | 0.116 | |
| | 1 | 0.048 | 0.160 | 0.456 | 0.691 | | 0.593 | | **0.039** | * |
| *Aspergillus amoenus* **DSM 1943** | 0 | 0.003 | 0.004 | 0.004 | | | | | | |
| | 0.5 | 0.033 | 0.145 | 0.239 | **0.035** | * | 0.085 | | 0.141 | |
| | 1 | 0.069 | 0.225 | 0.360 | **0.007** | ** | 0.066 | | 0.087 | |
| *Aspergillus parasiticus* **DSM 1300** | 0 | 0.008 | 0.290 | 0.791 | | | | | | |
| | 0.5 | 0.004 | 0.251 | 0.592 | 0.178 | | 0.561 | | 0.349 | |
| | 1 | 0.007 | 0.186 | 0.606 | 0.925 | | 0.101 | | 0.383 | |
| *Aspergillus glaucus* **DSM 16660** | 0 | 0.003 | 0.002 | 0.110 | | | | | | |
| | 0.5 | 0.016 | 0.020 | 0.136 | **0.006** | ** | 0.074 | | 0.848 | |
| | 1 | 0.050 | 0.188 | 0.799 | **0.001** | *** | 0.076 | | 0.081 | |
| *Eurotium rubrum* **DSM 62631** | 0 | 0.002 | 0.016 | 0.006 | | | | | | |
| | 0.5 | 0.016 | 0.019 | 0.159 | 0.141 | | 0.802 | | **0.001** | *** |
| | 1 | 0.051 | 0.109 | 0.547 | **0.024** | * | 0.162 | | 0.112 | |
| *Penicillium aurantiogriseum* **DSM 1250** | 0 | 0.004 | 0.115 | 0.781 | | | | | | |
| | 0.5 | 0.006 | 0.110 | 0.832 | 0.628 | | 0.821 | | 0.944 | |
| | 1 | 0.004 | 0.089 | 0.249 | 0.936 | | 0.305 | | 0.392 | |
| *Penicillium brevicompactum* **DSM 2215** | 0 | 0.000 | 0.001 | 0.018 | | | | | | |
| | 0.5 | 0.002 | 0.030 | 0.273 | **0.037** | * | 0.705 | | 0.214 | |
| | 1 | 0.001 | 0.066 | 0.168 | **0.008** | ** | 0.616 | | 0.481 | |
| *Penicillium chrysogenum* **DSM 895** | 0 | 0.007 | 0.140 | 0.373 | | | | | | |
| | 0.5 | 0.002 | 0.121 | 0.176 | **0.055** | * | 0.329 | | 0.128 | |
| | 1 | 0.002 | 0.085 | 0.137 | 0.068 | | **0.050** | * | 0.078 | |
| *Penicillium citrinum* **DSM 1179** | 0 | 0.002 | 0.004 | 0.005 | | | | | | |
| | 0.5 | 0.025 | 0.095 | 0.148 | **0.012** | * | 0.061 | | 0.145 | |
| | 1 | 0.067 | 0.144 | 0.202 | **0.001** | *** | **0.005** | ** | **0.017** | * |
| *Penicillium corylophylum* **DSM 62832** | 0 | 0.002 | 0.050 | 0.118 | | | | | | |
| | 0.5 | 0.001 | 0.033 | 0.486 | **0.027** | * | 0.336 | | 0.494 | |
| | 1 | 0.001 | 0.054 | 0.061 | **0.003** | ** | 0.815 | | 0.062 | |
| *Penicillium griseofulvum* **DSM 896** | 0 | 0.004 | 0.062 | 0.755 | | | | | | |

(*continued on next page*)

**Table 2** (*continued*)

| Indicator | TE supplementation | Absorbance (λ 620 nm) | | | *p*-value: 0.05*; 0.01**; 0.001*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | g:mL | 24 h | 48 h | 72 h | 24 h | | 48 h | | 72 h | |
| | 0.5 | 0.001 | 0.083 | 0.144 | 0.350 | | 0.549 | | 0.418 | |
| | 1 | 0.002 | 0.053 | 0.348 | 0.090 | | 0.438 | | 0.575 | |
| *Penicillium expansum* **DSM 1282** | 0 | 0.011 | 0.171 | 0.388 | | | | | | |
| | 0.5 | 0.010 | 0.153 | 0.769 | 0.833 | | 0.243 | | 0.279 | |
| | 1 | 0.036 | 0.159 | 0.485 | **0.001** | *** | 0.483 | | 0.372 | |
| *Penicillium roqueforti* **DSM 1079** | 0 | 0.001 | 0.054 | 0.209 | | | | | | |
| | 0.5 | 0.002 | 0.043 | 0.103 | 0.191 | | 0.526 | | 0.058 | |
| | 1 | 0.001 | 0.051 | 0.123 | 0.276 | | 0.616 | | **0.050** | * |
| *Penicillium viridicatum* **DSM 62878** | 0 | 0.051 | 0.324 | 1.624 | | | | | | |
| | 0.5 | 0.040 | 0.301 | 1.064 | 0.259 | | 0.391 | | 0.383 | |
| | 1 | 0.032 | 0.254 | 0.565 | 0.090 | | 0.080 | | **0.049** | * |
| *Hyphopichia burtonii* **DSM 3505** | 0 | 0.001 | 0.232 | 0.464 | | | | | | |
| | 0.5 | 0.128 | 0.188 | 0.230 | **0.000** | *** | 0.568 | | 0.201 | |
| | 1 | 0.207 | 0.263 | 0.300 | **0.001** | *** | 0.672 | | 0.325 | |
| *Moniliella suaveolens* **DSM 2400** | 0 | 0.001 | 0.002 | 0.001 | | | | | | |
| | 0.5 | 0.037 | 0.093 | 0.131 | **0.005** | ** | **0.006** | ** | **0.001** | *** |
| | 1 | 0.072 | 0.254 | 0.416 | **0.008** | ** | **0.011** | * | **0.018** | * |

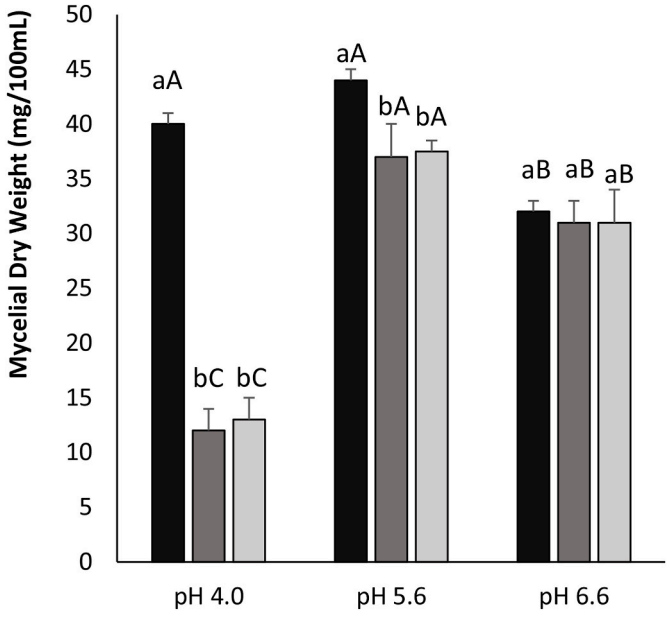


**Fig. 1.** Dry mycelial weight of *Penicillium roquefortii* DPPMAF1 cultivated for 48 h at 25 °C in PDB (black histogram), PDB supplemented with 10% (mL:mL) of TE (TE-PDB, dark grey histogram), and PDB supplemented with 10% (mL:mL) thermal treated TE (100 °C for 5 min) (tTE-PDB, light grey histogram), after correction of the substrate at pH 4.0, 5.6 or 6.6. Data are the means of three independent experiments ± standard deviations (n = 3). [a-b] Different lowercase letters, among different sample within the same pH-range mean significant differences at a $p < 0.05$. [A–C]Different uppercase letters among different pH levels of the same sample mean significant differences at a $p < 0.05$.

Compared to corresponding controls, the decreases were from −26 to −30% (Table 3). Compared to cYB and acYB, sourdough addition caused a marked increase of the concentration of the TFAA (+68%); while TE contributed to a lesser extent (from 8 to 14% compared to corresponding controls) (Table 3).

### 3.3.3. Textural and colour bread characterization

The addition of the TE caused an increase of the bread hardness in tYB and taYB (+74 and + 22% compared to corresponding controls cYB and acYB, respectively), while both the sourdough breads (produced without and with the TE) had similar ($p > 0.05$) hardness values (Table 4). The fracturability, which was the highest for cYB, significantly ($p < 0.05$) decreased with TE addition (−37% in tYB), while no significant ($p > 0.05$) differences were observed in the other bread samples (Table 4). Compared to the corresponding controls, a significant ($p < 0.05$) decrease of cohesiveness and adhesiveness was found in all bread samples added with TE, although sourdough breads were characterized by the highest values of both the parameters (Table 4). Springiness was also the highest for sourdough-containing breads compared to the others, although its values did not change ($p > 0.05$) when TE was used as ingredient. Contrarily to cohesiveness and adhesiveness, chewiness increased as the consequence of the TE addition, but also for this parameter, sourdough breads were characterized by the highest values (Table 4). Overall, the crust colour of the sourdough breads was characterized by the lowest L* and by the highest a* values. Accordingly, tSB and cSB were characterized by the highest values of C* and BI, followed by taYB, this latter characterized by slightly but significantly ($P < 0.05$)

**Table 3**
Main characteristics of the doughs after leavening at 28 °C for 1.5 h: control (cYB); chemically acidified control (acYB); control with sourdough (cSB); dough containing the TE (tYB); chemically acidified dough containing the TE (taYB); dough containing the TE and sourdough (tSB).

| | cYB | acYB | cSB | tYB | taYB | tSB |
|---|---|---|---|---|---|---|
| pH | 5.86 ± 0.22$^{a}$ | 4.00 ± 0.15$^{b}$ | 4.36 ± 0.20$^{b}$ | 5.71 ± 0.25$^{a}$ | 3.92 ± 0.14$^{b}$ | 4.24 ± 0.20$^{b}$ |
| TTA (ml NaOH 0,1 mol/L) | 4.42 ± 0.25$^{c}$ | 10.23 ± 0.18$^{a}$ | 7.91 ± 0.23$^{b}$ | 4.52 ± 0.28$^{c}$ | 10.30 ± 0.20$^{a}$ | 8.51 ± 0.15$^{b}$ |
| Lactic acid (mmol/kg) | 3.54 ± 0.51$^{c}$ | 45.73 ± 0.22$^{a}$ | 33.48 ± 1.60$^{b}$ | 3.61 ± 0.30$^{c}$ | 45.91 ± 2.40$^{a}$ | 34.10 ± 2.10$^{b}$ |
| Acetic acid (mmol/kg) | n.d | n.d. | 6.22 ± 0.21$^{a}$ | n.d | n.d | 6.5 ± 0.23$^{a}$ |
| Volume increase ΔV (mL) | 17.82 ± 0.21$^{b}$ | 15.04 ± 0.23$^{c}$ | 19.71 ± 0.30$^{a}$ | 12.96 ± 0.14$^{e}$ | 11.44 ± 0.23$^{f}$ | 14.13 ± 0.25$^{d}$ |
| TFAA (mg/kg) | 583 ± 12$^{c}$ | 590 ± 14$^{c}$ | 990 ± 20$^{b}$ | 630 ± 14$^{c}$ | 636 ± 15$^{c}$ | 1130 ± 10$^{a}$ |

The data are the means of three independent experiments ± standard deviations (n = 3).
[a-d]Values in the same row with different superscript letters differ significantly ($P < 0.05$).

**Table 4**
Main characteristics of the experimental breads: control (cYB); chemically acidified control (acYB); control with sourdough (cSB); bread containing the TE (tYB); chemically acidified bread containing the TE (taYB); bread containing the TE and sourdough (tSB).

| | cYB | acYB | cSB | tYB | taYB | tSB |
|---|---|---|---|---|---|---|
| *Hardness (N)* | 27.04 ± 1.20$^{d}$ | 50.04 ± 0.90$^{b}$ | 31.08 ± 1.05$^{c}$ | 47.12 ± 2.11$^{b}$ | 61.04 ± 0.90$^{a}$ | 35.18 ± 0.14$^{c}$ |
| *Fracturability (N)* | 17.69 ± 1.08$^{a}$ | 11.69 ± 1.08$^{b}$ | 12.49 ± 1.03$^{b}$ | 11.14 ± 1.11$^{b}$ | 10.69 ± 1.08$^{b}$ | 10.34 ± 1.00$^{b}$ |
| *Cohesiveness* | 0.30 ± 0.00$^{b}$ | 0.31 ± 0.10$^{b}$ | 0.42 ± 0.05$^{a}$ | 0.11 ± 0.04$^{c}$ | 0.10 ± 0.10$^{c}$ | 0.32 ± 0.02$^{b}$ |
| *Adhesiveness (J)* | 0.73 ± 0.25$^{d}$ | 0.74 ± 0.22$^{d}$ | 1.07 ± 0.03$^{c}$ | 3.18 ± 0.06$^{b}$ | 1.74 ± 0.22$^{c}$ | 4.61 ± 0.21$^{a}$ |
| *Springiness* | 4.34 ± 0.02$^{b}$ | 3.34 ± 0.02$^{c}$ | 7.69 ± 0.01$^{a}$ | 4.08 ± 0.03$^{b}$ | 3.04 ± 0.02$^{c}$ | 7.64 ± 0.10$^{a}$ |
| *Chewiness (N)* | 50.78 ± 3.37$^{d}$ | 80.55 ± 2.57$^{b}$ | 71.65 ± 0.95$^{c}$ | 71.65 ± 0.89$^{c}$ | 86.55 ± 2.57$^{a}$ | 87.32 ± 0.07$^{a}$ |
| Crust Color | | | | | | |
| L | 56.78 ± 2.88$^{a}$ | 56.66 ± 1.45$^{a}$ | 52.10 ± 0.61$^{a}$ | 52.63 ± 2.13$^{b}$ | 51.43 ± 1.93$^{b}$ | 50.06 ± 1.18$^{b}$ |
| *a* | 2.66 ± 1.85$^{d}$ | 2.64 ± 0.50$^{d}$ | 5.50 ± 0.46$^{b}$ | 4.32 ± 0.42$^{c}$ | 4.20 ± 0.33$^{c}$ | 7.48 ± 0.71$^{a}$ |
| *b* | 20.65 ± 2.65$^{b}$ | 20.70 ± 1.95$^{b}$ | 23.57 ± 0.21$^{b}$ | 22.01 ± 0.83$^{b}$ | 22.21 ± 0.91$^{b}$ | 27.00 ± 0.25$^{a}$ |
| ΔE | 40.65 ± 1.34$^{b}$ | 40.78 ± 1.64$^{b}$ | 46.40 ± 3.56$^{a}$ | 45.06 ± 1.85$^{a}$ | 46,20 ± 1.71$^{a}$ | 42.08 ± 2.61$^{ab}$ |
| *C** | 20.82 ± 1.62$^{d}$ | 20.86 ± 1.23$^{d}$ | 24.2 ± 0.25$^{b}$ | 22.43 ± 0.47$^{c}$ | 22.59 ± 0.48$^{bc}$ | 28.02 ± 0.38$^{a}$ |
| *H** | 82.66 ± 5.51$^{a}$ | 83.00 ± 5.24$^{a}$ | 76.87 ± 2.45$^{ab}$ | 78.90 ± 6.32$^{a}$ | 79.29 ± 7.01$^{a}$ | 74.52 ± 1.94$^{b}$ |
| BI | 3.49 ± 0.1$^{d}$ | 3.35 ± 0.16$^{d}$ | 7.64 ± 0.12$^{b}$ | 5.99 ± 0.03$^{c}$ | 5.96 ± 0.03$^{c}$ | 10.69 ± 0.10$^{a}$ |
| Crumb color | | | | | | |
| L | 60.62 ± 3.15$^{a}$ | 62.02 ± 2.00$^{a}$ | 52.98 ± 0.53$^{b}$ | 57.07 ± 2.53$^{a}$ | 57.02 ± 1.13$^{a}$ | 54.63 ± 2.17$^{b}$ |
| *a* | −4.09 ± 0.22$^{a}$ | −4.00 ± 0.25$^{a}$ | −2.32 ± 0.27$^{b}$ | −1.92 ± 0.30$^{c}$ | −1.80 ± 0.38$^{c}$ | −2.56 ± 0.21$^{b}$ |
| *b* | 9.56 ± 0.58$^{b}$ | 9.43 ± 0.32$^{b}$ | 12.49 ± 0.36$^{a}$ | 12.80 ± 0.87$^{a}$ | 12.50 ± 0.66$^{a}$ | 12.46 ± 0.13$^{a}$ |
| ΔE | 33.03 ± 3.47$^{c}$ | 31.65 ± 0.22$^{c}$ | 40.99 ± 0.17$^{a}$ | 37.16 ± 1.93$^{b}$ | 37.11 ± 0.35$^{b}$ | 39.42 ± 2.02$^{ab}$ |
| *C** | 10.4 ± 0.31$^{b}$ | 10.24 ± 0.2$^{b}$ | 12.70 ± 0.23$^{a}$ | 12.94 ± 0.46$^{a}$ | 12.63 ± 0.38$^{a}$ | 12.72 ± 0.12$^{a}$ |
| *H** | 113.16 ± 6.92$^{a}$ | 112.99 ± 5.20$^{a}$ | 100.52 ± 5.31$^{b}$ | 98.53 ± 7.10$^{b}$ | 98.19 ± 6.01$^{b}$ | 101.61 ± 3.18$^{b}$ |
| BI | −4.84 ± 0.01$^{b}$ | −4.63 ± 0.02$^{c}$ | −3.08 ± 0.08$^{b}$ | −2.34 ± 0.08$^{a}$ | −2.19 ± 0.09$^{a}$ | −3.3 ± 0.02$^{b}$ |

The data are the means of three independent experiments ± standard deviations (n = 3).
[a-d]Values in the same row with different superscript letters differ significantly (P < 0.05).

lower values for both parameters.

TE addition significantly ($p < 0.05$) increased the latter (Table 4). The presence of sourdough also affected crumb colour by significantly decreasing L*. TE addition corresponded to an increase of a* and b* values only in bread samples produced without sourdough, and conferred to the crumb of tYB, TaYB and tSB significant ($p < 0.05$) higher values of BI compared to the other samples (Table 4).

### 3.3.4. Sensory analysis

Among the breads produced without the addition of TE, the sensory profile of the cYB and cSB was characterized by the highest and similar ($p > 0.05$) score for the elasticity (Table 5 and Fig. 2). As expected, crust and crumb colour, salty and acidic taste and odor were all more intense in cSB compared to cYB. acYB was characterized by the highest score for the acidic taste. The addition of TE to the bread recipe was overall perceived with an increase of herbaceous taste and crumb colour. Astringency, that was perceived in tYB and taCB as the result of the TE addition, was not perceived in tSB (Table 5 and Fig. 2).

## 3.4. In-situ antifungal activity

Slices of the experimental breads were inoculated through nebulization of a conidia suspension of *P. roquefortii* DPPMAF1. The mycelia became visible in all the bread samples produced without the TE after the first three days of incubation at room temperature (Table 6). In particular, *circa* 25%, 15 and < 10% of the slice surface was covered by mycelia in CYB, acYB, and cSB, respectively. Only cYBcp and TE-containing bread appeared as mold-free at that time (Table 6). cYB and acYB showed the fastest growth of the indicator, indeed, after 6 and 9 days of incubation respectively, 50% of the slice surface was covered by the mycelia. cSB showed a limited growth of the indicator although from the 9th day of storage *circa* 30% of the slice surface was contaminated. taYB and tSB were characterized by the longest mold-free shelf-life; mycelia became visible after 6 and 9 days respectively (Table 6). The growth of the indicator in tSB follow the same evolution observed in cYBcp.

**Table 5**
Sensory profile of the experimental breads as assessed by a panel test. Control (cYB); chemically acidified control (acYB); control with sourdough (cSB); bread containing the TE (tYB); chemically acidified bread containing the TE (taYB); bread containing the TE and sourdough (tSB).

| Attribute | cYB | acYB | cSB | tYB | taYB | tSB |
|---|---|---|---|---|---|---|
| Elasticity | 7.12 ± 0.10$^{b}$ | 4.50 ± 0.25$^{d}$ | 7.00 ± 0.10$^{b}$ | 5.00 ± 0.21$^{c}$ | 5.00 ± 0.13$^{c}$ | 7.87 ± 0.13$^{a}$ |
| Friability | 5.75 ± 0.05$^{a}$ | 6.00 ± 0.20$^{a}$ | 6.12 ± 0.22$^{a}$ | 5.50 ± 0.20$^{b}$ | 5.40 ± 0.15$^{b}$ | 5.37 ± 0.25$^{b}$ |
| Crust color | 2.75 ± 0.12$^{d}$ | 3.00 ± 0.20$^{d}$ | 5.62 ± 0.15$^{b}$ | 5.50 ± 0.23$^{b}$ | 5.00 ± 0.20$^{c}$ | 7.37 ± 0.18$^{a}$ |
| Crumb color | 2.50 ± 0.10$^{c}$ | 2.30 ± 0.15$^{c}$ | 4.25 ± 0.15$^{b}$ | 4.25 ± 0.10$^{b}$ | 4.50 ± 0.25$^{b}$ | 5.50 ± 0.09$^{a}$ |
| Sweetness | 4.00 ± 0.20$^{b}$ | 4.00 ± 0.20$^{b}$ | 3.37 ± 0.13$^{c}$ | 4.50 ± 0.12$^{a}$ | 4.70 ± 0.26$^{a}$ | 3.50 ± 0.14$^{c}$ |
| Bitterness | 1.25 ± 0.25$^{c}$ | 2.00 ± 0.25$^{b}$ | 2.87 ± 0.23$^{a}$ | 2.25 ± 0.23$^{b}$ | 2.00 ± 0.20$^{b}$ | 2.25 ± 0.12$^{b}$ |
| Salty | 3.00 ± 0.15$^{d}$ | 2.00 ± 0.08$^{e}$ | 5.00 ± 0.24$^{b}$ | 3.87 ± 0.21$^{c}$ | 3.00 ± 0.20$^{d}$ | 6.37 ± 0.20$^{a}$ |
| Astringency | 2.25 ± 0.21$^{c}$ | 2.00 ± 0.10$^{c}$ | 3.00 ± 0.11$^{b}$ | 4.12 ± 0.08$^{a}$ | 4.50 ± 0.30$^{a}$ | 3.30 ± 0.10$^{b}$ |
| Herbaceous taste | 2.00 ± 0.08$^{c}$ | 2.00 ± 0.10$^{c}$ | 3.00 ± 0.09$^{b}$ | 4.50 ± 0.10$^{a}$ | 4.60 ± 0.15$^{a}$ | 4.75 ± 0.12$^{a}$ |
| Acidic taste | 1.25 ± 0.02$^{d}$ | 7.00 ± 0.35$^{a}$ | 5.00 ± 0.10$^{b}$ | 4.12 ± 0.10$^{c}$ | 7.30 ± 0.20$^{a}$ | 5.25 ± 0.18$^{b}$ |
| Acidic odor | 1.50 ± 0.10$^{d}$ | 4.00 ± 0.20$^{c}$ | 5.75 ± 0.11$^{a}$ | 5.00 ± 0.13$^{b}$ | 5.00 ± 0.24$^{b}$ | 6.00 ± 0.08$^{a}$ |

Data are the means of the scores collected in three independent tests ± standard deviations.
[a-e]Values in the same row with different superscript letters differ significantly (P < 0.05).

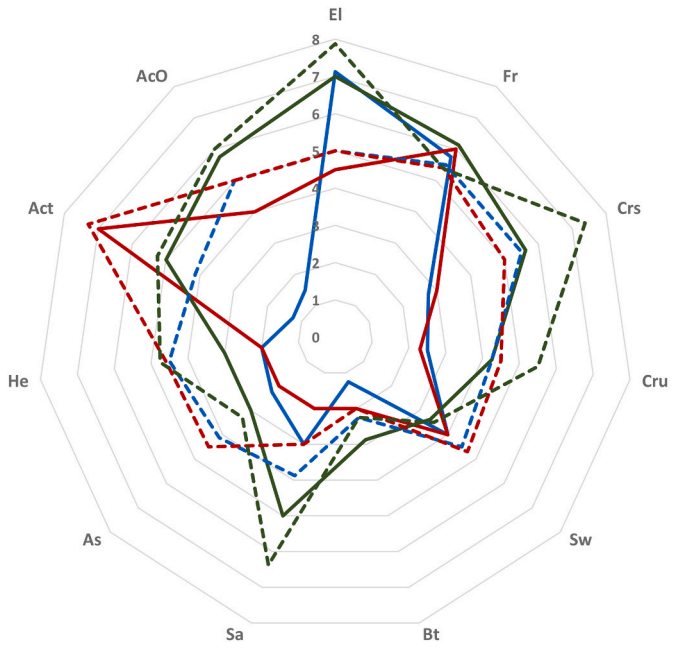


**Fig. 2.** Sensory profile of the experimental breads as assessed by a panel test: control (cYB, blue line); chemically acidified control (acYB, red line); control with sourdough (cSB, green line); bread containing the TE (tYB, blue dotted line); chemically acidified bread containing the TE (taYB, red dotted line); bread containing the TE and sourdough (tSB, green dotted line). Data are the means of the scores collected in three independent tests. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 6**
Fungal contamination of the experimental breads slices inoculated by nebulization with a suspension of $10^2$ conidia/ml of *Penicillium roqueforti* DPPMAF1 and incubated at room temperature for 15 days: control (cYB); chemically acidified control (acYB); control with sourdough (cSB); bread containing the TE (tYB); chemically acidified bread containing the TE (taYB); bread containing the TE and sourdough (tSB). The test was carried out in triplicate. A bread produced with the same protocol of the cYB but containing 0.1% (g:g) calcium propionate (cYBcp), was added to the experimental breads set.

| Days of storage | cYB | acYB | cSB | cYBcp | tYB | taYB | tSB |
|---|---|---|---|---|---|---|---|
| 3 | + | ± | ± | – | – | – | – |
| 6 | ++ | + | ± | – | ± | – | – |
| 9 | ++ | ++ | + | – | + | ± | – |
| 12 | +++ | +++ | + | ± | + | + | ± |
| 15 | +++ | +++ | ++ | + | ++ | + | + |

Contamination was scored on the basis of the slice surface covered by fungal mycelia as follows: –, 0%; +/–, <20%; +, 20–40%; ++, 50%; +++, 60–80%; ++++, >80%.

## 4. Discussion

For thousands of years, bread has been one of the main components of the human diet (Rosell, Bajerska, & El Sheikha, 2015). Nevertheless, due to their easily spoiled nature, the quality and palatability of baked goods quickly degrade during storage (Garcia et al., 2019). Indeed, the fungal spoilage is the primary cause of significant financial detriments also associated to the potential mycotoxin contamination (Ju et al., 2018) which poses financial losses for the bakeries and safety issues (Melikoglu & Webb, 2013; Melini & Melini, 2018). *Penicillium* spp., *Aspergillus* spp., *Wallemia* spp., and other species belonging to *Rhizopus*, *Chrysonilia*, and *Mucor* genera are the main fungi involved in the baked goods spoilage (Ju et al., 2020; Quattrini et al., 2019).

Based on the scientific evidence showing the antifungal potential of the *T. versicolor* extract (culture filtrate) against phytopathogenic fungi and its established use in food industry, the activity towards fungi commonly associated to baked good spoilage was investigated in this work, using an integrated approach including *in-vitro* and *in-situ* experiments.

Recent studies confirmed several bioactivities (e.g., immunomodulatory, antiviral, antioxidant activity, and antitumoral) of *T. versicolor* which mainly rely on protein-bound polysaccharides and exopolysaccharides and hence justify its use in traditional medicine and as food supplement (Angelova et al., 2022; Bains et al., 2021; Cerig, 2021). It has been moreover demonstrated that the necessity to survive in natural habitats in which they are in competition with numerous microbial species (dark places and high-humidity niches) (Bains & Chawla, 2020) makes higher fungi as *T. versicolor* able to synthesize antimicrobial compounds.

Overall, antibacterial activity of mushrooms is primarily due to bioactive phenolic compounds (e.g., phenolic acids, flavonoids, and terpenes) which damage cell wall and membrane or inhibit nucleic acid synthesis or enzyme activities (Asri, Yahya, Rehan, & Yahya, 2019; Matijašević et al., 2016), while fungitoxic activity is mainly due to protein/peptide fraction (exoproteome) (Parroni et al., 2019). A mixture of several proteins with molecular weight of 15 kDa (Ashraf et al., 2023) and from 40 to 75 kDa (Parroni et al., 2019) were identified as responsible for the antimicrobial activity of the *T. versicolor* culture filtrate.

Aiming at assessing the potential of the TE to inhibit fungi involved in baked goods spoilage, 46 strains, previously isolated from bakery products and bakeries, were included into the screening of the antifungal activity. An intense growth inhibition of most of the *Aspergillus* and *Penicillium* species tested was observed during the first 48 h of the incubation. Inhibition was evident until the third day of monitoring for several indicator strains. Antifungal activity was also found against *M. suaveolens, C. farinosa*, *H. burtonii* and, to a lesser extent, *Paec. variotii*.

Although the extract showed a very wide inhibitory spectrum of activity, few species or strains belonging to *P. echinulatum, P. lanosocoeruleum, P. albocoremium, P. aurantiogriseum,* and *P. griseofulvum* were not affected by *T. versicolor* metabolites (under these experimental conditions). A non-total inhibitory activity spectrum was already reported for many other antifungal "natural" matrices (Aldholmi, Marchand, Ourliac-Garnier, Le Pape, & Ganesan, 2019; Schlösser & Prange, 2018).

Since bread undergo the baking procedure, reaching a temperature 80–90 °C at the core of the product (Zanoni, Peri, & Pierucci, 1993), the thermal sensitivity of the extract was tested.

According to previous investigations that reported antifungal compounds of TE as highly thermostable (Ashraf et al., 2023), no differences were found in the antifungal activity of the thermal treated extract. Nevertheless, *in-vitro* assays showed that the inhibitory activity of the TE was more intense at acidic pH (4.00). Higher values of the pH (e.g., 5.6, that is typical of the wheat baked goods leavened with baker's yeast) caused a marked decrease of the activity.

The antifungal action of peptides and proteins can involve several mechanisms, such as perturbation of the fungal membrane integrity, translocation through membranes, alteration of fungal metabolisms. A positive charge of the antifungal proteins is necessary for interaction with the negatively charged cell membrane of target microorganisms (Thery, Lynch, & Arendt, 2019), therefore the pH of the matrix could be of key importance in the activation of such class of compounds. Being the antifungal proteins of the TE characterized by pI ranging from 4 to 5.5 (Parroni et al., 2019), the loss of their charge and, consequently, of the possibility to interact with membranes at high level of pH, can be hypothesized, thus explaining the pH-dependence of the TE activity.

The baking test was performed on bread samples obtained at different pH: two were produced according to the main protocols commonly employed for obtaining baked goods: with baker's yeast or with sourdough (this latter, including LAB, is characterized by a lower

pH compared to the first). A type-II sourdough was obtained through the inoculum of two selected LAB, aiming at obtaining a consistent, fast, and standardizable acidification level (De Vuyst et al., 2023). A chemically acidified dough was also produced, aiming at distinguishing the effect of the TE from that related to LAB. Sourdough breads are indeed characterized by a longer shelf-life thanks to the synergistic activity of antifungal organic acids (e.g., acetic, 4 phenyl-lactic) and peptides (Coda et al., 2011; 2013) released by LAB during fermentation.

It must be noted that the addition of the TE negatively affected the leavening performances, thus hypothesizing a weak, but significant, effect on the *Saccharomyces cerevisiae* growth or metabolism. Also, the structural parameters of the bread samples resulted moderately changed by the TE supplementation: compared to the corresponding controls produced without TE addition, hardness increased and fracturability decreased, respectively. Supplemented bread samples were indeed characterized by a more compact structure and by a lower elasticity. Also, the adhesiveness resulted markedly higher in bread samples obtained with TE. Overall, the use of sourdough determined the highest volume increase of the dough during proofing and counteracted the negative effect of the TE supplementation on all the structural features of the final product. There is considerable consensus of the scientific community with regard to the positive effects of sourdough addition for bread production, including improvements in bread volume and crumb structure (Arendt, Ryan, & Dal Bello, 2007; Arora et al., 2021). The sourdough microbiota has moreover been reported to have positive effects on bread staling. These effects are in part due to the improvement in loaf specific volume and gas cell volume, which is associated with a reduction in the rate of staling. Moreover, the dynamics associated with the biological acidification of dough and with the enzymatic activity of LAB on starch, affect the retrogradation phenomena thus slowing the staling during storage (Arendt et al., 2007).

Among the colorimetric coordinates of the bread samples, the major differences were observed for the crumb colour of the baker's yeast breads (tYB and taYB compared, respectively, to cYB and acYB), in which TE supplementation caused a decrease of lightness and an increase of the green-red index (a*). The extract is indeed characterized by a yellow-brown colour (data not showed). The presence of sourdough had a masking effect on the colour changes related to the TE addition, since it provides higher concentration of free amino acids compared to the other ingredients, compounds that contribute to the Maillard reaction and, consequently, to a uniform and slight darkening of the colour during baking. Accordingly, the Browning Index "BI", which is considered a very important quality parameter and indicator for the Maillard reaction in different conditions of treatments, processing, and storage (Abd El-Baset & Almoselhy, 2023), resulted 80–130% higher in sourdough breads crust compared to the other bread samples. The effect of the TE addition was more evident in crumb, whose BI resulting significantly higher in baker's yeast bread samples added with the extract compared to the controls.

TE, although added in relatively low amount to the bread samples, conferred perceivable herbaceous taste and astringency, nevertheless scores for these attributes were relatively low. The presence of a broad spectrum of natural flavour compounds and a pleasant aroma profile were already reported in different foods and beverages (Bains et al., 2021; Viswanath, Rajesh, Janardhan, Kumar, & Narasimha, 2014). For experimental sourdough bread, overall characterized by the highest intensity of the typical sourdough attributes (acidic flavour and taste, salty taste) the sensory modifications induced by the TE supplementation were much less perceived by the panellists. According to the recent literature, the sourdough bread sensory profile was largely recognized as the most appreciated by the consumer compared to that of baker's yeast baked goods (Arora et al., 2021).

Results of the monitoring the indicator growth on bread samples confirmed the antifungal activity *in-situ*. As expected, the activity was more intense in bread samples characterized by low pH level (either chemically or biologically acidified). Only the bread sample containing both TE and sourdough was however characterized by a growth inhibition comparable to that observed in bread sample added with the chemical preservative calcium propionate.

Today's food industry faces a tremendous problem in producing goods that are not only financially sustainable but also wholesome for customers, as well as much more long-lasting. The use of organic preservatives has the feasibility of meeting both needs (Ju et al., 2019; Luz et al., 2018). Natural antimicrobial preservatives have been the subject of extensive research, due to the growing evidence of the harmfulness of chemical preservatives and their impacts on consumer health (Dengate & Ruben, 2002; Erickson & Doyle, 2017). The restoration of chemical preservatives such as propionates and sorbates in bread and other bakery goods is of considerable interest (Luz et al., 2018). Chemical preservatives, such as calcium propionate, are commonly applied to expand the microbial lifespan of bread (Belz et al., 2012). However, prolonged exposure to chemical preservatives may pose a health risk. Thus, using biopreservation techniques on bread can aid in solving this issue and preventing economic loss caused by molds (Ghabraie, Vu, Tata, Salmieri, & Lacroix, 2016). The long history use of *T. versicolor* in traditional medicine and foods, together with recent studies on its food safety (Bains et al., 2021; Cerig, 2021), suggest a potential large-scale employment as ingredient into the modern food industry.

## 5. Conclusions

This is the first report showing the potential of a mushroom extract to be effective as natural biopreservative agent in baked goods. Thanks to the thermal stability and to a larger spectrum of activity on spoilage fungi compared to other "natural" antifungal ingredients recently proposed for similar applications (Aldholmi et al., 2019; Coda et al., 2008; Rizzello et al., 2015; Thery et al., 2019), TE represents a valid option for biopreservation within the bakery industry. Further investigations on both long-term safety and sensory profile of the extract, such as the sustainability of its production, are necessary for the evaluation of a potential large-scale use. Nevertheless, the cultivation of *T. versicolor* was extensively reported as sustainable in different application fields (enzyme production, food and feed production, wastewater treatment, and biofuel production), thanks to its capacity to grow in poor substrates and lignocellulosic byproducts (Tišma et al., 2021).

The combination with sourdough biotechnology, well-known to be responsible for the improvement of many nutritional, functional, and organoleptic features of breads, can be considered as a strategy for obtaining a biological acidification of the dough, needed to correctly exploit the antifungal potential of the extract, moreover improving leavening performances and technological features of bread samples supplemented with TE and allowing a synergistic effect on the shelf-life extension of the final product.

## Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## CRediT authorship contribution statement

**Andrea Torreggiani:** Formal analysis. **Marzia Beccaccioli:** Formal analysis. **Michela Verni:** Validation, Data curation. **Valentina Cecchetti:** Formal analysis. **Andrea Minisci:** Formal analysis, Validation. **Massimo Reverberi:** Resources, Supervision, Writing – original draft. **Erica Pontonio:** Validation, Writing – original draft. **Carlo Giuseppe Rizzello:** Resources, Supervision, Writing – original draft.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

## Data availability

No data was used for the research described in the article.

## Acknowledgments

Thanks to Alice Brannetti, Eleonora Iamele and Francesca Morgante for the support to the experimental activities.

## Appendix A. Supplementary data

Supplementary data related to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115467

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