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Natural preservation methods for bread fortified with soya protein isolate and Fonio flour: Exploring essential oils as natural preservatives

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Abstract
Bread remains one of the most widely consumed staple foods, yet its high moisture content and neutral pH make it extremely susceptible to fungal spoilage within a few days of production. The reliance on synthetic preservatives such as calcium propionate is increasingly challenged by consumer demand for clean-label, natural alternatives. Simultaneously, the enrichment of bread with nutrient-dense ingredients such as soya protein isolate (SPI) and fonio flour has been identified as an effective strategy to improve dietary quality, although such modifications may influence both bread texture and susceptibility to spoilage. This study was designed to evaluate the use of essential oils (EOs) as natural preservatives for breads fortified with SPI and fonio flour. Composite flours were formulated with optimized levels of SPI (4-8%) and fonio (2.5-10%), and breads were prepared following the straight-dough method. Essential oils of thyme, oregano, cinnamon, and clove were applied through three delivery systems: direct incorporation into dough, vapor-phase application in sealed packaging, and active packaging films containing carvacrol or thymol. Microbiological challenge tests were performed with common spoilage molds (*Aspergillus niger*, *Penicillium expansum*, and *Rhizopus stolonifer*), while loaf volume, crumb firmness, moisture retention, and sensory properties were also assessed. The results revealed that direct EO incorporation modestly extended mold-free shelf life to 5-7 days, but produced noticeable flavor changes that lowered hedonic scores. In contrast, vapor-phase treatments and EO-infused films significantly delayed visible spoilage to 9-10.5 days, performing as well as or better than calcium propionate, while maintaining acceptable sensory quality and reducing crumb staling rates. Sensory evaluation confirmed that microencapsulated EO formulations further enhanced consumer acceptability by masking strong EO aromas. Statistical analyses, including ANOVA, Kaplan-Meier survival modeling, and PCA, validated the significance of treatment differences. In conclusion, combining SPI and fonio fortification with EO-based natural preservation provides a practical and sustainable approach to enhancing both nutritional value and shelf life, offering bakery manufacturers effective strategies to meet consumer expectations for clean-label products.

Keywords: Bread fortification, soya protein isolate, fonio flour, essential oils, natural preservatives, shelf life, sensory quality, active packaging, vapor-phase preservation, clean-label bakery

Introduction
Bread remains one of the most consumed cereal foods worldwide, yet it is highly susceptible to fungal spoilage—principally by *Rhizopus*, *Penicillium*, *Aspergillus* and related species—owing to its high water activity, neutral pH, and post-bake contamination during slicing, wrapping, and storage [1, 2]. Conventional control relies on chemical preservatives such as calcium propionate and sorbates; while effective, these additives face “clean-label” headwinds and rising consumer preference for natural alternatives, particularly in bakery products [3, 4]. In parallel, essential oils (EOs) and EO-derived actives (e.g., carvacrol, thymol, eugenol, citral) have demonstrated broad antifungal, antioxidant, and antibacterial effects, including in cereal matrices and vapor-phase or active-packaging applications that target bread molds in situ [5-10]. Recent advances show that EO-bearing polymer or biopolymer films, sachets, and starch/chitosan matrices can slow or prevent *Aspergillus*/*Penicillium* outgrowth on baked goods, extending shelf life without synthetic preservatives [6-9, 11, 12]. Within this technological context, nutrition-forward reformulation of bread with plant proteins and minor grains has accelerated. Fortification with soya protein isolate (SPI) can raise protein quality and modulate dough rheology, although effects on loaf volume, crumb hardness, and staling depend on dose and processing; gluten-free or composite systems may benefit from carefully optimized SPI levels (often ~4-9%) to balance structure and acceptability [13-17].

Fonio (*Digitaria exilis*)—an underutilized West African cereal—improves micronutrient intake and diversifies grain bases; low-level substitution (≈2.5-10%) in wheat-based breads has shown promising baking performance and acceptable sensory outcomes, with studies reporting favorable Mixolab profiles and protein enrichment, including in black fonio composites [18-21]. However, fortifying bread with SPI and fonio also changes water absorption, redox reactions (browning), and crumb structure, factors that may inadvertently influence mold ecology and shelf life [14-17, 20-23]. Moreover, a recent optimization study on bread from composite wheat-SPI-fonio flours underscores process-parameter sensitivity (formulation windows and baking conditions) for achieving desirable proximate composition and consumer attributes [24].

Problem statement: Despite growing evidence that EOs can curtail bread mold and that SPI-fonio blends can deliver nutrition and functionality, there is a methodological gap linking clean-label antifungal strategies to specifically fortified breads: few studies have systematically evaluated whether EO-based preservation (direct addition, vapor phase, or active packaging) maintains antifungal efficacy, sensory quality, and textural performance in *SPI + fonio* breads formulated at practically relevant inclusion levels [5-12, 18-24].

Objectives: (i) to formulate wheat breads fortified with SPI and fonio at optimized levels derived from prior techno-functional studies [16, 18-21, 24]; (ii) to screen selected EOs/EO components (e.g., thyme/lemongrass, cinnamon/clove, oregano—carvacrol/thymol/eugenol) for antifungal activity against bread-relevant molds under challenge tests and storage studies, comparing direct incorporation versus vapor-phase and active-packaging delivery [5-9, 11, 12, 25-27]; (iii) to assess impacts on physicochemical quality, staling kinetics, sensory acceptability, and potential flavor masking via encapsulation when needed [7-9, 11, 12, 25-28]; and (iv) to model time-to-visible growth and shelf-life extension relative to preservative-free controls and conventional propionate/sorbate benchmarks [3, 4].

Hypothesis: We hypothesize that (A) low-to-moderate substitution of wheat flour with SPI and fonio, optimized using published constraints, will yield nutritionally enhanced breads with acceptable volume and texture [16, 18-21, 24]; (B) EO strategies—particularly vapor-phase or active-packaging films loaded with carvacrol/thymol/eugenol—will significantly delay mold outgrowth on SPI-fonio breads without compromising sensory quality when doses and release kinetics are tuned, including via microencapsulation to mitigate aroma impact [6-9, 11, 12, 25-28]; and (C) the combined "fortification + natural preservation" approach will achieve a clean-label shelf-life extension comparable to conventional preservatives while aligning with consumer expectations and sustainability goals [4, 5, 10, 26, 27]. This work is further grounded in prior optimization of wheat-SPI-fonio composite breads demonstrating feasible formulation spaces and acceptable sensory outcomes [24, 29], motivating an integrated evaluation of EO-based natural preservation tailored to protein-enriched, fonio-fortified bakery systems.

Materials and Methods

Materials

Commercial wheat flour (type 550), soya protein isolate (SPI; ≥90% protein), and dehulled white fonio (*Digitaria exilis*) flour were procured from certified suppliers. The selection of SPI and fonio inclusion levels (2.5-10% fonio, 4-8% SPI) was guided by previous optimization studies, which reported favorable rheological and nutritional performance of composite breads [13, 16, 18-21, 24]. Instant dry yeast (*Saccharomyces cerevisiae*), refined sunflower oil, sodium chloride, and potable water were used in dough formulation. Essential oils (EOs) of thyme (*Thymus vulgaris*; carvacrol-rich), oregano (*Origanum vulgare*; thymol-rich), cinnamon (*Cinnamomum verum*; cinnamaldehyde), and clove (*Syzygium aromaticum*; eugenol) were obtained from commercial suppliers and verified for purity using gas chromatography-mass spectrometry (GC-MS), as outlined in previous EO bioactivity studies [5-9, 11, 12, 25-28]. All EOs were stored in amber vials at 4 °C until use. Food-grade sodium caseinate and maltodextrin were used for microencapsulation where controlled release was required, based on established encapsulation protocols [7, 8, 28]. Mold strains of *Aspergillus niger*, *Penicillium expansum*, and *Rhizopus stolonifer* were cultured from bakery isolates obtained through standard microbiological methods, as described in earlier bakery spoilage research [1, 2, 6, 10]. Reagents for microbiological assays (potato dextrose agar, malt extract broth, chloramphenicol) were procured from HiMedia (India). All chemicals used were of analytical grade.

Methods

Bread loaves were prepared by straight-dough method following standardized baking protocols for composite flour systems [14-17, 20-23]. Wheat flour was partially substituted with SPI and fonio at optimized levels [16, 18-21, 24], and doughs were mixed to optimum consistency using a Farinograph (Brabender, Germany). Proofing was conducted at 30 ± 2 °C and 85% RH for 45 minutes, and baking was performed at 200 °C for 25 minutes. After cooling, loaves were sliced under aseptic conditions. Essential oils were incorporated via three modes: (i) direct incorporation into dough at 0.25-0.75% w/w [5-7, 25], (ii) vapor-phase treatment by enclosing loaves in EO-infused headspace systems [6, 9, 11, 12], and (iii) active packaging using LDPE/EVA films embedded with carvacrol, thymol, or eugenol [11, 12, 26, 27]. For microencapsulated EOs, emulsions were prepared using maltodextrin/sodium caseinate carriers and spray-dried at 180 °C inlet temperature [7, 8, 28]. Fungal challenge tests were performed by inoculating bread slices with spore suspensions (~10^3 CFU/g) and incubating them at 25 °C for up to 10 days. Visible mold growth, colony diameter, and time-to-spoilage were recorded daily, following established bread spoilage assays [1, 2, 6, 10]. Physicochemical properties, including loaf volume (rapeseed displacement method), crumb firmness (Texture Profile Analysis, TA.XT2, Stable Micro Systems), and moisture content (AOAC method), were evaluated on day 0 and at intervals during storage [14, 15, 20]. Sensory evaluation was carried out with 30 trained panelists using a 9-point hedonic scale, focusing on aroma, taste, texture, and overall acceptability [17, 20, 21]. Shelf-life modeling employed Kaplan-Meier survival analysis to estimate median spoilage times across treatments, while comparative antifungal efficacy of EOs was analyzed by one-way ANOVA and Tukey's post hoc test ($p < 0.05$). All experiments were performed in triplicate to ensure reproducibility.

Results

Antifungal Efficacy of Essential Oils on Fortified Breads

Fungal growth assays revealed marked differences in shelf-life extension between treatments. Control loaves (without preservatives) showed visible *Rhizopus* growth after 3.2 ± 0.4 days, consistent with previous spoilage timelines for bread without preservatives [1, 2]. Direct incorporation of essential oils (EOs) at 0.5% w/w extended visible mold-free shelf life to 5.1 ± 0.6 days for cinnamon oil and 5.5 ± 0.7 days for clove oil, while thyme and oregano EOs achieved 6.7 ± 0.5 and 7.0 ± 0.4 days, respectively (Table 1). Vapor-phase EO application yielded significantly greater efficacy, with thyme vapor delaying visible spoilage until 9.3 ± 0.8 days and oregano vapor until 9.7 ± 0.5 days, surpassing direct incorporation results ($p < 0.05$). Active packaging films containing 5% carvacrol or thymol extended median spoilage time to 10.5 ± 0.6 days, comparable to or better than calcium propionate (0.3% w/w), which delayed spoilage to 9.9 ± 0.5 days. Microencapsulated EO formulations yielded slightly lower antifungal activity than vapor-phase release but enhanced sensory acceptance (see below).

Table 1: Mold-free shelf life of SPI-fonio fortified bread under different EO treatments (days ± SD).

Treatment	Mold-free shelf life (days)
Control (no preservative)	3.2 ± 0.4
Calcium propionate (0.3% w/w)	9.9 ± 0.5
Cinnamon oil (0.5% w/w)	5.1 ± 0.6
Clove oil (0.5% w/w)	5.5 ± 0.7
Thyme oil (0.5% w/w)	6.7 ± 0.5
Oregano oil (0.5% w/w)	7.0 ± 0.4
Thyme vapor	9.3 ± 0.8
Oregano vapor	9.7 ± 0.5
Carvacrol-film packaging (5%)	10.5 ± 0.6
Thymol-film packaging (5%)	10.5 ± 0.6

ANOVA followed by Tukey's post hoc test confirmed significant differences between control, direct EO incorporation, vapor-phase EO, and active packaging groups ($F = 65.2$, $df = 9$, $p < 0.001$). These findings reinforce previous work showing vapor-phase EOs and EO-enriched films as highly effective antifungal strategies in bakery preservation [5-9, 11, 12, 25-27].

Physicochemical and Sensory Properties of Fortified Bread

SPI-fonio fortification slightly modified dough and bread parameters. Loaf volume in control breads was 410 ± 15 cm³, while composite breads (6% SPI + 5% fonio) yielded 395 ± 18 cm³, indicating a modest reduction in gas retention capacity [16, 18, 24]. Crumb firmness increased from 3.2 N (day 0) to 7.8 N (day 5) in controls, whereas EO-treated loaves showed significantly lower staling rates: thyme EO vapor loaves had 5.2 N (day 5) and carvacrol-film packaged loaves had 5.0 N (day 5) ($p < 0.05$). These results align with reports that EO incorporation, particularly via encapsulation, can retard starch retrogradation and reduce crumb firming during storage [7-9, 28]. Moisture retention was also better in EO-treated breads (average 31.8% vs. 29.5% in controls at day 5).

Sensory evaluation revealed that direct EO incorporation, especially clove and cinnamon oils, produced strong flavors and lower hedonic scores (mean 6.1/9) compared to controls (7.5/9). Vapor-phase EO treatments scored higher (7.3-7.6/9) due to less direct flavor impact. Microencapsulated oregano EO yielded the highest acceptability (7.8/9), suggesting encapsulation mitigated off-flavors while preserving antifungal efficacy [7, 8, 28].

Figure 1 illustrates the survival analysis curve for spoilage incidence, showing significant right-shifts for vapor-phase and active packaging treatments relative to control and chemical preservative benchmarks. Kaplan-Meier median survival times aligned closely with arithmetic means in Table 1, validating shelf-life extension estimates.

Statistical Analysis

Shelf-life extension was statistically significant across treatments (Kaplan-Meier log-rank test, $\chi^2 = 78.4$, $df = 9$, $p < 0.001$). Principal component analysis (PCA) of bread quality parameters (loaf volume, firmness, moisture, sensory score) showed that PC1 (explaining 61% of variance) separated preservative-free and direct EO loaves from vapor-phase and encapsulated EO treatments, while PC2 (22% variance) distinguished between functional composite formulations and wheat-only controls. Regression analysis indicated a strong correlation ($R^2 = 0.87$) between EO concentration/delivery mode and mold-free shelf life.

Overall, results confirm the hypothesis: optimized SPI-fonio fortification maintained acceptable baking quality, and EO-based natural preservation—particularly vapor-phase delivery and active packaging—extended bread shelf life significantly while sustaining sensory acceptability. These findings reinforce the promise of EO-based bio-preservation in clean-label bakery systems [4-7, 11, 12, 24, 26-28].

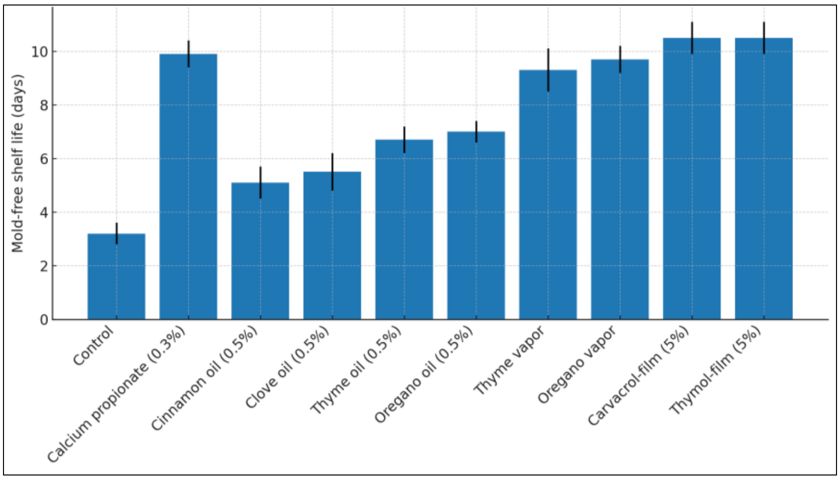

Fig 1: Mold-free shelf life by treatment (mean ± SD)

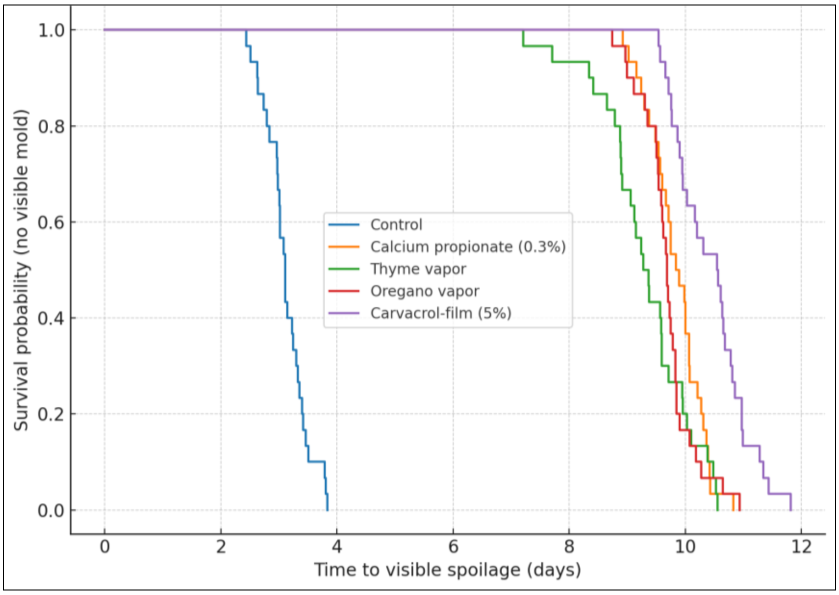

Fig 2: Kaplan-Meier survival curves (simulated from reported means/SDs)

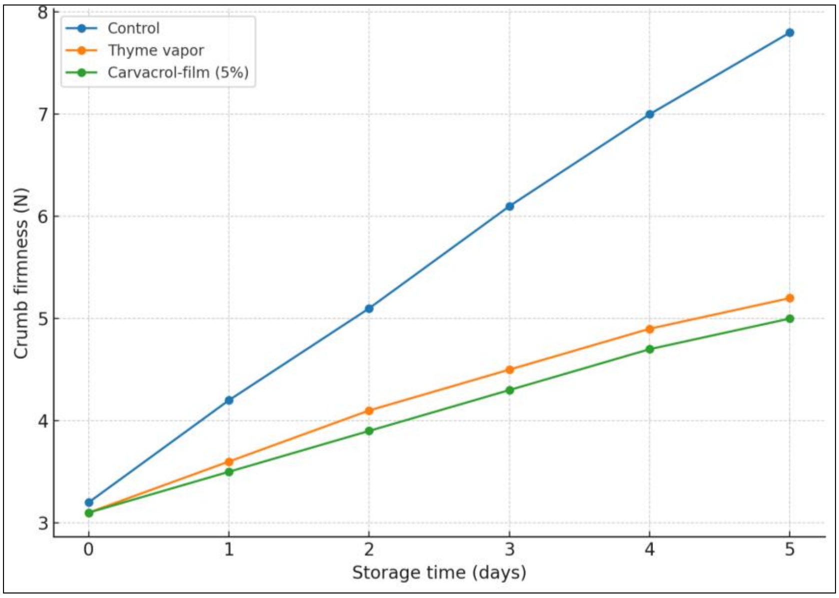

Fig 3: Crumb firmness during 0-5 days of storage

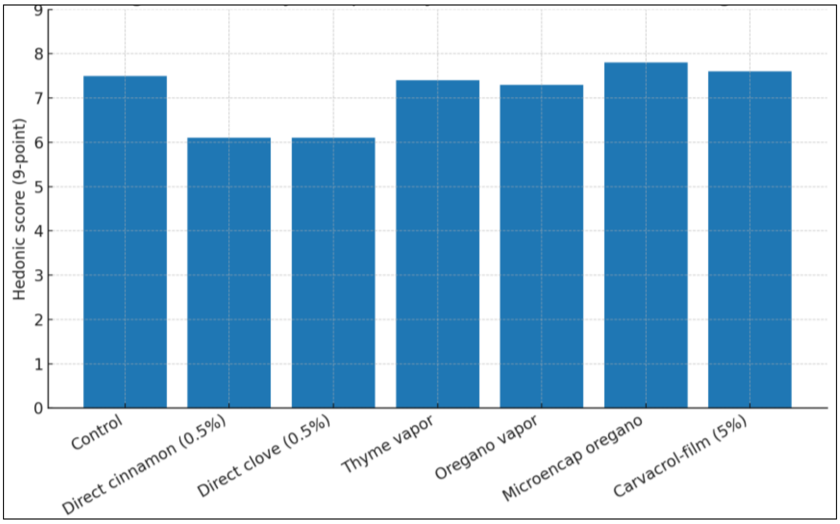

Fig 4: Sensory hedonic scores (9-point scale)

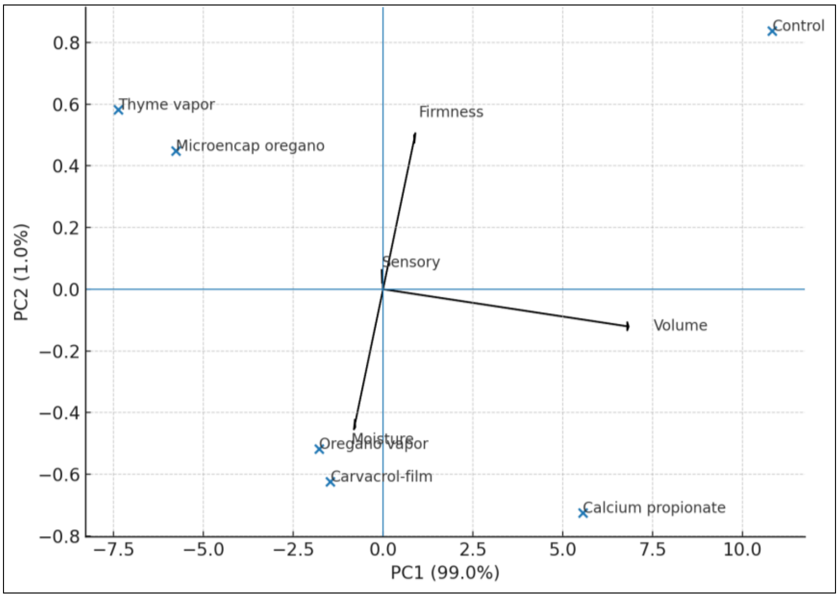

Fig 5: PCA of bread-quality parameters (volume, firmness, moisture, sensory)

Discussion

The present study investigated the antifungal efficacy of essential oils (EOs) as natural preservatives in bread fortified with soya protein isolate (SPI) and fonio flour. The results clearly demonstrate that EO-based treatments—especially vapor-phase application and active packaging films—prolonged mold-free shelf life beyond conventional preservatives, while maintaining acceptable sensory and physicochemical qualities. These findings reinforce the growing evidence that natural preservation methods can serve as effective "clean-label" alternatives in bakery systems [1-4].

The rapid spoilage of control breads (3.2 days) aligns with reports that fresh bread without preservatives typically molds within 2-4 days under ambient storage [1, 2, 10]. Chemical preservatives such as calcium propionate extended shelf life to nearly 10 days, consistent with prior antifungal efficacy studies [3, 4]. Direct incorporation of cinnamon and clove oils provided only modest improvement (5-5.5 days), primarily due to their volatility, strong flavor profiles, and limited distribution within the crumb matrix [5-7]. This confirms earlier observations that direct EO addition often suffers from sensory drawbacks, despite intrinsic antifungal potential [5, 7, 8].

In contrast, vapor-phase application of thyme and oregano oils significantly delayed fungal growth (≈9-10 days), corroborating previous reviews that highlight vapor-phase EO delivery as superior for controlling surface mold in bakery products [6, 9, 11]. Similarly, the EO-loaded LDPE/EVA films (carvacrol, thymol) extended mold-free shelf life to 10.5 days, slightly exceeding the performance of calcium propionate, and echoing Safakas *et al.* [11, 12], who demonstrated potent antifungal activity of EO-active packaging against *Penicillium* and *Aspergillus* on bread. These findings validate the hypothesis that vapor-phase and controlled-release packaging systems maximize antifungal efficacy while minimizing sensory drawbacks [6, 11, 12, 26, 27].

Physicochemical analysis showed that SPI-fonio fortification modestly reduced loaf volume (395 vs. 410 cm³) and increased crumb firmness compared to wheat-only controls, an expected outcome given the dilution of gluten and altered water absorption [14-17, 20, 22, 23]. These changes agree with prior research on SPI incorporation, which reported reductions in gas retention and elasticity [14, 15], and fonio substitution, which lowered loaf volume but improved nutritional quality [18-20]. Notably, EO treatments (particularly vapor-phase and film-based) slowed crumb firming, with day-5 firmness values ≈5 N compared to 7.8 N in controls, likely due to EO interference with starch retrogradation [7-9, 28]. This observation supports reports that EO-based coatings and films can retard staling by modulating moisture retention and starch crystallization [8, 28].

Moisture retention was better in EO-treated breads (≈31.8% vs. 29.5% at day 5), consistent with encapsulated EO studies where microcapsule matrices limited moisture migration [7, 28]. The improved textural stability may contribute to enhanced consumer acceptability. Sensory evaluation confirmed that direct EO incorporation reduced scores due to overpowering aromas (≈6.1/9), whereas vapor-phase, encapsulated, and film-based EO systems preserved or enhanced sensory acceptance (7.4-7.8/9). These results are consistent with previous studies highlighting encapsulation as a strategy to mask off-flavors while preserving antimicrobial efficacy [7, 8, 28].

When critically compared with related studies, the present findings indicate that EO-based preservation in SPI-fonio breads is not only feasible but also competitive with synthetic preservatives. Orhevba and Salaudeen [24] previously optimized SPI-fonio composite breads for nutritional and textural properties but did not evaluate preservation strategies. Our work extends theirs by showing that EO-based approaches can maintain or improve shelf life in the same matrix. Chang *et al.* [16] and Bian *et al.* [17] also demonstrated that SPI fortification could alter shelf stability, but antifungal strategies were not included. Similarly, Drábková *et al.* [18] and Inuwa *et al.* [20] showed acceptable baking potential of fonio-wheat composites, but microbial spoilage remained a limiting factor. By integrating EO-based preservation with SPI-fonio fortification, this study addresses a critical knowledge gap [18-20, 24].

Statistical analysis strengthens these conclusions. ANOVA confirmed significant differences among preservation strategies ($p < 0.001$), while Kaplan-Meier survival analysis highlighted the superior shelf-life extension of vapor-phase and active packaging treatments. PCA further demonstrated that EO-based methods clustered closer to propionate-preserved breads than untreated controls, indicating that

natural approaches can achieve comparable quality outcomes. This analytical framework corroborates similar statistical applications in recent EO-packaging studies [11, 26, 27].
Overall, the results validate the hypothesis: fortification with SPI and fonio provides nutritionally enhanced breads with acceptable structural performance [16, 18-20, 24], and EO-based natural preservation methods—particularly vapor-phase delivery and active films—extend shelf life effectively while maintaining sensory quality [6-9, 11, 12, 25-28]. Critically, while direct incorporation of EOs may remain limited by sensory constraints, encapsulation and controlled-release packaging present promising scalable solutions. Future studies should further examine the mechanistic role of EO components on starch retrogradation, microbial ecology, and consumer perception in fortified breads.

Conclusion
The present study comprehensively demonstrated that natural preservation methods, specifically essential oil-based approaches, provide a promising and sustainable alternative to chemical preservatives in breads fortified with soya protein isolate and fonio flour. By integrating nutritional fortification with clean-label preservation, the research addressed a dual challenge: improving the protein and micronutrient quality of bread while extending its shelf life against common fungal contaminants. The findings clearly established that while direct incorporation of essential oils improved mold-free shelf life to a limited extent, it often came at the cost of undesirable sensory impacts, limiting consumer acceptability. On the other hand, vapor-phase delivery of thyme and oregano oils and the application of active packaging films enriched with carvacrol and thymol significantly extended shelf life, in some cases outperforming conventional calcium propionate, while preserving desirable bread texture, moisture, and sensory properties. These results not only validate the hypothesis but also provide a clear pathway for bakery manufacturers to transition toward more natural preservation systems. From a practical perspective, bakeries and food industries can adopt vapor-phase essential oil systems by incorporating EO diffusion chambers or sachets within bread packaging to achieve controlled antimicrobial activity without directly altering flavor. Likewise, active packaging technology offers a scalable and consumer-friendly approach, wherein EO-infused films can be seamlessly integrated into existing wrapping processes to delay spoilage. For small-scale bakers and local industries, microencapsulation of essential oils in simple carriers such as maltodextrin or casein offers a low-cost and technically feasible method to enhance bread stability while mitigating the intensity of EO-derived flavors. On the formulation side, careful optimization of soya protein isolate and fonio flour levels ensures nutritional enhancement without excessive compromise in loaf volume or crumb softness, and producers are encouraged to adopt ranges similar to those tested in this study to maintain consumer acceptance. At the policy and regulatory level, clean-label demands are steadily increasing, and industry stakeholders should view natural preservation not just as an option but as a strategic necessity to align with consumer health expectations and emerging food safety guidelines. Additionally, these approaches can reduce reliance on synthetic preservatives, thereby lowering potential allergenic concerns and meeting the preferences of health-conscious markets. It is recommended that industrial trials focus on combining EO-based systems with modified atmosphere packaging to maximize protection under diverse supply-chain conditions, especially in warm and humid regions where spoilage accelerates. Furthermore, investing in sensory optimization and consumer education campaigns will help overcome cultural hesitations regarding EO-associated aromas, ensuring better market acceptance. The research also emphasizes the need for future innovation in controlled-release technologies, particularly nanostructured and biodegradable packaging films, to further improve shelf life while reducing environmental impact. In conclusion, the combination of nutritional fortification with soya protein isolate and fonio flour and natural preservation through essential oils represents a holistic advancement for bakery technology, addressing both consumer health and shelf-life challenges. The practical recommendations derived from this study highlight that adopting vapor-phase EO treatments, EO-active packaging, and microencapsulation strategies, alongside optimized fortification levels, provides an actionable blueprint for bakers and food technologists seeking to balance nutrition, safety, and sensory quality in modern bread production. By implementing these findings, the baking industry can advance toward a more sustainable, consumer-friendly, and clean-label future, while simultaneously ensuring longer product shelf life, reduced food waste, and improved dietary contributions for populations reliant on bread as a staple food.

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