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Clove Leaf Essential Oil in Vapor Phase as a Natural Alternative for Mold Control in Semi-Wholemeal Bread

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Abstract: The aims of this study were to characterize clove leaf essential oil (CLEO), evaluate its antifungal activity in the vapor phase, and determine its effects on the physicochemical and sensory properties of semi-wholemeal bread. Also, the physicochemical properties of the CLEO and the bread were determined, as well as the sensory properties of the bread. The CLEO was analyzed by chemical composition, refractive index, color, viscosity, and density. The antifungal activity was tested in vapor phase against *Aspergillus niger in vitro* and on bread at different concentrations during 10 days of storage. The formulated bread contained 60% of wholemeal flour. The CLEO analysis identified α-caryophyllene (32.2%) and eugenol (25.7%) as the major compounds. The minimum inhibitory concentration (MIC) of CLEO *in vitro* against *A. niger* was 300 μL/L_{air} while on bread was 1250 μL/L_{air}. A contaminating mold identified as *Penicillium chrysogenum* was more resistant than *A. niger*, and its MIC was 1750 μL/L_{air}. The pH of bread was 5.7, the water activity was 0.882, and its moisture content of 32.1%. The sensory scores obtained (6.3–6.7) indicate that the CLEO-treated bread remains within the moderate acceptance range. While CLEO inhibits mold growth, its high concentration needed in bread diminishes aroma and flavor acceptance. Future research should optimize exposure time and concentration to enhance shelf life without affecting sensory qualities.

Keywords: *Syzygium aromaticum*; eugenol; bread-biopreservation; antifungal activity; sensory acceptability

1. Introduction

Bread is the most common cereal-based staple worldwide, and its consumption is recommended in most international dietary guidelines due to its balanced mix of macronutrients and micronutrients [1]. There are several types of bread, classified by their primary ingredients and the breadmaking process. Wholemeal bread is a popular, healthier alternative to traditional white bread because it retains more dietary fiber, vitamins, and minerals [2], making it a better option for digestive health and blood sugar regulation. Dietary wholemeal bread intake is associated with beneficial effects, including the prevention of type 2 diabetes, cardiovascular disease, and colorectal, pancreatic, and gastric cancers. The potential benefits are suggested for 2 to consuming 3 servings per day (~45 g) [3]. In contrast, some concerns are associated with whole grains and their derived products due to contaminants that are present during production, manufacture, processing, preparation, treatment, packaging, transport, or holding of food. Therefore, their consumption also poses food safety risks due to contaminants accumulating in the grains' outer layers. These risks primarily include mycotoxins, toxic elements, and pesticide residues. Since these substances concentrate on the grain surface, whole and semi-whole flours tend to exhibit

higher contaminant levels than refined flours [4,5]. However, current scientific consensus and international food regulations suggest that rigorous quality control effectively reduces these risks. This means that the nutritional benefits of whole grains usually outweigh the potential chemical risks. As with other bakery products, the shelf life of wholemeal bread is limited (2-3 days at room temperature) when no preservatives are added [6].

Bread contains ingredients that can sustain fungal growth; it is the second leading cause of bread spoilage after staling. Molds are the primary cause of bread spoilage due to their pH (5.5–6.0) and water activity (a_w, 0.95–0.98) [7]. In bread, the most common molds that degrade it are *Aspergillus niger* and *Penicillium* spp. [6]. *Aspergillus flavus* and *A. niger* were the main molds in the air environment of a mechanized bakery [8]. Mold contamination, especially by *A. niger*, occurs in the production environment primarily in the slicing, packaging, and cooling areas, as reported by Chou et al. [9] for white bread produced at an industrial scale. Therefore, air quality, the velocity of cooling and packaging, and storage temperature could accelerate fungal contamination [7].

Essential oils (EOs) are recognized as biopreservative, flavoring, and decontaminating agents, attracting considerable interest in the food industry. EOs are volatile compounds associated with aromas of certain plants or their parts, such as flowers, leaves, shells, barks, and seeds. Various EOs have been demonstrated to have antifungal activity on bread, and those that contain carvacrol and eugenol as main compounds are highly effective. The clove leaf EO (CLEO) is obtained from the dried leaves of the clove tree, *Syzygium aromaticum*, and is commonly used as a flavoring in food and cosmetic products [10]. The main component of CLEO is eugenol (75–85%) [10], which confers strong antimicrobial activity against bacteria [11] and fungi [12]. The primary mechanisms of action of eugenol against *Fusarium* spp. are disruption of hyphae, destruction of cell membranes, and leakage of intracellular components (electrolytes, soluble proteins, nucleic acids, and malonydialdehyde) [13]. While in *A. niger*, eugenol alters the composition of fatty acids of the cell membrane, affects mitochondrial morphology, decreases the mitochondrial membrane potential, reduces the enzymatic activity of the six TCA cycle, and decreases energy metabolism, leading to loss of viability [14,15].

Because EOs are rich in aromatic and flavor compounds, they are well known to strongly modify the sensory properties of foods, thereby often challenging consumer acceptance and limiting their practical application [16–18]. To overcome these sensory alterations while maintaining antimicrobial efficacy, recent technological approaches focus on alternative methods, such as vapor phase delivery or encapsulation systems [19–21]. The inhibitory action of EOs is more effective in the vapor phase because volatile compounds (lipophilic molecules) are not associated with the aqueous phase (to form micelles), which restricts their interaction with the microorganisms [22,23], reducing the amount of EO to prevent fungal growth compared with direct addition [24]. The vapor phase of EOs, including clove, has demonstrated antifungal activity against mold strains isolated from cereal grains [25]. Furthermore, the use of EOs in the vapor phase did not modify the sensory quality of cheese (clove (62.5 μL/L) or litsea (125 μL/L)) [19] when compared with untreated ones. These features can be used to develop active packaging with EOs to effectively delay fungal growth in foods. Rusková et al. [20] encapsulated 5% lemongrass EO into poly(lactic acid) and poly(3-hydroxybutyrate) plasticized with acetyl tributyl citrate that inhibited bacteria, mold, and yeast growth for 21 days on strawberries while preserving its quality. Likewise, various studies have reported the antifungal activity of EOs or their components in vapor phase on bread through active packaging or releasing sachets/films/containers due to their diffusion capability. Passarinho et al. [26] found that 5% of oregano EO reduced yeast and mold counts in bread during 10 days of storage. In another study, 750 μL of lemongrass EO/L_{air} inhibited the growth of *Penicillium expansum* in bread for 21 days at 20 °C [27]. Mycelial growth inhibition (22–23%) of *Penicillium citrinum* on bread slices in vapor phase was obtained with *Hyperiucm perforatum* EO at 250 and 500 μL/L [28]. The vapors from eugenol and citral combined compounds loading in corn-starch into sachets inhibited the mold growth on bread loaves by 13 days at 25 °C and 9 days at 35 °C [29] and on bread slices packaged using three types of polymers (polypropylene, high density polyethylene, and low density polyethylene) the mold growth was retarded by 13, 13, and 15 days of storage, respectively, at 25 °C [30]. To the best of our knowledge, no previous reports on CLEO on bread or bakery products have been reported. However, CLEO (12%) helps preserve milkfish meat for 3 days by inhibiting bacterial growth [31]. Given the potential of CLEO's antifungal activity by direct addition, its GRAS status, the antifungal activity of eugenol, commercial availability, and its underexploited use in bakery products and bread, the present study aims to evaluate the chemical composition and physical properties of CLEO, its antifungal activity in the vapor phase against *Aspergillus niger in vitro*, and its effects on semi-wholemeal bread. Also, physicochemical and sensory properties of bread were evaluated. Therefore, we hypothesized that CLEO vapors could inhibit fungal growth in semi-wholemeal bread at concentrations that maintain acceptable sensory characteristics.

2. Materials and Methods

2.1. Clove-Leaf Essential Oil (CLEO) Characterization

The CLEO (*Syzygium aromaticum*) was purchased from Laboratorios Hersol S.A. de C.V. (obtained by steam distillation, batch number 543,901, manufacturing date 2022, San Mateo Atenco, Estado de Mexico, Mexico). The CLEO was chemically analyzed using an Agilent Technologies 6850N gas chromatograph (Palo Alto, CA, USA) equipped with an Agilent 5975C mass spectrometer (MS) (Palo Alto, CA, USA). The carrier gas flow rate, helium, was set to 1.1 mL/min. Compounds were separated on an HP 5 fused-silica column (Agilent, Palo Alto, CA, USA; 30 m, 0.25 mm ID, and 0.25 µm film thickness). The injector was maintained at 300 °C and operated in split mode (10:1). The column oven was held at 60 °C for 2 min, then heated to 250 °C at 10 °C/min. Retention indices (RI) were calculated using a homologous series of *n*-alkanes C8–C18 (Sigma-Aldrich, St. Louis, MO, USA). Compounds were identified by comparing their RI and mass spectra obtained with those of the main components (eugenol for clove EO) reported in the literature in the National Institute of Standards and Technology (NIST) mass spectra database.

The CLEO was also characterized by its color parameters (L^*, a^*, b^*) on the CIELab scale, using a colorimeter (model CR400, Konica Minolta, Tokyo, Japan) using the transmittance mode, and its refractive index was determined using a digital refractometer (PAL-BX/RI, ATAGO, Tokyo, Japan), according to the Official Method 920.141 of the AOAC [32], after prior calibration of the equipment. Viscosity was measured using a Cannon-Fenske viscometer (350, Thomas Scientific, Swedesboro, NJ, USA), and density was determined using a glass pycnometer, according to the Official Method 920.134 of the AOAC [32].

Regarding food safety, CLEO and one of its main components, eugenol, are well-regulated for consumption. CLEO and eugenol are recognized as safe (GRAS) by the FDA [33] and approved as a flavoring agent and preservative by EFSA and JECFA [34]. Despite the potential, high concentrations may cause cytotoxicity by disrupting cell membranes; the active doses employed in bakery vapor-phase antimicrobial treatments remain well below toxicological thresholds and the acceptable daily intake (ADI). Therefore, using CLEO in the vapor phase is a safe, non-contact biopreservation approach for human consumption.

2.2. Molds and Growing Conditions

The mold *A. niger* was obtained from the culture collection of the Food Microbiology Laboratory at the University of the Americas Puebla, Mexico. It was cultured on potato dextrose agar (PDA, Bioxon, BD, Cuautitlan, Estado de Mexico, Mexico) slants and incubated at 27 °C for 7 days. The strain was routinely plated and maintained in slants throughout the study period. The spores were harvested using a 0.1% Tween-80 (Sigma-Aldrich, St. Louis, MO, USA) solution, with thorough washing over the slant surface. The number of spores was counted using a Neubauer chamber, and the concentration was adjusted to obtain an initial count of 1×10^6 spores/mL.

2.3. Antimicrobial Activity Tests by the Inverted Petri Dish Technique

The methodology proposed by Reyes-Jurado et al. [35] was used with some modifications. PDA culture medium was prepared, and 7 mL was placed in 60 × 15 mm Petri dishes. The volume of air remaining in the Petri dish when it is closed was calculated (≈26.5 mL). Subsequently, 5 µL of the mold spore suspension (10^6 spores/mL) was inoculated into the center of the culture medium. Selected CLEO concentrations (0, 250, 300, 350, 400, 450, and 500 µL of CLEO/L_{air}) were achieved by applying specific volumes of the essential oil onto previously sterilized Whatman No. 1 filter paper (3 × 2 cm) attached to the center of the plate lid. The volume (µL) applied to each paper strip was calculated to match the target concentration (µL/L_{air}). Immediately after placing CLEO, the plates were inverted and sealed with Parafilm® to prevent volatile compounds from escaping and condensation from dripping into the medium. To ensure uniform volatilization, the selected filter paper, a cellulose fiber network (highly porous), was used as a capillary release matrix. The plates were incubated at 25 °C for 10 days. Each test was performed in triplicate.

During the incubation period, the mold's diameter was measured every 24 h in both directions at right angles using a digital caliper (Mitutoyo Corp., Kawasaki, Japan); growth controls were prepared in parallel to ensure the presence of viable organisms. The minimum inhibitory concentration (MIC) of CLEO was determined as the concentration that completely inhibited mold growth during the 10-day evaluation period. The colony diameter was plotted against incubation time. Each experiment was performed in triplicate.

The modified Gompertz equation (Equation (1)) was used to describe mold growth because it fits sigmoid behaviors. A nonlinear regression was performed on each dataset using Minitab 20 (Minitab LLC, State College, PA, USA) to estimate the equation parameters [36].

$$D = A \times exp\left[-exp\left(\frac{\mu}{A}\right)(\lambda - t) + 1\right] \quad (1)$$

μ is the maximum growth rate (mm/h), A is the maximum diameter observed, λ is the phase or lag time (h), and D is the colony diameter (mm) at time t (h).

2.4. Semi-Wholemeal Bread

Semi-wholemeal bread was prepared following the methodology described by Hernández-Figueroa et al. [37], with slight modifications, using the formulation presented in Table 1. All ingredients were mixed and kneaded in a Legacy HL200 mixer (Hobart, Troy, OH, USA) for 20 min. The dough was then allowed to rest for 20 min, degassed, and left to rest again for an additional 10 min. Subsequently, the dough was divided into 30 g portions and fermented at 35 °C for 30 min using a Mini combo fermenter chamber (Zucchelli Alpha, Trevenzuolo, Verona, Italy). After fermentation, the dough pieces were baked at 200 °C for 12 min using a Mini combo oven (Zucchelli Alpha, Trevenzuolo, Verona, Italy). Finally, the bread was cooled at room temperature for 1 h.

Table 1. Semi-wholemeal bread formulation.

Ingredients	Amount
Whole Wheat Flour	150 g
White Flour	100 g
Salt	3.5 g
Yeast	5 g
Water	160 g

Furthermore, various analyses of the final product were performed. Once the product cooled, water activity was measured in triplicate using an AquaLab 4TEV Series equipment (Meter Food, Pullman, WA, USA). Similarly, the moisture content of the samples was determined in triplicate by oven drying following the AOAC 930.15 method [32]. The pH of the bread was determined using a pH meter model HI2210 (Hanna Instruments, Woonsocket, RI, USA) by weighing 10 g of the crushed sample, dissolving it in 100 mL of distilled water that had been boiled and cooled to room temperature, and allowing it to stand for 30 min following the method AACC-02-52 [38]. The specific volume was also determined using the seed displacement technique, following AACC method 10-05 [38]. The bread was placed in a container of known volume and covered with turnip seeds until the empty spaces were filled; the displaced volume was recorded and divided by the sample weight to obtain the specific volume (mL/g). Bread texture (hardness) was assessed using a texture analyzer (EZ-SX, Shimadzu Corporation, Kyoto, Japan) on a 2.5 cm thick slice compressed to 50% using a stainless-steel cylinder probe (diameter = 25 mm) at a speed of 1 mm/s. Hardness was defined as the maximum force (N) recorded during the compression. According to the AACC 14–22 [38], crumb and crust color were measured using a colorimeter with the CIELab system. The parameters L^* (lightness), a^* (red-green), and b^* (yellow-blue) were recorded. Measurements were taken at different points on the surface to obtain representative values. The hue and chroma were calculated, while the yellowness index was determined using Equation (2) reported by Alam et al. [39]. All the analyses were performed in triplicate, and the average and standard deviation of the results were calculated and reported.

$$YI = 142.86\ \frac{b^*}{L^*} \quad (2)$$

2.5. Antifungal Activity in Semi-Wholemeal Bread

The airtight chamber technique was used to determine the CLEO MIC in the bread. The net volume of the gaseous phase in the used chambers was estimated by subtracting the specific volumes of three bread samples, yielding a net headspace volume of ≈1.64 L.

Three semi-wholemeal bread pieces were previously inoculated in the center of their upper surface with 5 µL of an *A. niger* spore suspension (1×10^6 spores/mL). Two small glass containers with wide mouths and short heights, serving as evaporation wells, were distributed evenly within each chamber, into which CLEO was loaded. The CLEO volume required for each treatment was determined to match the desired concentrations. Uniformity of volatilization and homogeneous atmospheric distribution within the system were achieved through natural passive diffusion. The hermetic sealing of the chambers prevented volatile leakage, allowing uniform vapor saturation throughout the chamber headspace during incubation. The sealed airtight chambers were kept at 25 °C for 10 days, and the bread samples were observed daily. After the evaluation period, the MIC was determined.

2.6. Fungal Isolation

In semi-wholemeal bread, the growth of molds other than *A. niger* was observed. Some colonies were detected on the surface of the bread and inoculated onto PDA at 25 °C for 5 days. The mold was repeatedly cultured on PDA until an isolated colony was obtained. All experiments were carried out in triplicate.

For the isolated colony, the mold identification was performed following the key clues described by Samson et al. [40] and Pitt & Hocking [41] using PDA, malt extract agar (Bioxon BD, Cuautitlan, Edo. de Mexico, Mexico), Czapek-Dox agar (Merck, Darmstadt, Germany), and an optical microscope (Zeiss, Mexico City, Cd. de Mexico, Mexico). Finally, the colonies were identified according to their morphological characteristics through microscopic observation and comparison with the morphology reported in the literature [40].

The MIC of CLEO was also determined for the isolated mold under the same conditions as *A. niger*; thus, the previously described method was followed.

2.7. Sensory Evaluation

A 9-point hedonic sensory scale test was conducted on semi-wholemeal bread, in which consumers were asked to rate their overall satisfaction with the following attributes: flavor, odor, texture, and acceptability. The evaluation was performed with 30 untrained panelists, who were informed of the test's purpose and rationale. Three bread samples, identical in size, were presented. One sample served as the control, while the other two were exposed to CLEO vapors at the MIC (1250 and 1750 $\mu L/L_{air}$) found in airtight chambers for 24 h. Prior to testing, the bread pieces were heated in a domestic microwave oven (800 W) for 10 s. This brief thermal pulse was intended to raise the internal core temperature of each bread piece to ~45 °C. This conditioning was standardized to promote the volatilization and optimal headspace release of volatiles (in this case, CLEO) for olfactory and flavor evaluation [42].

To standardize the interpretation of consumer compliance, an Acceptability Index (*AI*, %) was calculated using Equation (3):

$$AI\ (\%) = \frac{Mean\ score}{9} \times 100 \quad (3)$$

Informed consent was obtained from all subjects involved in the sensory evaluation. The panelists were informed about the use of wholemeal flour in bread formulations. Each participant signs an informed consent by responding affirmatively to the following statement: "I am aware that my responses are confidential, and I agree to participate in this sensory evaluation". They were informed that they could withdraw from the test at any time without providing a reason; we explicitly stated that "The products are safe for consumption" and that participants' data would not be disclosed without their knowledge.

2.8. Statistical Analysis

Experimental assays were executed in triplicate (n = 3), and results are expressed as mean values ± standard deviations. For the sensory analysis, the normality and homoscedasticity of the data were assessed prior to analysis using the Shapiro-Wilk test. The scores for each attribute for the different CLEO concentration treatments were statistically analyzed using One-way Analysis of Variance (ANOVA). Pairwise comparisons among treatment means were performed using Tukey's post-hoc test at a significance level of $p < 0.05$. All statistical evaluations were conducted utilizing the software Minitab 20 (Minitab LLC, State College, PA, USA).

3. Results and Discussion

3.1. Clove-Leaf Essential Oil Characteristics

The chemical composition of the CLEO was determined by gas chromatography, and the resulting identifications comparing mass spectra with the National Institute of Standards and Technology Mass Spectral database are presented in Table 2.

GC-MS analysis identified α-caryophyllene as the main volatile in CLEO, followed by eugenol (Table 2). As mentioned previously, eugenol is the component responsible for the EO's antimicrobial properties. In previous studies, eugenol has been reported as the main component of CLEO, ranging from 50–76% [10,43,44], while caryophyllene is often reported as the second component [10,44,45]. While eugenol typically dominates among *Syzygium aromaticum* derivatives, the phenylpropanoid-to-sesquiterpene ratio can vary due to technical and environmental factors. The chemical evaluation of CLEO highlights two critical precursor-derivative relationships that validate the EO's biological and processing history. Within the phenylpropanoid pathway, 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (coniferyl alcohol) acts as the direct metabolic precursor to eugenol via enzymatic acylation and downstream reduction by eugenol synthase [46]. Extraction kinetics during steam distillation are

critical; varying distillation times and temperatures can cause the loss or degradation of eugenol, thereby enriching less-volatile sesquiterpenes. The sesquiterpene fraction, α-caryophyllene, serves as the structural precursor to caryophyllene oxide, an oxygenated derivative formed via spontaneous thermal epoxidation, as the extraction temperature increased [47], such as during steam distillation. The chemical profile reflects phenological and environmental adaptations, with upregulation of the sesquiterpene pathway in leaves in response to climate, soil, or pests, and caryophyllenes acting as key phytoalexins [44,48]. The observed proportions of eugenol and α-caryophyllene reflect the specific agronomic history and extraction process of the raw material and indicate that it is an effective EO for bread preservation.

Table 2. Chemical composition of clove-leaf essential oil (*Syzygium aromaticum*).

Retention Index (RI)	Compound	Percentage (%)
1367	Eugenol	25.72
1434	α-Caryophyllene	32.16
1441	α-Farnesene	4.11
1524	Syn δ-cadiene	12.43
1550	Dipicedrene-1-oxide	3.28
1577	Caryophyllene oxide	3.37
1653	4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol	8.82

The physical properties of the CLEO are shown in Table 3. The physical data for CLEO indicate high purity, characterized by a pale yellow with a greenish hue (103.4°), chrome (16.9), and high luminosity. The density and refractive index are critical indicators confirming the predominant presence of eugenol, the compound responsible for the optical and physical concentration of this EO. These values align with previously reported values for CLEO: RI = 1.528 by Alighiri [49] and density 1.057 g/mL by Rukmawati and Said [31]. Furthermore, the viscosity of 7.9 cP indicates a stable molecular structure and a characteristic fluidity that distinguishes it from lighter EOs.

Table 3. Physical properties of clove-leaf essential oil (CLEO).

Property	CLEO
Color	
*L**	81.60 ± 0.20
*a**	−3.92 ± 0.03
*b**	16.43 ± 0.03
Refractive Index	1.535 ± 0.004
Viscosity (cP)	7.9 ± 0.01
Density (kg/L)	1.042 ± 0.020

3.2. Minimum Inhibitory Concentrations In Vitro

To determine the MIC of CLEO on *A. niger*, concentrations of 0, 250, and 500 μL/L_{air} were tested on PDA. Growth was observed in both the control and at 250 μL/L_{air}; however, no growth was detected at 500 μL/L_{air}. Subsequently, concentrations between 250 and 500 μL/L_{air} were evaluated, with further testing at 0, 250, 300, 350, 400, 450, and 500 μL/L_{air}. After a 10-day evaluation period, no mold growth was observed at concentrations ≥ 300 μL/L air. Figure 1 shows the average (and standard deviation) diameter of growth (mm) of *A. niger*. The control sample (0 μL/L_{air}) reached a diameter of ≈50 mm (the Petri dish's size). Therefore, the MIC of CLEO against *A. niger* was found to be 300 μL/L_{air}.

The growth pattern observed in *A. niger* is sigmoidal. Two characteristic growth phases can be identified in the curves obtained: the first is the adaptation phase, during which the mold adapts to the new growth conditions. The second is the exponential phase, in which cell division occurs, and therefore, growth is maintained at a constant rate [50,51]. A stationary phase was observed in the control sample; however, due to the growth conditions in the 60 × 15 mm Petri dishes, the mold sometimes reached the maximum diametral growth allowed by the dish. Using the modified Gompertz equation, a nonlinear regression was performed on the control sample and the sample with 250 μL/L_{air}. The model adequately fits the data, with R^2 values > 0.96. Table 4 shows the estimated parameters for the maximum diameter of growth (A), the lag time (λ), and the maximum growth rate (μm), as well as 95% confidence intervals of the parameters' estimation for the control and with a CLEO concentration of 250 μL/L_{air}. Mold inhibition occurred at 300 μL/L_{air}, indicating that this concentration is the MIC under the tested conditions, as it prevents germination. The antifungal activity of CLEO is evident in the mold growth parameters, as lag time

is prolonged and the maximum growth rate and diameter are reduced. Similar findings have been reported for thyme EO on *Aspergillus flavus* growth parameters [52].

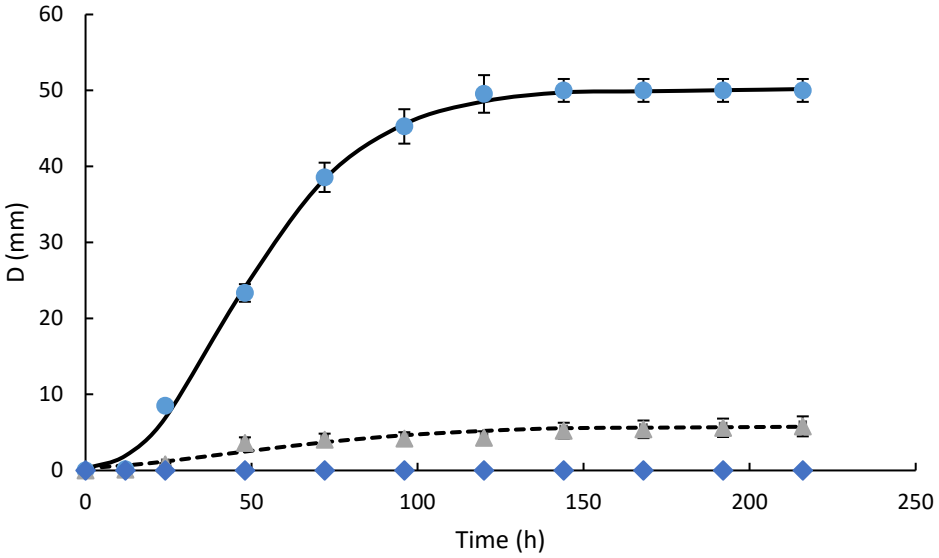

Figure 1. Effect of the different concentrations of clove leaf essential oil on the growth of *Aspergillus niger*. Control (●), 250 µL/L_{air} (▲), 300 µL/L_{air} (♦), and lines are the predicted values using the modified Gompertz equation.

Table 4. Estimated Gompertz parameters for *Aspergillus niger* growth at two clove leaf essential oil concentrations.

Parameter	Control			250 µL of oil/L_{air}		
	Estimate	SE	95% CI	Estimate	SE	95% CI
A (mm)	50.42	0.39	(49.64, 51.22)	7.11	0.15	(6.79, 7.48)
λ (h)	16.47	1.27	(13.98, 18.96)	27.08	1.97	(22.64, 31.47)
µm (mm/h)	2.085	0.079	(1.94, 2.24)	0.129	0.004	(0.12, 0.13)
R^2	0.998			0.968		

SE= Standard Error; CI = Confidence Interval.

3.3. Physicochemical Properties of Semi-Wholemeal Bread

The bread made with a mixture of whole wheat flour (60%) and refined flour had a pH of 5.71 ± 0.28, a characteristic value for breads baked with commercial yeast, in which alcoholic fermentation predominates over the production of organic acids, resulting in slightly acidic matrices (≈5.3–6.0) [53]. The water activity (a_w) and moisture content are within the typical range for fresh breads (Table 5), which favors an initial soft texture but also limits shelf life due to susceptibility to mold growth [54]. Similar moisture (~33%) and firmness (8–11 N) values were recorded for wholemeal bread containing different amounts of EOs in the dough [6]. In this regard, the estimated shelf life of 2–3 days under ambient conditions is consistent with that reported for preservative-free products with a_w values greater than 0.85.

Table 5. Physicochemical, structural, and color properties of the bread produced.

Parameter	Value	Value
pH	5.71 ± 0.28	
Water activity (a_w)	0.882 ± 0.018	
Moisture content (%)	32.10 ± 2.01	
Specific volume (mL/g)	2.4 ± 0.8	
Texture (Hardness, N)	9.5 ± 1.2	
Color (CIELab)	Crumb	Crust
*L**	59.43 ± 0.63	45.31 ± 0.48
*a**	5.52 ± 0.66	11.15 ± 0.13
*b**	18.32 ± 0.39	23.34 ± 0.26
Hue	73.23	64.46
Chrome	19.13	25.86
Yellow index (YI)	44.03	73.58

The specific volume obtained (2.4 mL/g), as shown in Table 5, was lower than that observed in bread pieces made exclusively with refined flour, which may be attributed to the diluting effect of the fiber in whole-wheat flour on the gluten network, thereby affecting gas retention and dough expansion [1]. This behavior was also reflected in the texture parameters, with a hardness of 9.5 N, indicating a relatively firm crumb. Previous studies have shown that incorporating whole-grain fractions increases firmness and decreases elasticity due to disruption of the continuous protein matrix, leading to a compact crumb [55].

Regarding color (Table 5), the crust values indicate adequate development of non-enzymatic browning reactions, primarily Maillard reactions, favored by the baking conditions (200 °C, 12 min) and the availability of reducing sugars and amino acids [56]. Meanwhile, the crumb showed higher lightness values, consistent with the presence of bran particles that generate beige to light brown tones. The color difference between the crumb and the crust shows a practical and appealing contrast. Increased a^* and b^* and decreased L^* confirm proper baking, with surface sugars and proteins reacting to form the crust. A YI of 73.58 indicates ideal coloration—sufficient starch and protein breakdown without burning. In the industry, higher YI correlates with better 'flavor' and crispiness. The crust gained 67% more 'yellowness/toast' than the crumb. Overall, the results suggest that although the formulation allows the production of a bread with acceptable physicochemical and sensory characteristics, there are limitations in volume and texture attributable to the high content of wholemeal flour and the processing conditions. Higher a_w (0.945–0.969) and specific volume (3.43–3.89), while lower L^* (65.57–66.23), a^* (4.13–4.59), and b^* (16.65–16.964) were reported for wholemeal bread added with different amounts of EOs (cinnamon, clove, and bay leaf) in the dough [6].

The semi-wholemeal bread developed in this study, formulated with a high proportion of wholemeal flour and characterized by a relatively compact structure (specific volume of 2.4 mL/g), suggests that the formulation may potentially contribute to a reduced glycemic response (lower glycemic index than conventional white bread); however, experimental validation is required. The presence of insoluble fiber and bran particles reduces the accessibility of starch to digestive enzymes, while lower crumb expansion may limit gelatinization and the surface area available for hydrolysis, both of which are associated with a more moderate glycemic response [57]. The processing conditions (moderate hydration and short fermentation) could favor a less aerated matrix, associated with slower starch digestion. This suggests that this semi-wholemeal bread would have a lower glycemic index than white bread, which is typically high (>70), perhaps falling within a moderate range, relevant for metabolic control and the prevention of type 2 diabetes [58]. However, this interpretation is an estimate based on compositional and structural characteristics, so experimental determination of the glycemic index through *in vivo* assays or validated *in vitro* methods is required to confirm these effects.

3.4. Antifungal Activity of CLEO on Semi-Wholemeal Bread

To determine the MIC of CLEO on the semi-wholemeal bread, different concentrations of CLEO (0–1750 $\mu L/L_{air}$) were tested. The MIC for *A. niger* was 1250 $\mu L/L_{air}$. Figure 2 exhibits the semi -wholemeal bread after 10 days of exposition to the vapor phase of the different CLEO concentrations. A notable increase was observed when comparing the *in vitro* MIC of CLEO against *A. niger* with the effective dose on semi-wholemeal bread. This aligns with other reports indicating that EO concentrations often need to be higher in complex food systems than in culture media due to the matrix's composition and structure. CLEO compounds may bind to proteins and lipids, limiting their vapor-phase availability needed to disrupt fungal cells. Similar trends are reported with vapor phase exposure of cinnamon, oregano, rosemary, lavender, mint, and thyme EOs, which require higher concentrations to control fungi on bread [59,60]. The higher *in vivo* MIC for CLEO emphasizes the importance of testing preservation systems within the food's structural/compositional context.

During the evaluation, a contaminating mold was observed on bread. Therefore, uninoculated bread was exposed to the same CLEO vapor phase concentrations to determine the MIC of the contaminating mold. The contaminating mold was presumptively identified as *Penicillium chrysogenum* based on its colony morphology (typically blue-greenish and white) and micro-structural features observed under optical microscopy (see Figure 3), according to the key clues described by Samson et al. [40] and Pitt and Hocking [41]. While this macro- and microscopic characterization provided a reliable baseline for monitoring fungistatic inhibition in the evaluated bread, molecular validation via multi-locus DNA barcoding would be necessary for a definitive genetic determination [61]. The MIC for *P. chrysogenum* was 1750 $\mu L/L_{air}$. Figure 4 shows the *P. chrysogenum* growth under different CLEO concentrations in the vapor phase on bread after 10 days. As mentioned before, *Penicillium* is one of the main fungal spoilage agents and a common contaminant in bakery products. *Penicillium spores* are highly ubiquitous and can contaminate bread during cooling, handling, or storage. The physicochemical characteristics of the semi-wholemeal bread (pH 5.71; a_w 0.882; moisture content 32.1%) create a favorable

condition for the growth of this microorganism, since a_w above 0.85 and a pH between 5 and 6 promote spore germination and mycelial development. From a shelf-life perspective, the presence of this mold explains the product's limited stability (2–3 days at room temperature). Once deposited on the surface, the relatively high a_w conditions facilitate rapid germination, resulting in the visible appearance of colonies in a short period.

Conventional bread preservation uses synthetic salts like calcium propionate or potassium sorbate in the dough to delay mold spoilage [62]. However, a direct comparison between these commercial additives and the evaluated CLEO system was not possible due to fundamental differences in their delivery mechanisms. While traditional organic acid salts operate strictly within the dough by reducing intracellular pH, the CLEO system functions via vapor phase contact, thereby reducing chemical migration into the bread. The viability of this approach is supported by our empirical shelf-life trials. While the untreated control bread exhibited visible mold development within 2 to 4 days, the samples treated with vapor-phase CLEO remained completely mold-free for over 10 days, representing a shelf-life extension of at least 150%. This performance aligns with emerging trends toward 'Clean Label', in which consumers reject synthetic preservatives [63,64].

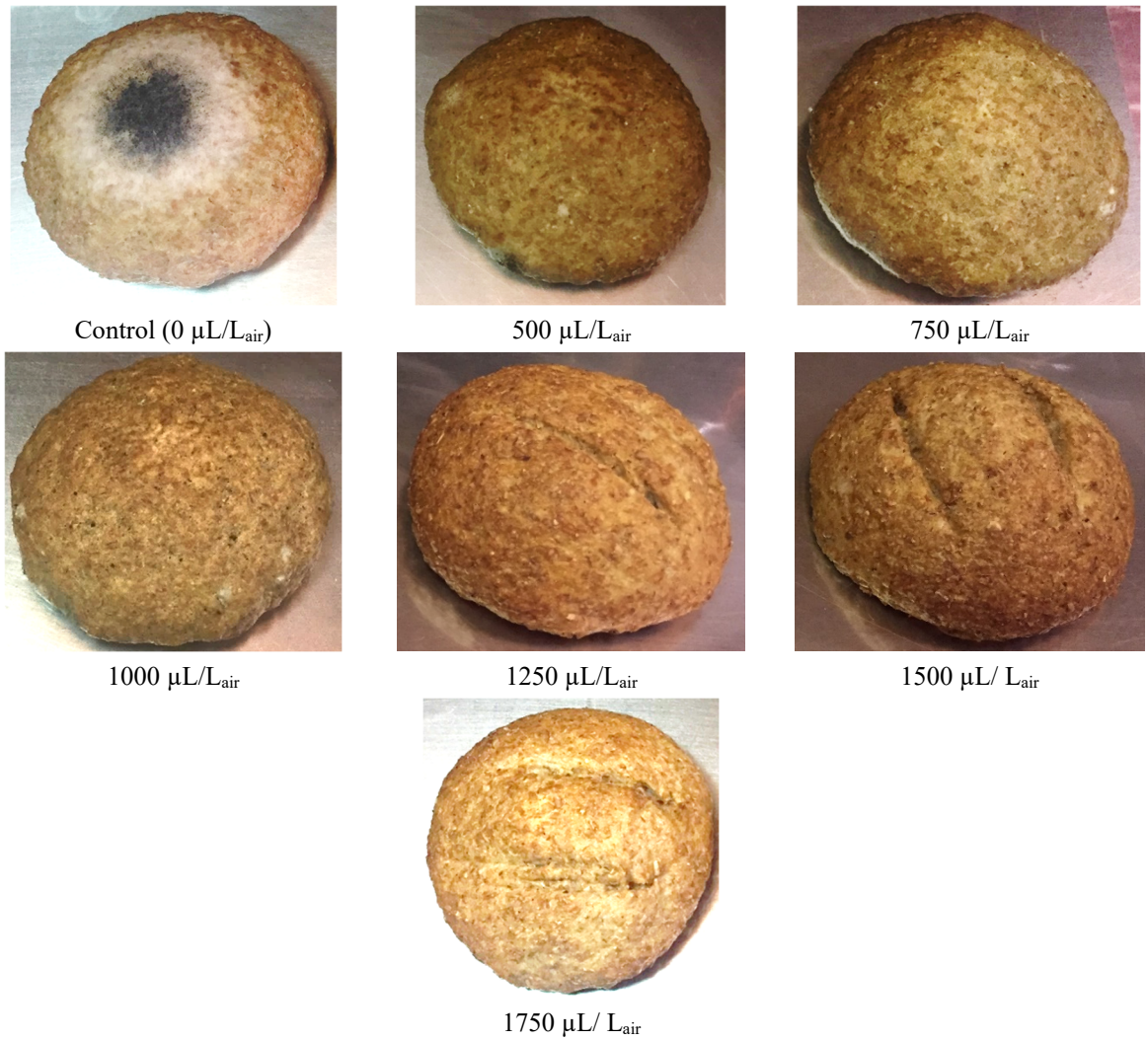

Figure 2. Fungal growth intentionally inoculated *Aspergillus niger* on bread exposed to different concentrations of clove leaf essential oil in vapor phase.

To the best of our knowledge, no previous reports on the antifungal activity of CLEO in the vapor phase on bread have been published. However, the vapor phase antifungal efficacy of several other EOs has been documented in bakery products. Specifically, the volatile fractions of cinnamon, thyme, oregano, and mustard EOs have been successfully utilized, either through direct headspace or active packaging systems, to inhibit common bread-spoilage molds such as *Penicillium*, *Aspergillus*, *Botrytis,* and *Rhizopus* [19,20,30]. Císarová et al. [59] reported lower minimum inhibitory doses to reduce by 90% the mycelial growth of *Aspergillus parasiticus* and *Aspergillus ochraceus* on bread at 25 °C, with clove EO (866.27 and 788.08 µL/L_{air}). Lower concentration of lemongrass EO in vapor phase (500 µL/L_{air}) was needed to inhibit the growth of *P. expansum* on bread for 18 days

at room temperature [27]. Compared to other EOs in the vapor phase on bread, higher amounts of CLEO are required to inhibit *A. niger* and *P. chrysogenum*. This was unexpected because the main components of CLEO (caryophyllene and eugenol) are recognized as potent antifungals when added directly to food products [29,49].

(**a**) (**b**)

Figure 3. *Penicillium chrysogenum* isolated from bread (**a**), colony morphology in potato dextrose agar (PDA), and (**b**) optical micrography (1000×).

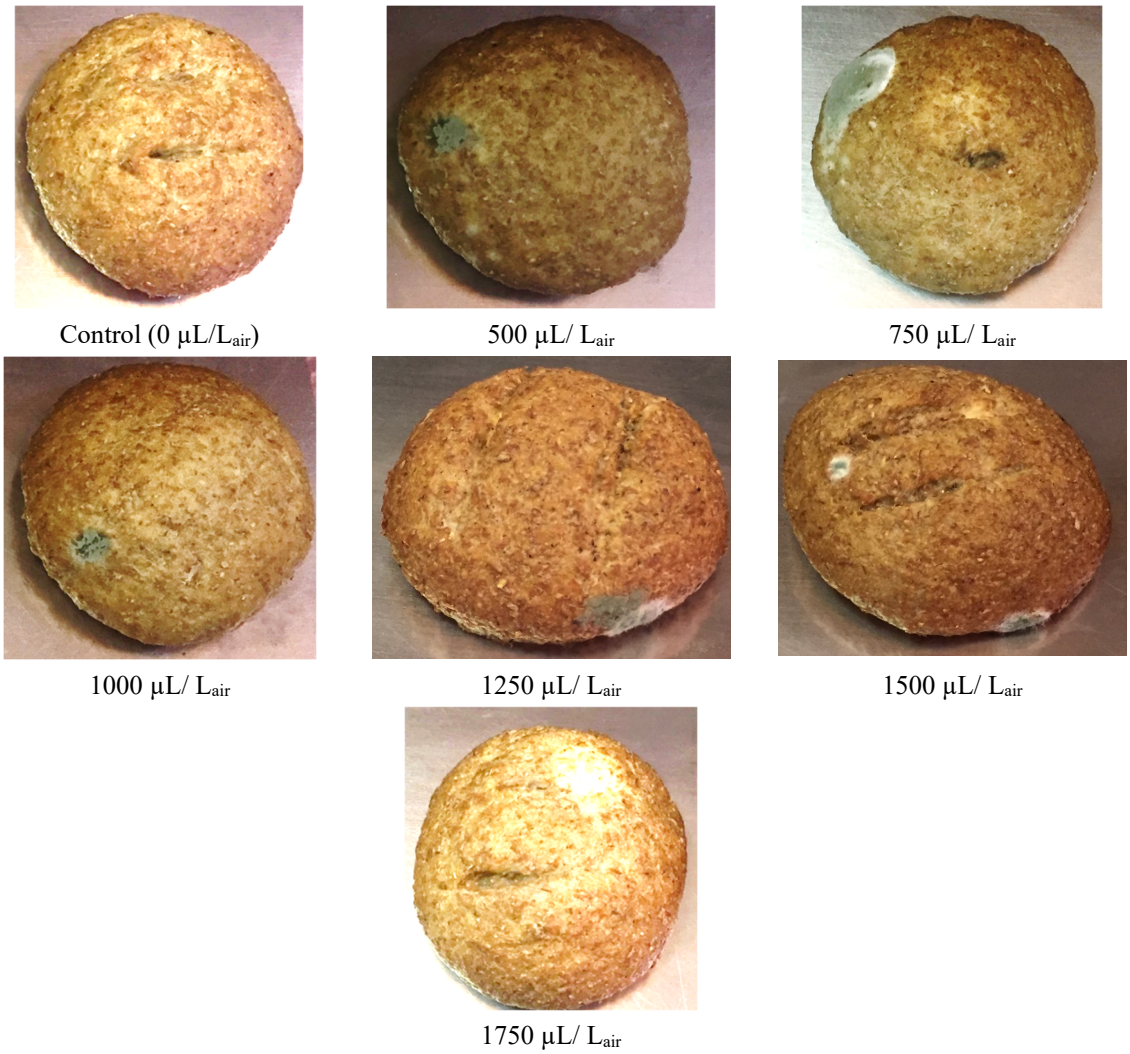

Figure 4. Fungal growth of contaminating mold (*Penicillium chrysogenum)* in bread exposed to different concentrations of clove leaf essential oil in vapor phase.

3.5. Sensory Evaluation of Semi-Wholemeal Bread

For the sensory evaluation, the panelists were asked to evaluate three samples: a control semi-wholemeal bread and bread treated with CLEO at 1250 µL/L_{air} and 1750 µL/L_{air}. The attributes to qualify were flavor, aroma, texture, and overall acceptability of the bread. The average scores are presented in Table 6. The sensory evaluation

results indicated that the control bread exhibited the highest values across all evaluated attributes, with average scores ranging from 6.80 to 7.57, corresponding to "moderately like" to "like very much". Texture did not show significant changes ($p > 0.05$) between treatments and remained within similar ranges (6.30–6.80), suggesting that the treatment did not affect the bread's structure. The incorporation of CLEO vapors tended to decrease sensory acceptance, particularly for flavor and aroma attributes, obtaining lower scores. This may be attributed to the characteristic aromatic intensity of compounds such as eugenol. Despite the antifungal efficacy of vapor phase CLEO, the primary constraint is its pronounced organoleptic impact. Phenylpropanoids such as eugenol exhibit a low human olfactory detection threshold, reported as <100 ppb in air (0.1 µL/L) [65]. Even when applied as a non-contact additive, the volatile saturation required to suppress mold development triggers a consumer perception of spicy, clove-like odor notes.

In food development, a threshold of AI ≈ 70% (corresponding to a mean hedonic score of ≈6, 'like slightly') is established as the baseline requirement for the successful new formulations [66,67]. Our sensory data reveal that while the untreated bread (control) exhibited the highest AI score (79.7%), the bread samples exposed to CLEO vapors maintained their organoleptic profiles near the commercial cutoff, achieving indexes of 72.6% (1250 µL/L_{air}) and 70.3% (1750 µL/L_{air}). This behavior indicates that the bran-derived flavor profile of the semi-wholemeal matrix helps attenuate the exogenous clove aromas. Regarding visual characteristics (color), the CLEO application in the vapor phase did not change the bread's crust or crumb color. The characteristic color of the semi-wholemeal bread remained consistent across samples, influenced only by the flour's bran pigments and thermal Maillard reactions. The CLEO MICs fulfilled their function of preventing fungal growth; however, the CLEO application to this baked goods product was not well received by the panelists.

In other studies, sensory evaluation revealed no differences between untreated and treated bread with thyme or clove EOs in vapor phase (500 µL/L_{air}) or direct addition (100–125 mg/kg, 15 ppm) at lower concentrations [52,59,68]. Bread exposed to 1000 µL/L_{air} of lemongrass EO in vapor phase did not modify the scores of sensory evaluations compared with untreated bread [27]. The compatibility between food's flavor and EO (direct addition or vapor phase) is critical in food sensory acceptance because odd-strong aromas/flavors typically induce the rejection of food. In this work, CLEO was expected to exhibit strong antifungal activity due to its components; however, high concentrations of CLEO were required to inhibit mold growth, resulting in less acceptable sensory attributes.

To further optimize these sensory parameters, advanced active packaging could be introduced. Recent innovations highlight that volatile release kinetics can be modulated through aroma-masking combinations or microencapsulation strategies, thereby restricting rapid headspace migration [17,19–21]. This controlled-release mechanism ensures that the minimum inhibitory concentration is continuously maintained at the crust interface to control mold spoilage while maintaining sensory attributes well above the critical 70% commercial acceptance threshold.

Table 6. Average scores of the sensory evaluation of the semi-wholemeal bread exposed or not to clove leaf essential oil vapor phase.

Sample	Flavor	Odor	Texture	Overall Acceptability	AI (%)
Control	7.57 ± 0.90 a	7.40 ± 1.00 a	6.80 ± 1.56 a	7.17 ± 0.83 a	79.7
1250 µL/L	6.70 ± 0.99 b	6.60 ± 1.77 ab	6.30 ± 1.09 a	6.53 ± 1.14 ab	72.6
1750 µL/L	6.47 ± 1.91 b	6.40 ± 1.65 b	6.73 ± 1.26 a	6.33 ± 1.24 b	70.3

AI = Acceptability Index. Different letters for the same attribute indicate significant differences ($p < 0.05$).

4. Conclusions

The study demonstrated CLEO's potential as an antifungal agent in semi-wholemeal bread. In vitro tests determined that the MIC of CLEO against *A. niger* was 300 µL/L_{air}, indicating the high sensitivity of this microorganism to the EO's volatile compounds. In bread, concentrations of 1250 µL/L_{air} were sufficient to inhibit the growth of *A. niger*, while a higher concentration (1750 µL/L of air) is needed to inhibit the growth of *P. chrysogenum* (the main contaminant mold of bread). In contrast, the treated bread scored lower for flavor, aroma, and overall acceptability than the untreated bread. This fact is attributed to the strong aroma of CLEO, dominated by the main components such as caryophyllene and eugenol, which can produce sensory notes that panelists perceive as intense and/or unfamiliar for bread. The scores (6.3–6.7) suggest that the treated bread remains within the moderate acceptance range. Overall, the results show a balance between antifungal effectiveness and sensory acceptability, with higher EO concentrations improving the bread's microbiological stability but tending to decrease its consumer acceptance. This suggests the need to optimize application conditions (concentration, exposure time, or combined strategies) to maximize shelf life without compromising the product's sensory quality.

Author Contributions

R.H.H.-F.: Conceptualization, Methodology, Data curation, Formal analysis, Writing—Reviewing and Editing; P.Y.S.-B.: Investigation, Methodology, Data curation, Formal analysis, Writing—Original draft preparation; A.L.-M.: Conceptualization, Data curation, Resources, Supervision, Writing—Reviewing and Editing. E.M.-L.: Methodology, Formal analysis, Data curation, Visualization, Writing—Original draft preparation, Writing—Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The Research and Ethics Committee on Sensory Evaluation of Foods of the doctoral program in Food Science of the Universidad de Las Américas Puebla approved the protocol for the sensory evaluation carried out in this work on 9 February 2026, SEDCL-2026/010.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the sensory evaluation. The panelists were informed about the use of wholemeal flour in bread formulations. Each participant signed an informed consent by responding affirmatively to the following statement: “I am aware that my responses are confidential, and I agree to participate in this sensory evaluation”. They were informed that they could withdraw from the test at any time without providing a reason; we explicitly stated that “The products are safe for consumption” and that participants’ data would not be disclosed without their knowledge.

Data Availability Statement

The datasets used during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

Artificial intelligence (AI) tools were used solely for basic grammar correction and language refinement in preparing this manuscript. Specifically, OpenAI’s ChatGPT and Grammarly were used to improve the readability and linguistic clarity of the English text. All scientific content, data interpretation, and conclusions were developed independently by the author. The authors have thoroughly reviewed and edited the AI-assisted text to ensure its accuracy and accept full responsibility for the manuscript's content.

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