



Exploring lactic acid bacteria diversity of hop plant by-products to develop a multi-strain starter culture to be used in hop-supplemented sourdough bread

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ABSTRACT

The hop plant is gaining interest in the food, pharmaceutical, and cosmetics industries due to its abundance of secondary metabolites. However, branches and leaves, despite their antioxidant potential, are typically discarded. To valorize these components as functional ingredients they were dried, milled into hop powder (HP), and used to enrich bread. Polyphenol-resistant lactic acid bacteria (LAB) isolated in this study from HP, *Leuconostoc pseudomesenteroides* and *Enterococcus lactis*, were selected for their acidification capacity and combined with sourdough LAB to create a multi-strain starter culture for hop bread (HB) production. Three trials were conducted: HB-0 (control bread without HP), HB-2.5 (bread with 2.5 % HP), and HB-5 (bread with 5 % HP). All samples were evaluated for quality traits, phenolic content, antioxidant activity, and profiles of polyphenols and volatile organic compounds (VOCs). HP-supplemented breads showed reduced weight loss, increased firmness, and darker crust and crumb. Bioactive compounds such as xanthohumol, lupulone, and VOCs like β -myrcene and α -humulene were retained. Sensory analysis revealed that HP addition enhanced aroma intensity, color, astringency, and bitterness, with HB-2.5 receiving the highest overall rating. Shelf life tests showed mold appeared on day 11 in HB-0, but was delayed to day 13 in HP breads. Staling, assessed via differential scanning calorimetry, showed increasing melting enthalpy (ΔH) over time in all samples, confirming starch retrogradation, with no significant differences among formulations. This study validates the use of HP as a functional ingredient, enhancing sensory profiles and bioactive compound content. The application of selected LAB and HP supports sustainable practices in the food sector.

1. Introduction

Waste from agriculture and agri-food processing, often disposed of in costly and environmentally harmful ways, contains valuable compounds with significant market potential. By recovering and utilizing functional components like polyphenols, essential oils, and bioactives, these by-

products can be transformed into resources for sectors such as food, pharmaceuticals, cosmetics, and packaging. This approach aligns with sustainability goals and supports the SDGs agenda 2030, highlighting the potential of biomass valorization to enhance market and environmental outcomes (Ahmad et al., 2024; Panzella et al., 2020).

In hop cultivation, after cone harvesting, large quantities of

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vegetative biomass, including leaves and stems, approximately 2.6 kg per plant, need to be disposed. Usually, hop growers destine this biomass to compost production, instead it has potential applications, still largely unexplored (Abram et al., 2015; Sabbatini et al., 2024).

In the last years, several studies focused at characterizing and valorising hop leaves potentially bioactive compounds (Calvert et al., 2024; Chiancone et al., 2023; Leto et al., 2024; Macchioni et al., 2022; Sabbatini et al., 2024). Hop leaves are, indeed, a rich source of phenolic compounds, including flavonols, prenylflavonoids, and proanthocyanidins, related to potential antioxidant, antimicrobial, and estrogenic activities (Censi et al., 2021; Santarelli et al., 2021). These bioactive compounds are synthesized as part of the plant's natural defence mechanisms against biotic and abiotic stresses (Kunej et al., 2020).

From a practical and applicative standpoint, extending the focus beyond just the leaves to include a comprehensive characterization of the entire hop vegetative biomass could be beneficial. This approach may discover potential commercial uses for materials previously considered waste from hop cultivation.

This research focuses on isolating ad hoc lactic acid bacteria (LAB) to repurpose hop plant (HP) by-products as new ingredient for producing healthy, innovative foods. Specifically, the primary objective of this study was to evaluate the potential application of HP by-product particularly vegetative biomass formed by branches, leaves, and discarded cones, in sourdough bread production. HP was characterized for its polyphenolic profile, microbiological safety, and the presence of LAB with sourdough-fermenting capabilities. HP was subsequently incorporated into sourdough bread production at two percentages, and the fermentation process was monitored both chemically and microbiologically. The final breads were assessed for quality parameters, polyphenol content, shelf life, and aromatic profile.

2. Materials and methods

2.1. Plant materials

Hop plants pertaining to Cascade variety were cultivated at Azienda Agricola Ludovico Lucchi in Campogalliano (MO), located in the Emilia-Romagna region of Italy (44°42'19.9" N, 10°50'26.1" E). Cascade is an American aromatic hop variety developed in 1972 at Oregon State University and USDA (United States Department of Agriculture) (Horner et al., 1972; Padgett-Cobb et al., 2021). This variety emerged from a breeding program involving Fuggle and the cross (Serebrianka x Fuggle-seedling) followed by open-pollination (Henning et al., 2018). Its floral and fruity aroma, particularly noted for grapefruit and floral notes, is highly valued by the brewing industry.

2.2. Hop plant by-product powder production

Branches, leaves, and discarded cones of fresh hop were dried at 35 °C using a low-temperature dryer (model PKT 2022, Packtin Srl, 42122 Reggio Emilia, RE, Italy), until reaching a relative humidity of 6 ± 0.5 %. After drying, the matrix was processed through a hammer mill (model Rapide 200Z, Nuova Guseo S.r.l., 29010 Villanova sull'Arda, PC, Italy) and then passed through a 1000 µm sieve to create a fine HP by-products powder. HP was subsequently packaged in low-density polyethylene bags and stored at room temperature.

2.3. Biochemical characterization of hop plant by-product powder

Prior to determining the total phenolic content (TPC) and antioxidant activity (AO) of HP, hydroalcoholic extracts were prepared with an ethanol/water solution (80/20) at a dilution ratio of 1:20. During extraction, the mixture was shaken on a digital shaker (HS 501, IKA-Werke GmbH & Co, Staufen, Germany) at 200 rpm for 2 h at room temperature, following the protocols of Carbone et al. (2020) and Martelli et al. (2020). After shaking, the samples were centrifuged at

5000 rpm for 10 min at room temperature in a Centrifugette 4206 centrifuge (Alc International, Pévy, France). The supernatants were diluted 1:5 with distilled water for all assays. Each extraction procedure was performed in triplicate to ensure reliability and consistency.

TPC and Antioxidant Activity (AO) of the samples were determined through different spectrophotometric assays, according to the protocol described by Chiancone et al. (2023). The TPC was determined through the Folin-Ciocalteu test while, about AO, to evaluate the radical scavenging activity, two tests were used as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and, lastly to determine the reducing capacity, the ferric reducing antioxidant power (FRAP) test was used.

The spectrophotometer JASCO V-530 (Easton, MD, USA) has been used for sample evaluations; for the calibration curve of TPC five points were used into a range of 10–100 mg/kg of gallic acid and the results reported in mg/g GAE (Gallic Acid Equivalents). The same process was used for AO determinations, but the curve was constructed using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard and the results expressed in mg/g TEAC (Trolox Equivalent Antioxidant Capacity). Instrument has been set with feature absorbance for each assay: at 760 nm for the TPC, 517 nm for DPPH, 734 nm for ABTS and 593 nm for the FRAP (Chiancone et al., 2023).

2.4. Microbiological analysis of hop plant by-product powder

HP (10 g) was suspended in 90 mL of Ringer's solution (Sigma-Aldrich, Milan, Italy) through magnetic stirring in a 250-mL Pyrex Erlenmeyer flask. The resulting cell suspensions were then serially diluted (1:10) in Ringer's solution and plated on the following media: plate count agar (PCA) incubated aerobically at 30 °C for 72 h for total mesophilic microorganisms (TMM); Baird-Parker (BP) agar supplemented with rabbit plasma fibrinogen (RPF) incubated aerobically at 37 °C for 48 h for coagulase-positive staphylococci (CPS); violet red bile glucose agar (VRGBA) incubated aerobically at 37 °C for 24 h for members of the Enterobacteriaceae family; kanamycin esculin azide (KAA) agar incubated aerobically at 37 °C for 24 h for enterococci; *Pseudomonas* agar base (PAB) supplemented with cephaloridine sodium fusidate cetrimide (CFC) incubated aerobically at 25 °C for 48 h for pseudomonads; malt agar (MA) added with chloramphenicol (0.1 g/L) incubated aerobically at 28 °C for 48 h and 7 days for yeasts and moulds. Respectively; hektoen enteric agar (HEA) incubated for 1 d at 37 °C for *Escherichia coli*; *Listeria* selective agar base (LSAB) added with SR0140E supplement incubated for 1 d at 37 °C for *Listeria monocytogenes*; xylose lysine deoxycholate (XLD) agar incubated for 1 d at 37 °C for *Salmonella* spp.; de Man-Rogosa-Sharpe (MRS) incubated anaerobically for 48 h at 30 °C and 44 °C for mesophilic and thermophilic LAB rods respectively; M17 incubated anaerobically for 48 h at 30 °C and 44 °C for mesophilic and thermophilic LAB cocci, respectively. Spore-forming aerobic bacteria were also investigated: all dilutions of cell suspensions were heated at 85 °C for 15 min; aliquots of 0.1 mL were spread onto Nutrient Agar (NA) plates which were incubated for 48 h at 32 °C. All media and supplements were purchased from Oxoid (Basingstoke, UK). Anaerobiosis occurred in hermetically sealed jars with the AnaeroGen AN25 system (Oxoid). Microbiological counts were carried out in triplicates.

2.5. Identification and characterization of LAB

2.5.1. Isolation, typing, and identification of LAB

If no growth of presumptive LAB was observed on MRS and M17 agar plates, the following enrichment procedure was applied: 10 g of HP were inoculated in 50 mL of modified-MRS (mMRS) broth. mMRS was prepared from standard MRS which was supplemented with 1 % (w/v) maltose, 1 % (w/v) lactose, and 10 % (v/v) fresh yeast extract and the final pH was adjusted to 5.6 with 5 M lactic acid. Additionally, 10 g of HP were also inoculated into 50 mL of San Francisco Medium (SFM) prepared as described by Vogel et al. (1994). After 96 h of incubation at

30 °C, the pH was measured. If acidification occurred, the broth was streaked onto the corresponding agar media to promote the development of putative LAB colonies. To ensure maximum LAB biodiversity, all colonies with similar appearance in terms of shape, diameter, color, edge, surface characteristics, and elevation were isolated. All isolates were initially characterized by Gram staining, using the method described by Gregersen (1978) and by a catalase test as reported by Koneman et al. (1997). Gram-positive cultures that were catalase-negative were considered putative LAB and were further investigated for cell morphology.

Random amplification of polymorphic DNA (RAPD)-PCR analysis was conducted to differentiate the isolates at the strain level. To this purpose, LAB cultures were grown overnight at 30 °C in MRS or M17 broth media, centrifuged at 5000 rpm for 2 min and the pelleted cells were subjected to DNA extraction using the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Crude cell extracts were directly used as templates for PCR using the single primers M13, AB111, and AB106. The resulting amplicons were separated by agarose gel electrophoresis and analyzed by Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium).

Species identification occurred through 16S rRNA gene sequencing using the primers fd1 (5'-AGAGTTTGATCCTGGCTCAG-3')/rD1 (5'-AAGGAGGTGATCCAGCC-3'). PCR reactions were performed following the method described by Weisburg et al. (1991) and the resulting amplicons of approximately 1600 bp were purified using the QIAquick purification kit (Quiagen S.p.a., Milan, Italy). The Sanger method was applied to process PCR products at BMR Genomics s.r.l. (Padova, Italy). The sequence outputs were aligned with those available in the GenBank/EMBL/DBJ (Altschul et al., 1997) and EzTaxon-e databases (Chun et al., 2007) to perform a comparison for identification.

2.5.2. Acidification capacity of LAB

The identified LAB were evaluated for their acidification capacity. LAB were reactivated and centrifuged as per DNA extraction, but the pelleted cells were washed in Ringer's solution and inoculated at 1 % (v/v) in sterile semolina extract (SSE). The pH decrease was monitored at intervals 0, 2, 4, 6, 8, and 24 h.

2.6. Fermenting bacteria and sourdough propagation

LAB identified in this study were considered intrinsically resistant to hop polyphenols and used in combination with *Fructilactobacillus sanfranciscensis* RC-UNIPASAAFM01100, *Weissella cibaria* RC-UNIPASAAFM01109, and *Leuconostoc citreum* RC-UNIPASAAFM01118, originating from sourdoughs used in Sicilian bakeries and belonging to the culture collection of SAAF Department – University of Palermo – to initiate the fermentation of the sourdough.

All strains were reactivated from glycerol stocks stored at –80 °C and propagated twice in modified de Man-Rogosa-Sharpe (mMRS) medium following the protocol of Corsetti et al. (2008). Subsequently, the bacteria were washed as reported above and inoculated individually and propagated in SSE. After three sub-cultivation steps, all LAB strains were used to prepare a multi-strain starter culture by mixing all the fermented SSE together.

The resulting cell suspension was diluted in sterile tap water to a final volume of 187.5 mL. This cell suspension was then added to 312.5 g of commercial semolina ("Cuore Mediterraneo", SIMEC, Santa Giusta, Italy) to create a 500 g dough with a dough yield (DY = weight of the dough/weight of semolina × 100) of 160 with a cell density of approximately 10⁶ to 10⁷ CFU/g. The dough was then fermented for 16 h at 28 °C and refreshed daily for seven days to obtain a mature sourdough ready for use in baking.

2.7. Bread making process

The breadmaking was carried out using sourdough starter initiated

with LAB as the sole fermentative agent. Although the absence of salt is not common in breads from southern Italy, no salt was added to better evaluate the effect of HP on the persistence of LAB inoculums during hop bread (HB) production. The bread dough (1 kg) was prepared with a DY ~175 following this control (HB-0) test recipe: 571.50 g of semolina; 142.80 g of 7-day-mature sourdough, and 285.70 mL of sterile tap H₂O. The experimental trials contained HP at 2.5 % (HB-2.5) or 5.0 % (HB-5) (w/w) on the weight of semolina. Therefore, HB-2.5 and HB-5 trial doughs were prepared like the control dough but with 557.22 g and 542.92 g of semolina, respectively. The planetary mixer model XBM10S (Electrolux Professional, SpA, Pordenone, Italy) equipped with a paddle was used to mix the ingredients at speed 4 for 15 min.

Six doughs (100 g each) per trial were placed in stainless steel baking trays with trapezoidal dimensions [143 × 79 mm (top inner part), 129 × 64 mm (bottom outer part), 57 mm (inner depth)] according to the American Association of Cereal Chemists – Method 10-10B of AACCC (2000). The trays, covered with aluminum foil, were incubated at 28 °C for 8 h to allow fermentation. The rest of dough from each trial was transferred into sterile glass jars (Vetzeria Borgonovo Spa, Borgonovo Val Tidone, Italy) and fermented in the same t/T conditions. All bread productions were performed in duplicate (two technical repeats) and repeated twice (two independent replicates) after 1 month.

The baking process was conducted in a semi-industrial oven (Compact Combi, Electrolux, Pordenone, Italy) applying a 2-phase program consisting of an initial exposure of the doughs to hot air/steam at 200 °C for 5 min followed by 15 min of convection heat (hot air only).

2.7.1. Monitoring of sourdough evolution

The monitoring of the fermentation of refreshed sourdough until inoculation and the doughs for each bread formulation was carried out by determining the pH, total titratable acidity (TTA), and the density of LAB cells. The first two parameters were measured immediately after inoculation and during fermentation. Measurements were taken at 0, 2, 4, 6, and 8 h by immersing the pH meter probe (XS Instruments, Carpi, Italy) directly in SSE test tubes (10 mL) or in 10 g of doughs collected aseptically. The pH of was also measured: 10 g of milled bread were dispersed in 90 mL of distilled water (H₂O) using a magnetic stirrer before inserting the pH meter probe. TTA was measured at the same times as the pH determinations. Specifically, the same samples subjected to pH measurements were transferred to stomacher bags, added with 90 mL of distilled water, and homogenized using the stomacher BagMixer® 400 (Interscience, Saint Nom, France) at maximum speed for 2 min before titration with 0.1 N NaOH. The TTA results were expressed as mL of 0.1 N NaOH/10 g of dough.

All samples were homogenized as described above to perform the viable count of fermenting groups, but sterile Ringer's solution (Sigma-Aldrich, Milan, Italy) was used instead of distilled water. The homogenates were subjected to serial decimal dilutions and inoculated as follows: on PCA incubated aerobically at 30 °C for 72 h to enumerate TMM; on mMRS agar, incubated anaerobically at 30 °C for 48 h for mesophilic rod-shaped LAB; on M17 agar, incubated anaerobically at 30 °C for 48 h for mesophilic coccus-shaped LAB; on sourdough bacteria (SDB) agar (Kline & Sugihara, 1971) incubated aerobically at 30 °C for 48 h for sourdough LAB. Additionally, the number of Enterobacteriaceae family members was determined on VRBGA, incubated at 37 °C for 24 h, and total coliforms on violet red bile agar (VRBA), incubated at 37 °C for 24 h. All culture media were purchased from Oxoid. Microbiological analyses were performed in duplicate, and the final results were expressed as Log CFU/g.

2.8. Determination of quality parameters of breads

All baked breads were cooled at room temperature for 30 min before analyzing quality parameters.

Regarding morphogeometric parameters, the weight loss (WL) due to

water evaporation during baking was calculated using the following formula: $WL = \left(\frac{wd - wb}{wd} \right) \times 100$.

where “wd” is the weight (g) of the dough and “wb” is the weight (g) of the bread (Purlis & Salvadori, 2007).

The bread volume was determined using a 2 L volumeter for bakery products, with flax seeds placed in the appropriate container (ErreCi srl, Merate, Italy).

The color of the breads was evaluated on both crust and the crumb using the Chroma Meter CR-400 (Minolta, Osaka, Japan) based on the Hunter's scale parameters (L^* , a^* and b^*). Specifically, three crumb points were taken from the central slice, while four points were analyzed on the upper surface of each bread.

The hardness of the bread was determined by measuring the compression resistance (N/mm^2) using the Instron-5564 (Instron Corp., Canton, MA), as described by Corsetti et al. (2000).

The central slice of each bread was subjected to image analysis. Each slice was examined for the void fraction (the percentage of the total area corresponding to the alveoli), cell density (number of alveoli/ cm^2), and the average area of the alveoli (in mm^2). The image analysis was performed after scanning the bread slices at a resolution of 350 dpi (Epson Perfection 4180 Photo, Seiko Epson Corp., Japan). The images, saved in TIFF format, were processed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All images were cropped into squares of 207×207 pixels (representing an area of the bread slice of 15×15 mm), converted to grayscale (8-bit), and binary images were obtained by applying Otsu's thresholding algorithm.

The breads were also examined for the presence of aerobic spore-forming bacteria. Twenty-five grams of each bread were serially diluted in Ringer's solution as described for the dough samples. All cell suspensions were treated as reported above (par. 2.4).

2.9. Biochemical characterization of breads

Breads were grounded and then the same extraction protocol was used as for HP with minor modifications. The breads were analyzed for TPC and AO using, also in this case, DPPH, ABTS and FRAP assays. Hydroalcoholic extracts were also prepared for these samples using an ethanol/water solution (80/20), but with a dilution ratio of 1:10 for extraction. Furthermore, an additional dilution of 1:5 was required for the TPC assay (resulting in a final dilution of 1:50), while a further dilution was added for the AO assays (final dilution of 1:10).

2.10. Determination of polyphenolic profile of hop plant by-product powder and breads

Polyphenolic compound profiles were determined in HP and HB samples. A total of 200 mg of each sample was extracted with 2 mL of an 80:20 methanol-water mixture containing 0.1 % formic acid using an ultrasonic bath (40 kHz) at 40 °C for 30 min (Altemimi et al., 2015). After extraction, the samples were centrifuged (8000 rpm, 15 min), filtered (0.45 μm), then 5 μL of the extract was injected into the LC-MS/MS system. Polyphenol measurement was performed using an Ultimate 3000 UHPLC system coupled with a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific, San José, CA, USA) following the methodology described by Indelicato et al. (2023), with further optimization of the LC-MS/MS method for this study. Chromatographic separation was performed by Hypersil GOLD C18 reversed-phase analytical column (2.1×50 mm, 1.9 μm particle size, Thermo Fisher Scientific). The column temperature was maintained at 30 °C, and the mobile phases used were water with 0.1 % formic acid (Eluent A) and methanol (Eluent B), both LC/MS grade (Sigma-Aldrich). The gradient program applied started with 5 % of mobile phase B for the first 2 min, followed by a linear increase to 70 % B over the next 8 min. Between 10 and 12 min, the gradient continued to rise linearly, reaching 100 % B, which was then maintained for 5 min. Afterward, from 17.0 to

17.1 min, the percentage of phase B was reduced to 1 %, and this condition was held for an additional 1.9 min. Throughout the entire process, the flow rate was kept constant at 300 $\mu L/min$. Detection was carried out using Selected Reaction Monitoring (SRM) in negative ion mode with a heated electrospray ionization (HESI) source. Pure standards of isoxanthohumol, xanthohumol, lupulone, gentisic acid, catechin, caffeic acid, epicatechin, hydroxycinnamic acid, rutin, quercetin, kaempferol, L-mandelic acid, syringic acid, and resveratrol were used for mass optimization and quantification. Detection conditions were optimized by infusing standard solutions of each analyte (1 ppm in methanol) into the mass spectrometer. Key operating parameters for mass spectrometry were as follows: spray voltage at 2500 V, auxiliary gas pressure at 10 a. u., sheath gas pressure at 50 psi, sweep gas at 1 a.u., ion transfer tube temperature at 325 °C, and vaporizer temperature at 350 °C. Additional parameters included a dwell time of 100 ms, Q1 resolution at 1 Da, Q3 resolution at 0.4 Da and a collision-induced dissociation (CID) gas (argon) pressure of 2.0 mTorr. An external calibration curve in the range of 5 ppb to 1 ppm was used for the quantification of phenolic compounds. Data acquisition and processing were conducted using the Quan/Qual Browser and TraceFinder softwares (Thermo Fisher Scientific, San José, CA, USA). Each calibration point was based on the average of three independent injections.

2.11. Determination of volatile organic profile hop plant by-product powder and breads

Volatile Organic Compounds (VOCs) were identified in HP and HB samples by HS-SPME/GC-MS. An aliquot (3 g) of HP or grounded HB samples was transferred to a 40 mL glass vial with a silicone septum. The sample was placed in a thermostatic water bath and equilibrated for 30 min at 60 °C. Extraction of the aromatic component was performed using a 50/30 μm divinylbenzene/carboxyl/polydimethylsiloxane tri-phasic fiber (DVB/CAR/PDMS, Supelco, Bellefonte, PA) by exposing it to the sample headspace for 30 min. After extraction, the fiber was removed from the vial, transferred to the injector port of GC, and desorbed for 5 min at 230 °C. Before use, the fiber was conditioned in a GC injector at 270 °C for 1 h. Analyses were performed using a Thermo Fisher Scientific (Waltham, MA, USA) TSQ 800 triple quadrupole mass spectrometer and a Thermo Fisher Scientific Trace 1310 Gas chromatographic analyzer. Chromatographic separation was performed using a TG XLBMS column (20 m $\times$ 0.18 mm ID $\times$ 0.18 μm , Thermo Scientific GC column) and the injector port was maintained at 230 °C. The column carrier gas was helium (99.99 %) at a constant flow rate of 1.2 mL/min. The oven temperature was held at 35 °C for 5 min, increased to 100 °C at a rate of 5 °C/min and held for 2 min, then increased to 180 °C at a rate of 6 °C/min and held for 2 min, and finally increased to 230 °C at a rate of 8 °C/min and held for 2 min. Mass analysis was performed under electron ionization (EI) conditions with an ionization energy of 70 eV and the source temperature was set to 300 °C. Full-scan acquisition was used in the range of 30 to 350 m/z . Each component was identified using its mass spectral data (NIST05 library). The results are expressed as a relative percentage of the total identified peak.

2.12. Sensory bread analysis

The breads underwent a descriptive sensory analysis by a panel of 17 judges (11 women and 6 men, aged 20 to 67). Initially, the judges were trained using commercial bread to familiarize themselves with bread attributes. Two-centimeter-thick slices were cut from each bread kept at room temperature (Panirani et al., 2023) and, after about 5 min, were placed on plastic plates labeled with three-digit codes (Moretton et al., 2023). The panelists were not allowed to see the entire bread before tasting. They were specifically asked to evaluate the appearance, texture, odor, and taste of the breads (Comendador et al., 2012; Martins et al., 2015; Rodrigues et al., 2014) using a 9-point scale (1 = extremely

bad; 9 = extremely good) for each attribute. An overall assessment based on all attribute scores was also requested. The sensory evaluation sessions were conducted in individual chambers following ISO 13299 (2003) guidelines.

2.13. Shelf life tests

Experimental breads were assessed for mold-free shelf life according to the method described by Syrokou et al. (2022). After cooling at room temperature for 3 h, the breads were sliced, and each slice (approximately 20 g) was individually packed in sterile BagLight® 400 Multi-layer® bags (Interscience, Saint Nom, France) made of PolySilk®. The bags were thermally sealed using a Laica VT3112 hot bar (Vicenza, Italy). The bread slices were then stored at 25 °C (Ju et al., 2020) until visible mold growth was observed. The experiment was conducted in triplicate.

Thermal analysis was performed using a DSC131 evo differential scanning calorimeter (Setaram, France). Approximately 20 mg of each bread sample were accurately weighed and sealed in aluminum pans. All measurements were carried out under an inert nitrogen atmosphere. The samples were subjected to an heating ramp from 30 °C to 160 °C at a rate of 5 °C/min. The endothermic transition associated with the melting of retrograded amylopectin was identified and integrated to determine the melting enthalpy (ΔH), which was used as an indicator of the degree of staling.

DSC measurements were performed in triplicate for each formulation and storage time (0, 24, 48, 72, and 96 h). Results were expressed as average values of ΔH (J/g of sample).

2.14. Statistical analysis of data

Data obtained from chemical and microbiological analysis were statistically analyzed resorting to a one-way analysis of Variance. Tukey's test ($p \leq 0.05$) was used for mean separation (SYSTAT 13).

3. Results and discussion

3.1. Total phenolic content and antioxidant activity of hop plant by-product powder

First of all, HP was tested for its total content in (poly)phenols and antioxidant compounds. The tests confirmed the richness of the matrix in potentially bioactive components. In particular, the Folin-Ciocalteu test gave a value of 7.25 ± 0.04 mg GAE/g, which is in agreement with Sabbatini et al. (2024), who reported a slightly lower value but, in that study, only hop leaves were analyzed. Similarly, Keskin Çavdar (2019) obtained lower results, likely due to analyzing only leaves and employing a slightly different extraction method.

Antioxidant activity assays highlighted the richness of HP with results of 8.07 ± 0.31 mg TEAC/mL for DPPH, 19.11 ± 0.18 mg TEAC/mL for ABTS and 12.15 ± 0.04 mg TEAC/mL for FRAP. All tests yielded higher values than those reported in similar studies with comparable matrices (Arruda et al., 2022; Muzykiewicz et al., 2019; Sabbatini et al., 2024).

3.2. Microbiological characterization of hop plant by-product powder and LAB species identified

HP underwent a thorough culture-dependent microbiological investigation to identify pro-technological, spoilage, and pathogenic microorganisms commonly found in fruit and vegetables (Miceli & Settanni, 2019). This analysis is crucial when applying plant by-products and/or agri-food wastes in food production due to their natural microbial contamination from soil, water, and air, making them potential vectors of microbiological hazards (Socas-Rodríguez et al., 2021). Exceptionally, all microbial groups investigated were below the

detection limit. The absence of spoilage (*Pseudomonas* spp., unicellular and filamentous fungi) and pathogenic (aerobic spore-forming bacteria, CPS, *L. monocytogenes*, members of the *Enterobacteriaceae* family, *E. coli*, and *Salmonella* spp.) microorganisms, which could indicate poor hygiene of raw materials (Sahoo et al., 2022), demonstrates the high hygienic quality of HP, making it suitable for food production.

LAB are essential for sourdough production (Valmorri et al., 2006). Authors hypothesized that LAB in HP are naturally resistant to hop polyphenols, and intended to use these LAB along with those from the sourdough LAB strain collection of the University of Palermo. However, since no colonies were detected from HP in plate counts, an enrichment procedure to promote the growth of undetectable LAB to above the detection limit was used. After incubating at 30 °C for a few days, the pH of the liquid media inoculated with HP decreased, with mMRS dropping to 4.0 and SDB to 3.7, indicating the presence of LAB. The LAB responsible for this acidification were then isolated from the supplemented broths by streaking onto the corresponding agar media.

Twenty colonies of Gram-positive and catalase-negative, presumed to be LAB and all coccus-shaped, were randomly isolated from both mMRS and SDB plates (10 colonies per medium). These bacteria were purified and analyzed using RAPD-PCR, a common technique for strain typing of LAB from food sources. The agarose gel electrophoresis revealed only two distinct DNA band patterns (results not shown). Since isolates with identical polymorphic profiles are considered the same strain, this analysis confirmed the presence of two distinct presumptive LAB strains in HP. To verify their classification as LAB, both strains underwent 16S rRNA gene sequencing and were identified as *E. lactis* RC-UNIPASAAFM01339 (Ac. No. PQ628117) and *L. pseudomesenteroides* RC-UNIPASAAFM01340 (Ac. No. PQ628118).

Enterococcus lactis was first isolated from dairy products (Morandi et al., 2012) and has also been found in association with shrimps (Braiek et al., 2017). In the context of sourdough, *E. lactis* can produce acids from maltose (Morandi et al., 2012), which is the main fermentable sugar in this cereal-based product (Vrancken et al., 2008). Even though the presence of *Enterococcus* species generally poses a risk to consumers due to their potential to acquire and transfer genes responsible for antimicrobial resistance and virulence traits (Krawczyk et al., 2021), *E. lactis* has demonstrated probiotic properties (Braiek, Morandi, et al., 2018; Braiek, Smaoui, et al., 2018), highlighting its beneficial aspects in food production.

Leuconostoc pseudomesenteroides is commonly isolated from sourdoughs (Dertli et al., 2016). It exhibits acidifying properties and biotechnological potential (Gumustop & Ortakci, 2023), making it valuable for sourdough production. Due to their high adaptation and performance in the sourdough environment (Montemurro et al., 2020), leuconostocs are considered non-conventional LAB with significant potential as starter cultures in sourdough fermentation (Müller et al., 2021).

3.3. Fermentation process

The acidification carried out by the starter LAB was monitored in SSE broth, where all selected strains dropped the initial pH from 5.6 to levels below 4.1. Specifically, *E. lactis* RC-UNIPASAAFM01339 decreased the SSE pH to 4.1, while *L. pseudomesenteroides* RC-UNIPASAAFM01340 brought it down to 3.7. This latter pH value aligns with those observed for the other strains used in this study (*F. sanfranciscensis* RC-UNIPASAAFM01100, *W. cibaria* RC-UNIPASAAFM01109, and *L. citreum* RC-UNIPASAAFM01118). After three propagation cycles, the five acidified SSE broths were mixed (forming a direct liquid inoculum) and added to semolina to develop the sourdough, which was used as the sole leavening agent in bread production. The sourdough prepared with the five selected strains exhibited a pH of 3.86 ± 0.07 and TTA value of 13.35 ± 0.21 mL NaOH 0.1 N/10 g. Both parameters (pH and TTA) were highly comparable to those recorded in studies that developed sourdough using direct inoculation of selected lactobacilli, leuconostocs, and

Table 1
Chemical parameters of control and hop plant by-product added doughs.

Samples	0 h		4 h		8 h	
	pH	TTA	pH	TTA	pH	TTA
HB-0	5.64 ± 0.06 ^b	7.08 ± 0.07 ^b	4.61 ± 0.03 ^b	8.88 ± 0.32 ^b	4.15 ± 0.01 ^c	12.10 ± 0.07 ^b
HB-2.5	5.74 ± 0.06 ^b	7.00 ± 0.11 ^b	4.70 ± 0.06 ^b	8.48 ± 0.18 ^b	4.24 ± 0.04 ^b	11.73 ± 0.32 ^b
HB-5	5.99 ± 0.07 ^a	6.13 ± 0.18 ^λ	5.15 ± 0.04 ^a	7.48 ± 0.11 ^λ	4.40 ± 0.04 ^a	11.15 ± 0.14 ^λ
p-value	< 0.001	< 0.001	< 0.0001	< 0.001	< 0.001	< 0.01

Results indicate mean values ± S.D. (standard deviation) of six determinations (carried out in three technical repeats for two independent experiments). Data within a column followed by different letters (pH) or symbols (TTA) are significantly different according to Tukey's test. Abbreviations: TTA, total titratable acidity expressed in mL NaOH 0.1 N/10 g; HB-0, control bread, HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP.

weissellas (Syrokou et al., 2020). This sourdough underwent seven daily refreshments and was then considered mature enough for use in bread production.

The changes in pH and TTA values during the fermentation of the experimental doughs are presented in Table 1. At the start (T_0), the HB-0 dough had a lower pH (5.64) compared to the HP-added doughs (5.74 for the HB-2.5 trial and 5.99 for the HB-5 trial). The higher pH in the experimental doughs could be attributed to the pH of HB (6.78), whereas common bread raw materials such as flour and/or semolina typically have a pH range of 5.90–6.10. Measurements taken over time (at t_4 and t_8) showed a decrease in pH for all trials, and by the end of fermentation, the pH values were quite similar, with 4.15, 4.24, and 4.40 for HB-0, HB-2.5, and HB-5, respectively. TTA, which evolves inversely to pH (Siepmann et al., 2019), increased over time, reaching almost comparable values for the control and supplemented breads (11.15–12.10 mL NaOH 0.1 N/10 g), with the highest value observed in the HB-0 dough.

The fermentation process was also monitored microbiologically by enumerating LAB from control and HP-added doughs (Table 2). The sourdough, developed with the five selected LAB strains, showed a cell density of 8.90 ± 0.39 Log CFU/g on SDB, 9.08 ± 0.21 Log CFU/g on mMRS, and 8.74 ± 0.19 Log CFU/g on M17 after maturation. Regarding bread trials, at t_0 , LAB levels were slightly higher than 10^7 CFU/g on all three media used for plate counts. These values were comparable to those recorded for TMM (7.27–7.34 Log CFU/g), indicating a clear dominance of LAB in all doughs. The fermentation, carried out over 8 h, resulted in a nearly two Log cycle increase in LAB for all doughs. This level of increase is commonly reported for LAB during dough acidification leavened by sourdough inoculum (Suo et al., 2021). Differences

Table 2
Microbiological analysis of control and hop plant by-product powder added doughs.

Samples	Media											
	TMM			Sourdough LAB			Mesophilic LAB rods			Mesophilic LAB cocci		
	0 h	8 h	p-value	0 h	8 h	p-value	0 h	8 h	p-value	0 h	8 h	p-value
HB-0	7.34 ± 0.16 ^B	9.08 ± 0.12 ^A	< 0.01	7.26 ± 0.13 ^B	9.28 ± 0.12 ^A	< 0.001	7.74 ± 0.08 ^B	8.85 ± 0.10 ^{aA}	< 0.01	7.34 ± 0.16 ^B	9.15 ± 0.09 ^{aA}	< 0.001
HB-2.5	7.27 ± 0.12 ^B	8.93 ± 0.16 ^A	< 0.01	7.22 ± 0.10 ^B	9.22 ± 0.09 ^A	< 0.001	7.65 ± 0.08 ^B	8.37 ± 0.12 ^{bA}	< 0.01	7.18 ± 0.09 ^B	8.90 ± 0.14 ^{abA}	< 0.001
HB-5	7.28 ± 0.14 ^B	8.81 ± 0.11 ^A	< 0.01	7.15 ± 0.15 ^B	9.15 ± 0.07 ^A	< 0.001	7.65 ± 0.12 ^B	8.30 ± 0.09 ^{bA}	< 0.05	7.08 ± 0.14 ^B	8.78 ± 0.13 ^{ba}	< 0.01
p-value	0.782	0.120		0.670	0.307		0.467	< 0.01		0.171	< 0.05	

Results indicate mean values ± S.D. (standard deviation) of six determinations (carried out in three technical repeats for two independent experiments). Data within a column followed by different lowercase letters and data within a row followed by different uppercase letters are significantly different according to Tukey's test. Abbreviations: TMM, total mesophilic microorganisms; LAB, lactic acid bacteria; HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP.

in LAB levels among HB-0 and supplemented doughs were negligible. Thus, adding HB at 2.5 % and 5 % did not affect normal LAB development during sourdough bread fermentation. The inclusion of *E. lactis* RC-UNIPASAAFM01339 and *L. pseudomesenteroides* RC-UNIPASAAFM01340 did not alter the typical LAB evolution seen in the multi-strain starter culture, which includes *F. sanfranciscensis* RC-UNIPASAAFM01100, *W. cibaria* RC-UNIPASAAFM01109, and *L. citreum* RC-UNIPASAAFM01118. Previous studies have monitored the acidification capacity of these strains during the production of sourdough breads with the inclusion of by-products (Gaglio et al., 2023).

3.4. Bread characterization

The characteristics of the final breads were evaluated after baking (Table 3). Weight loss (WL), specific volume, and firmness of the HB-0 and HB-2.5 breads were nearly identical, whereas the HB-5 trial breads exhibited lower WL and specific volume, and higher firmness compared to the others. Due to the absence of studies reporting data on breads produced with HP addition, direct comparisons of these three morphogeometric parameters with literature data are not possible. However, the WL, specific volume, and firmness observed in this study followed a similar pattern to those seen in sourdough semolina breads made with powdered almond skin (Viola et al., 2024) or powdered raspberry exhausted seeds (Gaglio et al., 2023). Regarding WL reduction, the trend observed with increasing fiber percentages from powdered by-products could be due to the greater hygroscopicity limiting water migration from breads. HP impacted the color of both the crust and crumb of the bread. Specifically, the L^* value decreased with the addition of HP in both sections. As for the a^* and b^* parameters, both decreased for the crust, whereas an opposite trend was observed in the crumb. The b^* value ranged from 19.61 in HB-0 to 27.90 in HB-5, while the a^* value went from −3.15 in HB-0 to −1.51 in HB-5. This darkening effect had previously been noted with the addition of powdered almond skin (Viola et al., 2024). As expected, the addition of HP resulted in a reduction in the specific volume, and image analysis showed reduced alveolation in the bread crumb (Table 4). The digital images of the crumb areas, converted to grey scale (Fig. 1), illustrate the differences in the number and size of the alveoli.

3.5. Total polyphenol content and antioxidant activity of breads

Folin-Ciocalteu test revealed that the HB-5 had the highest total polyphenol content (TPC) with a value of 2.16 ± 0.05 mg GAE/g, compared to the HB-0 (0.81 ± 0.04 mg GAE/g) and the HB-2.5 (1.36 ± 0.16 mg GAE/g) (Table 5). To the best of authors' knowledge, derived from HP by-products and presented in powder, has been used for the first time in bread production, and therefore, no direct comparisons with findings from previous studies are available. However, the increasing

Table 3
Quality attributes of the control and hop plant by-product powder-supplemented breads.

Breads	Weight loss		Specific volume		Firmness		Crust color		Crumb color	
	(%)		(cm ³ /g)		(N/mm ²)		L*	a*	L*	a*
HB-0	14.72 ± 1.00 ^a		3.38 ± 0.15 ^a		0.090 ± 0.005 ^b		46.33 ± 1.97 ^a	13.42 ± 2.20 ^a	68.79 ± 1.17 ^a	-3.15 ± 0.12 ^c
HB-2.5	14.73 ± 0.89 ^a		3.33 ± 0.10 ^a		0.087 ± 0.008 ^b		41.92 ± 1.64 ^{ab}	10.23 ± 0.91 ^{ab}	54.26 ± 0.77 ^b	-2.01 ± 0.04 ^b
HB-5	12.21 ± 1.17 ^b		2.79 ± 0.09 ^b		0.155 ± 0.026 ^a		38.25 ± 2.01 ^b	8.68 ± 1.03 ^b	50.34 ± 2.09 ^b	-1.51 ± 0.10 ^a
p-value	< 0.05		< 0.01		< 0.01		< 0.01	< 0.05	< 0.001	< 0.001

Results indicate mean values ± S.D. (standard deviation) of six determinations (carried out in three technical repeats for two independent experiments). Data within a column followed by different letters are significantly different according to Tukey's test. Abbreviations: HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP.

Table 4
Digital image analysis of crumb structure of the breads supplemented with hop plant by-product powder.

Bread slices	Void fraction		Cell density	
	(%)		(n cm ⁻²)	Mean cell area (mm ²)
HB-0	43.58 ± 3.83 ^b		57.33 ± 2.91	0.77 ± 0.14
HB-2.5	50.25 ± 0.53 ^a		60.22 ± 5.46	0.76 ± 0.05
HB-5	52.56 ± 2.35 ^a		62.52 ± 5.97	0.71 ± 0.10
p-value	< 0.05		0.484	0.771

Results indicate mean values ± S.D. (standard deviation) of six determinations (carried out in three technical repeats for two independent experiments). Data within a column followed by different letters are significantly different according to Tukey's test. Abbreviations: HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP.

amount of research, especially in recent years, shows that more and more plant-based ingredients are being used to improve the nutritional value of various foods, including bread (Amoah et al., 2022; Lachowicz et al., 2021).

The results of the DPPH, ABTS and FRAP tests support the positive impact of the addition of HP on the bread antioxidant activity. The HB-5 showed a higher antioxidant capacity in all tests. In the DPPH assay, the HB-5 reached a value of 9.74 ± 0.44 mg TEAC/mL, significantly higher than HB-0, which yielded 2.84 ± 0.23 mg TEAC/mL, and the HB-2.5 (6.50 ± 0.50 mg TEAC/mL). Similarly, the ABTS assay revealed that the HB-5 had 9.39 ± 0.27 mg TEAC/mL, significantly higher than the control (6.72 ± 0.17 mg TEAC/mL) and the HB-2.5 (8.11 ± 0.28 mg TEAC/mL). In the FRAP assay, the HB-5, again, showed the significantly highest value with 11.40 ± 0.39 mg TEAC/mL, followed by HB-0 (2.42 ± 0.05 mg TEAC/mL) and HB-2.5 (7.40 ± 0.26 mg TEAC/mL) (Table 5).

These results show a significant increase in antioxidant activity at higher concentrations of HP and emphasize the effectiveness of HP as an antioxidant ingredient in bread production. Although no direct comparisons can be made with other studies using this matrix, the promising results are consistent with the findings of similar researches that have investigated the use of herbal ingredients to improve the antioxidant properties of bakery products (Amoah et al., 2022; Cardinali et al., 2024; Lachowicz et al., 2021).

3.6. (poly)phenolic profile of hop-plant by-product powder and breads

(Poly)phenols identified in both bread samples and HP are reported in Table 6. First, to evaluate the impact of adding HP to bread, the polyphenolic profile was investigated. In HP 11 compounds were detected; specifically, 3 of them (isoxanthohumol, xanthohumol, and lupulone) were characteristic of hop (Chiancone et al., 2023; Kao & Wu, 2013; Magalhaes et al., 2007), while other 8 compounds (kaempferol, quercetin, rutin, hydroxycinnamic acid, epicatechin, caffeic acid, catechin, and gentisic acid) are commonly found in both hop and edible plants (Issaoui et al., 2020; Jan et al., 2022; Mahmud et al., 2023; Míean & Mohamed, 2001; Mikyška & Jurková, 2019; Murshid et al., 2022; Sun & Shahrajabian, 2023;). (Poly)phenols identified in this study are frequently found in hop plants (Chiancone et al., 2023; Sabbatini et al., 2024), albeit in varying concentration ratios (Čeh et al., 2007). These differences could depend to different factors as hop variety, growing region, and the specific plant parts analyzed (Knez Hrnčič et al., 2019). This highlights the importance of detailed phenolic profile characterization prior to the exploitation of HP as a functional food additive. Among the hop's characteristic compounds, xanthohumol stands out and remains a key research focus for various applications (Biendl & Pinzl, 2013; Jiang et al., 2018; Knez Hrnčič et al., 2019). Several studies have reported that xanthohumol exhibits several bioactive properties, including antimicrobial antioxidant, sedative, antiinflammatory, chemoprotective activities (Biendl & Pinzl, 2013; Dietz et al., 2005;

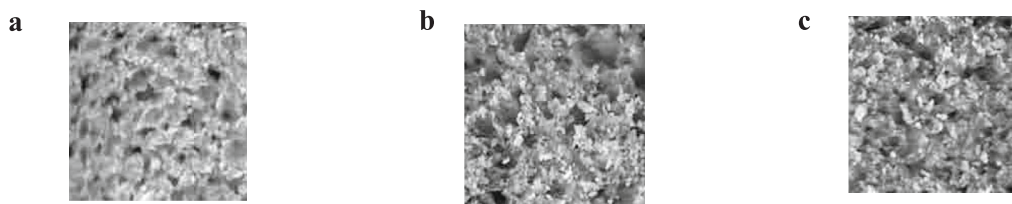


Fig. 1. Digital images (15 × 15 mm crumb area) converted to grey-level image (8 bit) of bread samples. a) HB-0, control bread; b) HB-2.5, experimental bread supplemented with 2.5 % (w/w) of hop powder (HP); c) HB-5, experimental bread supplemented with 5 % (w/w) of HP.

Table 5

Polyphenol content and antioxidant activity of breads supplemented with hop plant by-product powder.

Treatment	TPC	DPPH	ABTS	FRAP
	mg GAE/g	mg TEAC/mL	mg TEAC/mL	mg TEAC/mL
HB-0	0.81 ± 0.04 ^c	2.84 ± 0.23 ^c	6.72 ± 0.17 ^c	2.42 ± 0.05 ^c
HB-2.5	1.36 ± 0.16 ^b	6.50 ± 0.50 ^b	8.11 ± 0.28 ^b	7.40 ± 0.26 ^b
HB-5	2.16 ± 0.05 ^a	9.74 ± 0.44 ^a	9.39 ± 0.27 ^a	11.40 ± 0.39 ^a
p-value	< 0.0001	< 0.0001	< 0.001	< 0.0001

Results indicate mean values ± S.D. (standard deviation) of six determinations. Data within a column followed by different letters are significantly different according to Tukey's test. Abbreviations: HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP.

Table 6

(Poly)phenolic compounds identified in hop plant by-product powder, control bread and experimental breads.

Compounds	HP	HB-0	HB-2.5	HB-5	p-value
Isoxanthohumol	4.90 ± 0.40 ^a	n.d.	0.020 ± 0.001 ^b	0.050 ± 0.003 ^b	< 0.0001
Xanthumolol	55.59 ± 2.66 ^a	n.d.	0.52 ± 0.02 ^b	0.68 ± 0.04 ^b	< 0.0001
Lupulone	622.09 ± 36.70 ^a	n.d.	2.42 ± 0.17 ^b	5.09 ± 0.33 ^b	< 0.0001
Gentisic Acid	9.75 ± 0.97	n.d.	n.d.	n.d.	n.d.
Catechin	68.08 ± 3.40 ^a	n.d.	0.88 ± 0.07 ^b	1.57 ± 0.11 ^b	< 0.0001
Caffeic Acid	23.07 ± 1.84 ^a	0.30 ± 0.02 ^d	4.75 ± 0.35 ^c	11.81 ± 0.83 ^b	< 0.0001
Epicatechin	55.82 ± 2.94 ^a	n.d.	0.78 ± 0.04 ^b	1.28 ± 0.09 ^b	< 0.0001
Hydroxycinnamic Acid	7.62 ± 0.68 ^a	0.58 ± 0.68 ^c	3.22 ± 0.18 ^b	7.19 ± 0.51 ^a	< 0.0001
Rutin	675.01 ± 40.50 ^a	0.16 ± 0.013 ^b	10.63 ± 0.95 ^b	14.99 ± 0.77 ^b	< 0.0001
Quercetin	180.66 ± 9.75 ^a	0.02 ± 0.001 ^d	35.24 ± 1.83 ^c	64.26 ± 4.75 ^b	< 0.0001
Kaempferol	27.47 ± 0.96 ^a	n.d.	7.03 ± 0.52 ^c	14.79 ± 1.19 ^b	< 0.0001

(Poly)phenolic compounds were analyzed by HPLC-MS/MS. Results indicate mean values (µg/g) ± S.D. (standard deviation) of six determinations. Data within a row followed by different letters are significantly different according to Tukey's test. Abbreviations: HP, hop powder; HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP. n.d.: not detected. The limit of quantification (LOQ) for catechin, epicatechin, caffeic acid, rutin, resveratrol, quercetin, and kaempferol was 0.05 µg/g; for gentisic acid, trans-hydroxycinnamic acid, isoxanthohumol, xanthumolol, and lupulone it was 0.02 µg/g. The LOD values correspond to one-third of the LOQ for each compound.

Gerhäuser et al., 2002; Harikumar et al., 2009; Miranda et al., 2000; Rudzitis-Auth et al., 2012; Stracke et al., 2011). Isoxanthohumol, an isomer of xanthohumol, was also detected in HP. This biologically active

flavonoid exhibits both antimicrobial and antioxidant activities (Ameh et al., 2015; Biendl & Pinzl, 2013; Zolnierczyk et al., 2015). Lupulone was the most abundant bioactive compounds detected among the hop's characteristic components, which is also noteworthy (Li et al., 2023; Mouratidis et al., 2013). Several studies indicate that lupulone exhibits antibacterial activity (Lamy et al., 2010, 2011), as well as anti-inflammatory (Hall et al., 2008) and antioxidant properties (Yamaguchi et al., 2009). Among the bioactive compounds commonly found in plants, in HP significant levels of rutin and quercetin were individuated. It is important to highlight that these compounds are known to potentially contribute to human health. Quercetin has been associated with cardioprotective and antihypertensive benefits in humans (Espley et al., 2014). The reducing properties of rutin against different oxidizing species, such as superoxide, peroxy, and hydroxyl radicals, confer substantial antioxidant effects (Mauludin et al., 2009). Pioneering work has also suggested potential synergistic effects of rutin and quercetin with antibiotics, such as gentamicin (an aminoglycoside) and ceftriaxone (a third-generation cephalosporin), against drug-resistant superbugs (Alnour et al., 2022). These results suggest the potential of using this HP as a food additive.

In bread samples with HP addition were detected six potentially bioactive compounds (isoxanthohumol, xanthohumol, lupulone, kaempferol, epicatechin, caffeic acid, and catechin) that were not detected in the HB-0, indicating that their presence results from the addition of HP. Additionally, the incorporation of HP generated a significant increase in the levels of compounds already present in the control bread, such as quercetin, rutin, hydroxycinnamic acid, and caffeic acid. It is noteworthy that the polyphenolic profile of the supplemented bread reflects the profile of hops; in fact, the overall enrichment of the bread was proportional to the amount of HP added. Previous reports have shown that bread supplemented with plant leaves, exhibited significant increases in total water-soluble (poly)phenol contents (Alashi et al., 2018, 2019). Therefore, the increase in antioxidant properties observed in this study may be attributed, in part, to the higher polyphenol contents of the supplemented breads. These results underscore the potential of this by-product to enhance heat property of supplemented HP breads.

3.7. Volatile profile of hop-plant by-product powder and breads

The volatile aromatic compounds emitted from HP and bread samples (HB-0, HB-2.5, HB-5) were identified using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME/GC–MS), and the results are reported in Table 7. The analysis of HP reveals a complex volatile profile, with 32 compounds identified across different classes, including alcohols, carbonyl compounds, hydrocarbons and terpenes. The results indicate that terpene compounds represent the most prominent class, aligning with previous research on hop aroma (Ou et al., 2024; Vázquez-Araújo et al., 2013). In particular, terpenes in HP constitute 86.3 % of the total identified compounds. These compounds are noteworthy not only for imparting distinctive aromatic notes (Ou et al., 2024; Surendran et al., 2021), but also for introducing potential functional benefits, as many terpenes are known for their bioactive properties (Cho et al., 2017; Huang et al., 2012; Thoppil & Bishayee, 2011). This highlights the potential of HP as a

Table 7

Volatile organic compounds identified in hop plant by-product powder, control bread and experimental breads.

Volatile Compounds	HP	HB-0	HB-2.5	HB-5	p-value
Acids					
Acetic Acid	n.d.	11.29 ± 1.67	10.41 ± 1.64	9.71 ± 1.43	0.680
Alcohols					
1-Octen-3-ol	n.d.	1.61 ± 0.24 ^b	4.52 ± 0.76 ^a	4.01 ± 0.59 ^a	< 0.0001
1-Nonanol	n.d.	7.56 ± 1.11	n.d.	n.d.	n.d.
1-Hexanol.2-ethyl	n.d.	1.33 ± 0.23	1.43 ± 0.21	1.22 ± 0.21	0.601
3-Buten-2-ol-2 methyl	4.34 ± 0.68	n.d.	n.d.	n.d.	n.d.
6-Methyl-1-octanol	n.d.	4.26 ± 0.77	n.d.	n.d.	n.d.
Carbonylic Compounds					
Hexanal	n.d.	27.09 ± 3.47 ^{ab}	27.53 ± 2.97 ^a	19.63 ± 2.51 ^b	< 0.0001
Heptanal	n.d.	1.12 ± 0.15	n.d.	0.86 ± 0.12	0.066
Benzaldehyde	0.34 ± 0.04	n.d.	n.d.	n.d.	n.d.
2-Heptenal	n.d.	5.19 ± 0.82 ^b	7.74 ± 1.30 ^a	3.36 ± 0.43 ^b	< 0.001
Octanal	n.d.	1.54 ± 0.27	n.d.	n.d.	n.d.
3-Octen-2-one	n.d.	8.82 ± 1.25 ^a	5.28 ± 0.77 ^b	3.29 ± 0.59 ^b	< 0.0001
2-Octenal	n.d.	2.89 ± 0.47	n.d.	n.d.	n.d.
5-Hepten-2-one. 6 methyl-	1.82 ± 0.29 ^c	0.47 ± 0.05 ^c	6.15 ± 0.77 ^b	10.51 ± 1.88 ^a	< 0.0001
3.5-Octadien-2-one	n.d.	6.97 ± 1.09	9.45 ± 1.69	6.73 ± 1.06	0.144
Nonanal	1.00 ± 0.10 ^c	9.43 ± 1.39 ^a	4.46 ± 0.48 ^b	3.74 ± 0.55 ^b	< 0.0001
Decanal	0.57 ± 0.04 ^b	1.83 ± 0.31 ^a	1.02 ± 0.13 ^b	0.61 ± 0.10 ^b	< 0.001
Esters					
Octanoic Acid. Ethyl Ester	n.d.	0.35 ± 0.05	n.d.	n.d.	n.d.
Furan Compounds	n.d.	n.d.	n.d.	n.d.	n.d.
Furan-2-pentyl-	n.d.	2.39 ± 0.30 ^b	n.d.	5.44 ± 0.89 ^a	< 0.0001
Hydrocarbons					
Decane.2.4.6-trimethyl-	n.d.	2.69 ± 0.29	2.28 ± 0.37	2.45 ± 0.34	0.427
Undecane	0.66 ± 0.09	n.d.	n.d.	n.d.	n.d.
Dodecane 4.6-dimethyl	0.47 ± 0.04 ^b	0.8 ± 0.14 ^a	0.49 ± 0.07	n.d.	< 0.0001
Dodecane 2.6.11 trimethyl	1.61 ± 0.22	1.98 ± 0.32	2.38 ± 0.35	1.79 ± 0.3	0.121
Dodecane	2.89 ± 0.30 ^a	0.32 ± 0.05 ^b	n.d.	n.d.	< 0.0001
Monoterpenes					
Limonene	2.31 ± 0.13 ^a	n.d.	0.1 ± 0.01 ^b	n.d.	< 0.0001
α-Pinene	3.55 ± 0.23 ^a	n.d.	0.32 ± 0.06 ^c	0.91 ± 0.16 ^b	< 0.0001
β-Myrcene	13.48 ± 1.22 ^a	n.d.	4.24 ± 0.53 ^b	5.67 ± 0.6 ^b	< 0.0001
Citronellal	0.09 ± 0.007	n.d.	n.d.	n.d.	n.d.

Table 7 (continued)

Volatile Compounds	HP	HB-0	HB-2.5	HB-5	p-value
Geranylacetone	0.6 ± 0.06 ^a	n.d.	0.41 ± 0.05 ^b	0.59 ± 0.05 ^a	< 0.0001
Alcohol Monoterpenes					
Endo-borneol	0.20 ± 0.03	n.d.	n.d.	n.d.	n.d.
α-Terpineol	0.64 ± 0.08 ^a	n.d.	n.d.	0.03 ± 0.006 ^b	< 0.0001
α-Linalool	3.14 ± 0.7 ^a	n.d.	n.d.	0.13 ± 0.02 ^b	< 0.001
Sesquiterpenes					
Ylangene	1.18 ± 0.15	n.d.	n.d.	n.d.	n.d.
α-Copaene	3.02 ± 0.40 ^a	n.d.	0.39 ± 0.06 ^b	0.57 ± 0.09 ^b	< 0.0001
α-Bourbonene	1.00 ± 0.09 ^a	n.d.	0.28 ± 0.04 ^b	0.32 ± 0.04 ^b	< 0.0001
β-Caryophyllene	19.86 ± 1.88 ^a	n.d.	3.58 ± 0.6 ^b	6.76 ± 1.05 ^b	< 0.0001
α-Farnesene	0.58 ± 0.15	n.d.	n.d.	n.d.	n.d.
Humulene	16.40 ± 1.79 ^a	n.d.	5.15 ± 0.81 ^b	7.92 ± 1.31 ^b	< 0.0001
Murolene	5.41 ± 0.54 ^a	n.d.	0.16 ± 0.02 ^b	0.23 ± 0.04 ^b	< 0.0001
α-Curcumene	2.19 ± 0.47 ^a	n.d.	0.79 ± 0.14 ^b	0.63 ± 0.11 ^{bc}	< 0.001
Valencene	3.73 ± 0.51 ^a	n.d.	0.51 ± 0.06 ^c	2.68 ± 0.32 ^b	< 0.0001
α-Selinene	3.60 ± 0.49 ^a	n.d.	0.21 ± 0.04 ^b	0.13 ± 0.02 ^b	< 0.0001
α-Bisabolene	1.79 ± 0.32 ^a	n.d.	0.26 ± 0.04 ^b	0.25 ± 0.04 ^b	< 0.0001
Cadina-1(10.4)-diene	1.63 ± 0.27 ^a	n.d.	0.20 ± 0.03 ^b	0.23 ± 0.03 ^b	< 0.0001
α-Sesquiphellandrene	1.24 ± 0.33 ^a	n.d.	0.14 ± 0.02 ^b	0.11 ± 0.015 ^b	< 0.001
δ-Cadinene	0.43 ± 0.02 ^a	n.d.	0.06 ± 0.006 ^b	0.07 ± 0.01 ^b	< 0.0001
Ar-tumerone	0.20 ± 0.03 ^a	n.d.	0.02 ± 0.003 ^b	0.04 ± 0.01 ^b	< 0.0001

Volatile organic compounds were analyzed by GC-MS. Results indicate mean percentage ± S.D. (standard deviation) of three replicates, expressed as (peak area of each compound/total area of significant peaks) x100. Data within a row followed by different letters are significantly different according to Tukey's test. Abbreviations: HP, hop powder; HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP. *n.d.*: not detected.

valuable functional additive in food applications. Although, as in other plants, the aromatic profile of hop can vary based on a range of factors (Eyes & Dufour, 2009; Guimarães et al., 2021;), HP shares compounds often abundant in hop, such as β-myrcene, α-humulene, and β-caryophyllene (Aberl & Coelho, 2012; Ou et al., 2024; Vázquez-Araújo et al., 2013). β-Myrcene is recognized as a major contributor to the “fresh hoppy” aroma characteristic of hops, imparting herbal and woody undertones (Surendran et al., 2021); moreover, it acts as an aroma precursor, capable of generating compounds like linalool and geraniol through oxidation, which further enrich the hop's overall aroma profile (Behr & Johnen, 2009). Beyond its sensory role, β-myrcene has been widely studied for its antioxidant and anti-inflammatory properties and its role in inhibiting lipid oxidation in food products (Surendran et al., 2021). Equally noteworthy is the high presence of α-humulene (also known as α-caryophyllene) and its isomer β-caryophyllene, both of which are recognized for their antimicrobial and antioxidant properties (Haro-González et al., 2021).

Considering breads, in HB-0 22 compounds belonging to different classes, including one acid, four alcohols, ten carboxylic compounds, one ester, one furan compound, one monoterpene and four

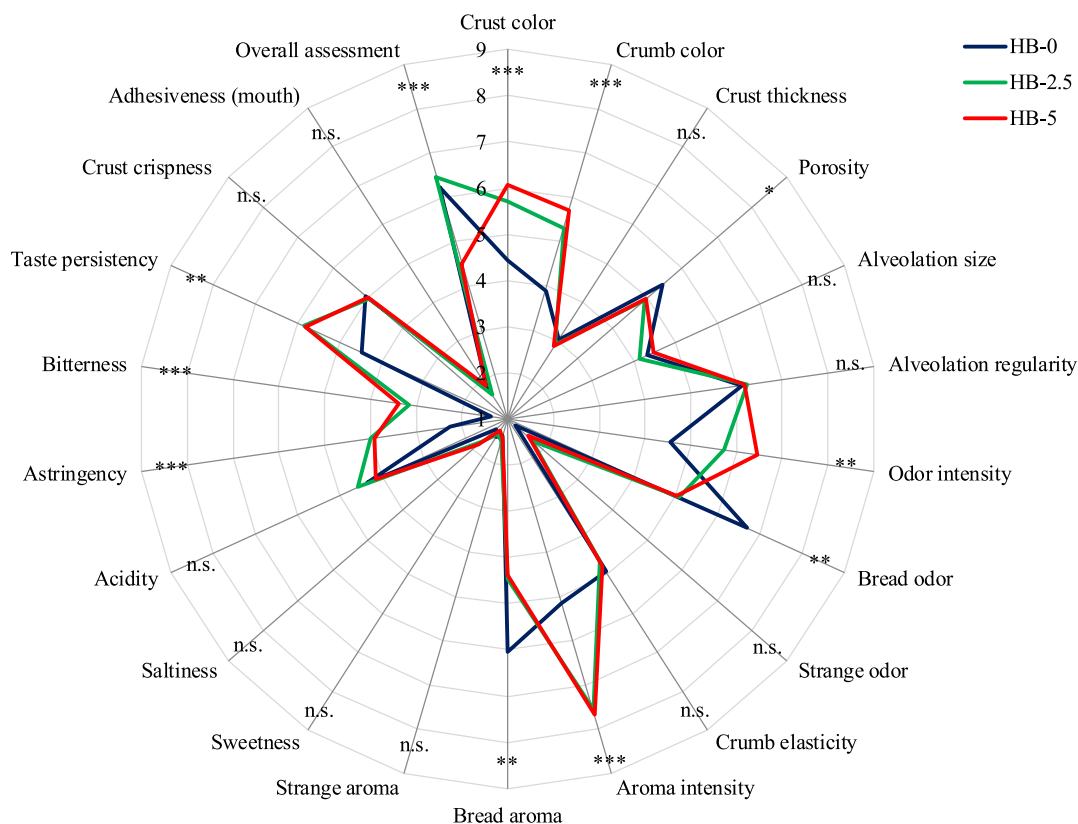


Fig. 2. Spider chart of descriptive sensory evaluation of experimental breads. Abbreviations: HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP.

hydrocarbons were identified. Similar aromatic profiles have been reported by several studies (De Luca et al., 2021; Giannone et al., 2018; Ruiz et al., 2003). Among the most abundant compounds, hexanal, followed by acetic acid, and nonanal can be mentioned. Acetic acid is responsible for the unique sour taste that enhances flavor (De Luca et al., 2021). Hexanal and nonanal also contribute significantly to the aromatic component of bread, originating from lipid oxidation and may provide a pleasant herbaceous odor (Sánchez-García et al., 2019). Interestingly, in breads sampled with HP addition, more than 70 % of the terpenes identified in HP were detected. These compounds were not found in the control bread, indicating that they specifically result from the addition of HP. Overall, the aromatic profile of the supplemented bread was supplemented by 16 % in the HB-2.5 sample and by 27.8 % in the HB-5 sample. The results demonstrate that the addition of HP has significantly enhanced the aromatic profile of the bread by contributing to the terpene content. Since terpene compounds not only impart scent and flavor to plants and fruits, but also possess numerous interesting properties, including antibacterial and anti-inflammatory effects (Ge et al., 2022), these findings further reinforce the potential of HP as a functional food additive due to its combined aromatic complexity and health-promoting properties.

3.8. Sensory profile of breads

Sensory analysis is crucial to evaluate consumer acceptance of novel food products; it is mandatory for a new product before commercialization (Swiąder & Marczevska, 2021). Results indicated that enriching bread with HP changes its sensory attributes (Fig. 2). The spider plot from the sensory evaluation showed the highest significant differences for aroma intensity, crust and crumb color, astringency, bitterness, and overall assessment. Specifically, breads with HP had a darker crust and crumb, higher aroma intensity, astringency, and bitterness compared to control bread. The differences increased with higher percentages of HP

addition, consistent with findings from other studies on breads added with powdered agri-food wastes or by-products (Gaglio et al., 2023; Gailite et al., 2008). Interestingly, the overall assessment of HB-2.5 was higher than that of HB-0. Regarding attributes typically negatively affected by powdered by-products, such as porosity and alveolation, only porosity showed significant differences among HB samples. This effect is due to the reduced gluten content from adding powdered ingredients that do not contain gluten (Rathnayake et al., 2018).

3.9. Shelf life of breads

The shelf life was assessed based on the appearance of visible mold development. Fig. 3 illustrates the bread slices over time. The first mold spot was observed on day 11 in the HB-0 trial. At the same time, no detectable mold growth was noted in either of the trials containing HP. In both supplemented bread samples, mold spots became clearly visible from day 13 onwards. According to Ju et al. (2020), the shelf life is determined as the day before the first noticeable sign of mold appears on the bread slices. Therefore, the shelf life of HB-0 was 10 days, while the shelf life of both HB- samples was slightly longer of 12 days.

To assess the staling behavior of the bread samples, DSC analyses were carried out after different storage times (0, 24, 48, 72, and 96 h). The endothermic transitions corresponding to the melting of retrograded amylopectin were used as indicators of starch retrogradation, a key factor in the staling process of bakery products (Gray & Bemiller, 2003). The melting enthalpy (ΔH) was determined by integrating the area under the relevant endothermic peak, observed in the 80–110 °C range. The evolution of ΔH values as a function of storage time for all bread formulations is shown in Fig. 4. In all samples, ΔH values progressively increased over time, indicating enhanced starch retrogradation during storage. This trend is in good agreement with previous studies and confirms the development of staling phenomena in all formulations (Del Vecchio et al., 2025; Fessas & Schiraldi, 1998). In

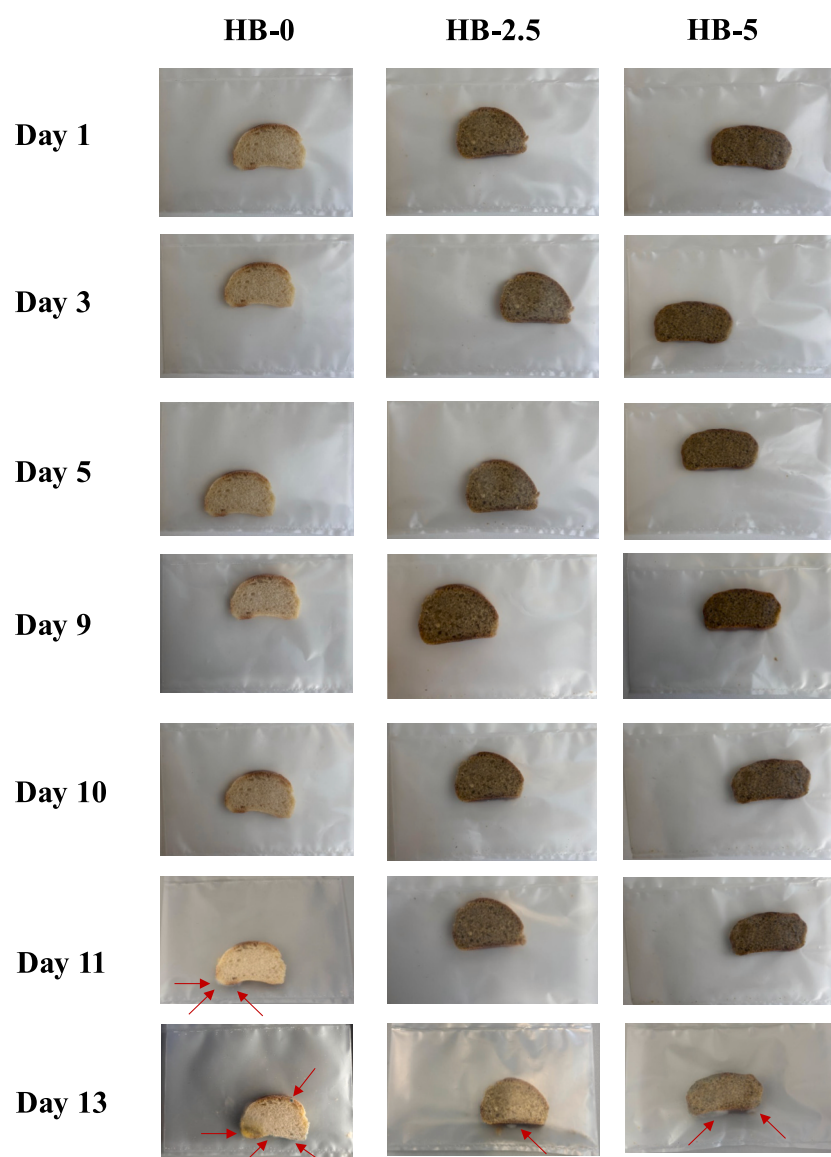


Fig. 3. Visual appearance of fungal growth on sliced breads. Abbreviations: HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP.

addition, the peak temperature of the endothermic transition showed a slight shift toward higher values with increasing storage time, regardless of the formulation. This behavior, also consistent with literature findings, is commonly attributed to increased crystallinity and thermal stability of the retrograded amylopectin structures as they reorganize and form more ordered crystalline domains during storage (Chakraborty et al., 2023). No significant differences were observed among the three formulations in terms of either ΔH values or peak temperature trends. This suggests that the presence of HP did not significantly affect the rate or extent of starch retrogradation under the tested conditions.

4. Conclusions

In this work, HP was utilized as a sustainable ingredient in sourdough bread to enhance the bioactive (poly)phenols and antioxidant activity of the final products. Firstly, the microbiological suitability of HP in food production was assessed through the absence of undesired groups. LAB strains were directly isolated from HP, which was essential for developing selected starter cultures for functional sourdough bread production with HP. Generally, starter LAB for (poly)phenol-

supplemented breads should be tested for their resistance to the primary (poly)phenols present in food by-products, or they can be selected starting from plant by-products. This latter approach was adopted in this study and proved to be promising. In the bread trials, breads supplemented with 2.5 % and 5 % of HP showed improved moisture retention, firmness, and darker appearance. HP enriched flavor, aroma, extended shelf life by delaying mold growth, but did not significantly affect the rate or extent of starch retrogradation. Thus, the study promotes sustainable practices by valorizing agro-industrial by-products and meeting the demand for functional, bioactive-supplemented foods. Future research should focus on large-scale fermentation performance and the impact on flavor development. Furthermore, exploration of the health benefits of HP is necessary. Finally, the application of HP in diverse food systems to maximize its potential in circular economy initiatives will be investigated.

CRediT authorship contribution statement

Enrico Viola: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Leandra Leto:** Writing – review &

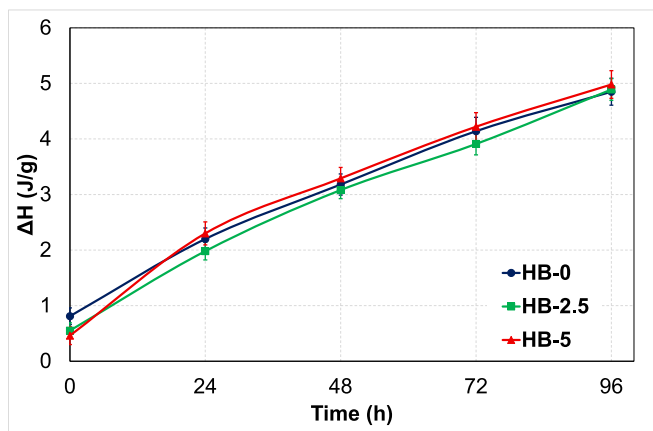


Fig. 4. Melting enthalpy (ΔH) associated with starch retrogradation in bread samples as a function of storage time, as measured by Differential Scanning Calorimetry (DSC). Abbreviations: HB-0, control bread; HB-2.5, experimental bread supplemented with 2.5 % (w/w) of HP; HB-5, experimental bread supplemented with 5 % (w/w) of HP.

editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Daniela Piazzese:** Writing – review & editing. **Serena Indelicato:** Formal analysis, Data curation. **David Bongiorno:** Writing – review & editing. **Claudia Lino:** Formal analysis. **Giuliana Garofalo:** Methodology, Data curation. **Francesco Bigi:** Resources, Investigation, Formal analysis. **Anna Agosti:** Investigation. **Valeria Guarrasi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis. **Erika Indovino:** Formal analysis. **Luigi Botta:** Data curation, Formal analysis. **Martina Cirilini:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Funding acquisition, Formal analysis. **Raimondo Gaglio:** Formal analysis, Data curation. **Marcella Barbera:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Formal analysis, Data curation. **Luca Settanni:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Benedetta Chiancone:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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.

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Data availability

Data will be made available on request.

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