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FROM RESEARCH TO ENTERPRISES: MODELS OF SCALE-UP IN FOOD SCIENCE

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Summary (English)

This doctoral thesis is related to the Mission 4, Component 2 "M4C2" of the National Recovery and Resilience Plan, which aims to support investments in research and development, to promote innovation and its transfer to food companies. The latter represent the largest manufacturing sector worldwide, facing global challenges, such as the increase of non-communicable diseases (NCDs), pollution and waste, climate change and population growth. Innovation in products, technologies and services has a pivotal role in maintaining food companies competitive and able to pursue the Sustainable Development Goals of the 2030 Agenda. Innovation, indeed, is widely accepted as one of the keys to being successful. Usually, universities or research centres drive innovation by generating scientific breakthroughs; however, their outputs are often confined to laboratory scale, resulting in minimal influence on the industrial food system. Therefore, translating these scientific outcomes into practical applications (scale-up) is essential to achieve innovation in the food sector. Collaboration between food companies and universities is essential, as research centres play an important role in the advancement of a product or a technology, from basic research, up to a laboratory scale validation. Subsequently, food companies validate and demonstrate innovations in a relevant environment, progressing towards the commercialization of the products.

This PhD project therefore analyses and validates models of technology transfer and scale-up in the food industry, demonstrating how scientific innovations proposed by research can be effectively translated from the laboratory to industrial practice and validation. The thesis is composed of three chapters, each presenting one or more case studies exploring different models of scale-up and prototypes of food innovative solutions, which could be easily transferred on an industrial scale. Moreover, each chapter addresses global challenges, including pollution and waste, the rising prevalence of NCDs and concerns related to food safety. Specifically, Chapter I – Innovation in fresh pasta formulation and shelf-life extension: a strategy to promote healthier lifestyles and reduce food waste; Chapter II – Innovation in the formulation of traditional foods: a strategy to address non-communicable diseases; Chapter III – Innovation in craft beer production: a strategic approach to address food safety concerns. Although focused on different case studies, the three chapters are linked by a common Technology Readiness Level approach (a scale used to assess the maturity of a process), covering different stages of technology transfer and scale-up in the food sector. What follows is a brief overview of the content of the subsequent chapters.

The first chapter dealt with several societal challenges, such as: i) the reduction of pollution and waste, upcycling a by-product, useful to improve the quality of fresh pasta during storage; ii) the prevention of NCDs, producing a food product with higher antioxidant activity. With consumers generating approximately 60% of food waste along the food chain in industrialized nations, the responsibility of food companies in waste prevention becomes pivotal. Employing products, process

and packaging innovation could be a valuable strategy for food companies to address pollution and waste challenges, while pursuing the 12th Sustainable Development Goal of the 2030 Agenda, which aims to reduce food waste by 50% by 2030. Designing foods with longer shelf-life, indeed, gives consumer more time to plan their consumption, thereby reducing product return and food waste. Considering that the household storage accounts for more than 12% of total pasta losses, it is important to develop strategies aimed at extending its shelf-life. The use of natural bioactive compounds with antimicrobial and antioxidant properties, recovered from by-products, has gained considerable attention from scientific researchers, as a consequence of increasing consumer demand for more natural and high-quality foods. Among bioactive compounds, polyphenols stand out not only for their role as natural preservatives, but also for their beneficial effects on consumers health. Indeed, fortifying staple foods, such as pasta, with bioactive compounds offers a promising strategy to encourage healthier diets, without changing consumers' eating habits. Among vegetable by-products, olive pomace (OP) is a valuable source of phenolic compounds with antimicrobial and biological properties. Thus, transforming OP into a valuable resource reduces the environmental footprint of olive oil production and creates economic opportunities, as high added value foods can be designed at laboratory scale and validated on a relevant environment, showing that innovation depends on the collaboration among researchers and companies.

As several authors tested OP as natural preservatives and for food functionalization at laboratory scale, the aim of this first chapter of the thesis was to validate and demonstrate at industrial level the production of fresh pasta with higher antioxidant activity and improved quality during storage, supporting food companies in efforts to reduce food waste, pollution and promote healthier lifestyle. Therefore, fresh pasta samples were produced at industrial scale, using phenolic olive pomace extract (OPE) as such (F-OPE) and in its microencapsulated form (M-OPE), as source of antioxidants and antimicrobial compounds. Samples were characterized immediately after production and subsequently stored at 4°C. Further analyses (microbiological and physicochemical) were performed at regular time intervals from the time of production, up to 120 days of storage. Results showed that microencapsulation preserved total phenolic compounds (TPC) during pasta manufacturing, as just after production, fresh pasta samples with M-OPE had higher TPC and antioxidant activity, compared to the corresponding samples with F-OPE. However, especially at higher concentrations, M-OPE led to a partial loss of the gluten matrix, causing higher losses of TPC during cooking. However, cooked pasta samples with M-OPE had higher TPC and antioxidant activity. OPE reduced moisture, a_w and pH compared to the control (CP). Moisture and a_w showed a decreasing trend during storage, as a consequence of water release into the surrounding atmosphere. Oxidation led to a reduction of TPC and antioxidant activity during storage, which also affected volatile profile, particularly for CP and for the sample with the highest F-OPE content. As regards colour, OPE caused an increase of *a** values, whereas a reduction of *L** and *b** values, was observed increasing OPE content in pasta formulation. A decreasing trend of *b** values was observed during storage; however, samples with M-OPE had higher *b** values (appreciated by consumers), compared to the corresponding samples with F-OPE. Notably, OPE reduced growth of spoilage microbes, especially moulds, yeast and total aerobic mesophilic bacteria, showing its potential to extend the shelf-life of fresh pasta.

In conclusion, the industrial validation of fresh pasta fortified with OPE demonstrated the feasibility of upscaling natural antioxidant and antimicrobial solutions, offering a sustainable strategy in improving total phenolic compounds, antioxidant activity and fresh pasta microbiological quality during storage, reducing food waste and promoting healthier diet.

The potential of preventing NCDs through healthier food products was the cornerstone of the second chapter of this thesis. NCDs, representing a global public health challenge, account for about 38 million deaths each year. Cardiovascular diseases (CVDs) are one of the most prominent NCDs, associated with unhealthy diet, rich in saturated fats. Therefore, efforts are necessary to reduce the content of highly unsaturated fatty acids in food products, in order to pursue the 3rd Sustainable Development Goal of the 2030 Agenda, of reducing NCD-related deaths by a third by 2030. Cheese bread (*pão de queijo)* is a gluten-free traditional Brazilian bread, currently available on foreign markets. It is commonly consumed at multiple times during the day. However, it is produced with cassava starch, salt, oil, cheese and eggs, resulting in a product rich in saturated fatty acids and with a low content of dietary fibre. These findings highlight the need to improve the nutritional profile of this traditional bread, that is widely consumed and could be associated with increased risk of CVDs for consumers. Considering that this product is already obtained at an industrial scale, there is a clear necessity to find solutions that can be easily adapted to a large-scale production. Hence, in collaboration with the Nutrition Institute of Rio de Janeiro State University, experimental cheese breads with improved nutritional profile were produced on a small scale, substituting cheese with avocado pulp and emulsion-filled gel (EFG), produced with inulin and olive oil. This research assessed the effects of these alternative ingredients on physicochemical, nutritional and rheological properties of cheese breads. Moreover, a consumer test was performed, to evaluate the sensory perception of the products. Results from rheological analysis proved that doughs had a dominant elastic-like behaviour and shear-thinning properties, pivotal for sensory perception and for food processing applications. Specifically, as the doughs become less viscous under shear stress, less mechanical force is required during mixing and pumping operations, thereby decreasing overall energy consumption. Temperature sweep analysis showed that control sample and formulation with partial cheese replacement with avocado pulp exhibited a decreasing trend of dynamic moduli, as a consequence of fat melting. A subsequent increase of storage and loss modulus was related to starch gelatinization and protein denaturation. As expected, plant-based ingredients increased fibre concentration and diminished fat content. As a consequence, an improvement of fatty acid composition was observed in experimental samples. Interestingly, alternative formulations were characterised by lower atherogenic and thrombogenic index, and higher level of PUFA/SFA ratio, highlighting their beneficial effects on cardiovascular health. Breads with partial substitution of cheese with avocado pulp and EFG had higher springiness and lower chewiness values, properties preferred by consumers. Check All That Apply (CATA) results obtained in this research suggested that including avocado and structured lipid systems altered the sensory characteristics of the product, resulting in formulations with plant-based attributes. Although the traditional formulation remains the most widely accepted, the preference mapping results suggest that selected plant-based and functional formulations may appeal to a subset of consumers, indicating opportunities for further consumer-oriented research and providing a solid starting point for product improvement.

On the whole, this study was limited to laboratory-scale production, while pilot- and industrial-scale validation are necessary steps for future development. These plant-based ingredients, indeed, could be easily adapted to a large-scale production and therefore, future assessments should produce these innovative formulations with an automatic *pão de queijo* forming machine, which involves kneading, forming and possible filling phases, validating and demonstrating the innovation in a relevant environment. Furthermore, for large-scale production of emulsion-filled gels, it is essential for companies to conduct trials using turboemulsifiers. These apparatuses employ counter-rotating blades alongside homogenizing turbines to generate stable and high-quality emulsions. Such equipment is particularly well-suited for the food sector and specifically for manufacture of gels, creams, and sauces.

The third chapter of this thesis dealt with food safety, which represents a global public health challenge, contributing to 600 million cases of foodborne diseases and 420,000 deaths every year. The most significant health concerns are related to foodborne intoxication, caused by natural occurring toxins, such as biogenic amines (BAs), toxic compounds mainly produced by decarboxylation of free amino acids. These compounds could induce digestive, circulatory and respiratory disorders, which could be enhanced by alcohol. These findings highlight the need to investigate on the content of BAs in alcoholic beverages, such as craft beers, in order to propose strategies, effective for the reduction of these toxic molecules. Such strategies should be validated and demonstrated on an industrial scale, aligning with the 2nd objectives of the 2030 Agenda, whose purpose is to guarantee universal access to food that is safe, nutritious, and sufficient for every individual. This research activity, in collaboration with the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, began with the investigation of BAs in beers produced in Italian and Romanian microbreweries, to collect data and identify critical steps in BAs accumulation. Proposing corrective actions to be applied during the brewing process, the objective was to produce craft beers that do not pose any health risks to all types of consumers. Although the industrial validation phase was not completed due to time constraints, the research identified key control points in BAs accumulation, laying the groundwork for future scale-up strategies, to enhance the safety of craft beers. Results showed that tyramine, tryptamine, putrescine and cadaverine were the main BAs detected in craft beers. Despite histamine was found in low concentrations, 86% of the samples had histamine content above the detectable level, representing a health hazard for people with histamine intolerance. Craft beers consumption in combination with other fermented foods should be carefully considered by susceptible consumers, as 35% of the samples analysed had a total BAs content higher than the limit suggested by literature for this type of consumers (20 mg/L). Healthy individuals face a lower risk, because only 10% of beer products had a total BAs level higher than 50 mg/L, regarded as harmful for this type of consumer. Moreover, results highlighted that some practices applied in the production of craft beers could pose a high food safety risk. The selection of raw materials, especially unconventional ingredients, malt baking, mashing, fermentation process and the selection of starter cultures and generally hygiene conditions have been identified as critical points for the accumulation of BAs in craft beers. Future research should test, validate and demonstrate strategies for BAs control and reduction at laboratory level and subsequently on industrial scale.

In conclusion, this doctoral thesis demonstrates the potential of scaling-up food innovations from laboratory to industrial environments, through collaborative research-industry models and illustrates innovative food prototypes which could be easily transferred to food companies. By addressing sustainability, nutrition, and safety, the case studies illustrate how scientific research can be translated into tangible industrial outcomes, contributing to achieve innovation in the food sector, bridging the gap between basic research and industrial validation and supporting the goals of the 2030 Agenda.

Summary (Italian)

Tesi di dottorato in Scienze del Suolo e degli Alimenti

Dalla ricerca all'impresa: modelli di scale-up in ambito food science

Questa tesi di dottorato è in linea con l'obiettivo "M4C2" del Piano Nazionale Ripresa e Resilienza Italia, che prevede di sostenere gli investimenti in ricerca e sviluppo, di promuovere le innovazioni e il loro trasferimento alle aziende alimentari. Quest'ultime costituiscono il più grande settore manifatturiero a livello mondiale ed affrontano diverse sfide, quali l'incremento delle malattie croniche non trasmissibili, l'inquinamento e l'aumento degli scarti alimentari, i cambiamenti climatici e la crescita della popolazione mondiale. L'innovazione nelle tecnologie, nei prodotti e nei servizi ha un ruolo fondamentale nel fare in modo che le aziende alimentari siano competitive, sostenibili e capaci di raggiungere gli obiettivi dell'agenda 2030. L'innovazione, infatti, è accettata come un punto chiave per raggiungere il successo. Generalmente, le università o i centri di ricerca sono motori di innovazione, grazie alle loro scoperte scientifiche; tuttavia, i risultati prodotti restano spesso confinati al solo ambito laboratoriale, incidendo poco sul sistema industriale alimentare. Per questo, il trasferimento di queste scoperte scientifiche in contesti pratici (scale-up), è necessario per raggiungere l'innovazione nel settore alimentare. La collaborazione tra le aziende del settore alimentare e l'università è necessaria, poiché i centri di ricerca hanno un ruolo importante nell'avanzamento dell'innovazione, dalla ricerca di base, fino alla validazione a livello laboratoriale. Successivamente, le aziende validano e dimostrano le innovazioni in un contesto di riferimento. Pertanto, questa tesi di dottorato si propone di analizzare e validare modelli di trasferimento tecnologico e di scale-up nel settore alimentare, dimostrando come le innovazioni scientifiche proposte dalla ricerca possano essere efficacemente traslate dalla scala di laboratorio, alla pratica industriale.

Questa tesi è divisa in tre capitoli, ciascuno dei quali presenta casi studio che riguardano modelli di scale-up o prototipi sperimentali di innovazioni alimentari orientati alla scalabilità reale, e facilmente trasferibili e applicabili su larga scala. Ciascun capitolo, inoltre, affronta sfide di interesse globale, quali, gli sprechi alimentari, l'aumento di malattie non trasmissibili (NCDs) e preoccupazioni riguardo la sicurezza alimentare. In particolare: Capitolo I- Innovazione nella formulazione di pasta fresca e nell'estensione della shelf-life, come strategia per promuovere alimenti più sani e ridurre gli sprechi alimentari; Capitolo II- Innovazione nella formulazione di alimenti tradizionali, per fronteggiare la sfida globale delle malattie croniche non trasmissibili; Capitolo III- Innovazione nella produzione di birra artigianale, per mitigare preoccupazioni relative alla sicurezza alimentare. Pur riguardando casi studio differenti, i tre capitoli sono collegati da un comune approccio basato sui Technology Readiness Levels (una scala utilizzata per valutare il livello di maturità di una tecnologia) e descrivono un percorso coerente dal laboratorio all'applicazione industriale, attraverso il

trasferimento tecnologico e lo scale-up. Ciò che segue fornisce una panoramica dei contenuti dei capitoli successivi.

Il primo capitolo ha affrontato diverse sfide di rilevanza sociale, tra le quali: i) la riduzione dell'inquinamento e degli sprechi, mediante la valorizzazione di un sottoprodotto, funzionale al miglioramento della qualità della pasta fresca durante la conservazione; ii) la prevenzione delle malattie croniche non trasmissibili, attraverso lo sviluppo di un prodotto alimentare caratterizzato da una maggiore attività antiossidante.

Poiché i consumatori sono responsabili di circa il 60% degli sprechi alimentari lungo la filiera nelle nazioni industrializzate, la responsabilità delle aziende alimentari nella prevenzione degli sprechi diventa cruciale. L'impiego di innovazioni nei prodotti, nei processi e nel packaging potrebbe rappresentare una strategia preziosa per le aziende alimentari per far fronte a sfide globali, quali lo spreco alimentare e l'inquinamento, in linea con il dodicesimo obiettivo dell'Agenda 2030, che prevede di dimezzare gli sprechi alimentari entro il 2030. Progettare alimenti con una shelf-life più lunga offre, infatti, ai consumatori più tempo per organizzarsi e consumarli, evitando lo spreco alimentare. Considerando che i consumatori sono responsabili di più del 12% delle perdite totali di pasta, è importante sviluppare strategie volte a migliorarne la qualità durante la conservazione. L'uso di composti bioattivi naturali con proprietà antimicrobiche ed antiossidanti, recuperati da sottoprodotti agro-alimentari, ha attirato notevole attenzione nella comunità scientifica, in risposta alla crescente domanda da parte dei consumatori di alimenti più naturali e di alta qualità. Tra i composti bioattivi, i polifenoli si distinguono non solo per il loro ruolo come conservanti naturali, ma anche per i loro comprovati effetti benefici sulla salute dei consumatori. Fortificare alimenti consumati abitualmente, come la pasta, con composti bioattivi, rappresenta una strategia promettente per incoraggiare diete più sane, senza modificare le abitudini alimentari dei consumatori.

Tra i sottoprodotti vegetali, la sansa di oliva (OP) è una fonte preziosa di composti fenolici con proprietà antimicrobiche e biologiche (antiossidante, antidiabetica, anticancro, neuroprotettiva). Trasformare la OP in una risorsa da riutilizzare, riduce l'impronta ambientale della produzione di olio d'oliva e crea opportunità economiche, poiché alimenti ad alto valore aggiunto possono essere progettati a livello di laboratorio e validati in un ambiente rilevante, dimostrando che l'innovazione dipende dalla collaborazione tra ricercatori ed aziende. Poiché diversi autori hanno testato la OP come conservante naturale e per l'ottenimento di alimenti funzionali solo a livello di laboratorio, l'obiettivo del primo capitolo di questa tesi è stato validare e dimostrare a livello industriale la produzione di pasta fresca con maggiore attività antiossidante e migliore qualità (chimico-fisica e microbiologica) durante la conservazione, impiegando estratti fenolici di sansa di oliva (OPE), supportando le aziende alimentari negli sforzi per ridurre gli sprechi alimentari e promuovere stili di vita più sani. Pertanto, i campioni di pasta fresca sono stati prodotti su scala industriale, utilizzando l'estratto fenolico di sansa di oliva (OPE), liofilizzato (F-OPE) e microincapsulato (M-OPE), come fonte di antiossidanti e composti antimicrobici. I campioni sono stati caratterizzati subito dopo la produzione; successivamente sono stati conservati a 4°C e analizzati (dal punto di vista fisico-chimico e microbiologico) a intervalli regolari fino a 120 giorni. I risultati hanno mostrato che la microincapsulazione ha preservato i composti fenolici totali (TPC) durante la produzione della pasta; subito dopo la produzione, infatti, i campioni con M-OPE erano caratterizzati da valori di TPC e attività antiossidante superiori rispetto a quelli con F-OPE. Tuttavia, specialmente a concentrazioni più elevate, M-OPE ha causato una parziale perdita della maglia glutinica, con conseguenti maggiori perdite di TPC durante la cottura. Nonostante ciò, i campioni cotti con M-OPE mostravano valori più elevati di TPC ed attività antiossidante. L'impiego di OPE ha ridotto umidità, attività dell'acqua (a_w) e pH rispetto al controllo (CP). Umidità e a_w hanno anche mostrato un andamento decrescente durante la conservazione, a causa del rilascio di acqua nell'atmosfera all'interno della confezione. L'ossidazione ha portato a una riduzione di TPC e attività antiossidante nel tempo, influenzando anche il profilo volatile, in particolare per il campione CP e quello con il più alto contenuto di F-OPE. Per quanto riguarda il colore, OPE ha causato un aumento dei valori dell'indice di rosso (a^*), mentre si è osservata una diminuzione dell'indice di luminosità (L^*) e dell'indice di giallo (b^*), con l'aumentare del contenuto di OPE nella formulazione. Durante la conservazione, i valori dell'indice di giallo hanno mostrato una riduzione; tuttavia, i campioni con M-OPE presentavano valori più alti (maggiormente apprezzati dai consumatori) rispetto ai corrispondenti campioni con F-OPE. È importante evidenziare, inoltre, che OPE ha ridotto la crescita di microrganismi alteranti, quali, muffe, lieviti e batteri aerobi mesofili totali, rispetto a CP, mostrando il suo potenziale nell'estendere la shelf-life della pasta fresca.

In conclusione, questa ricerca ha validato e dimostrato con successo, in un ambiente rilevante, l'uso dell'estratto fenolico di sansa di oliva come fonte naturale di composti con proprietà antimicrobiche ed antiossidanti, evidenziandone il promettente potenziale per lo sviluppo di approcci sostenibili nella promozione di diete più sane e nella conservazione degli alimenti.

La possibilità di prevenire malattie non trasmissibili attraverso prodotti alimentari più sani è stato il fulcro del secondo capitolo della tesi. Queste patologie croniche rappresentano una sfida globale per la salute pubblica; causano, infatti, circa 38 milioni di morti ogni anno. Le malattie cardiovascolari sono una delle principali malattie non trasmissibili, associate a diete poco salutari e in particolare, ricche di grassi saturi. È quindi necessario ridurre il contenuto di acidi grassi saturi negli alimenti, per perseguire il terzo Obiettivo di Sviluppo Sostenibile dell'Agenda 2030, che prevede una riduzione di un terzo delle morti correlate alle malattie non trasmissibili, entro il 2030. Il *pão de queijo* (pane al formaggio), pane tradizionale brasiliano senza glutine, è ampiamente diffuso anche sui mercati esteri ed è spesso consumato più volte nell'arco della giornata. Questo pane al formaggio, tuttavia, è prodotto con amido di manioca, sale, olio, formaggio e uova, risultando ricco di acidi grassi saturi. Queste premesse evidenziano la necessità di migliorare il profilo nutrizionale di questo pane tradizionale, e potenzialmente associato ad un maggior rischio di malattie cardiovascolari per i consumatori. Considerando che questo pane al formaggio è già prodotto a livello industriale, vi è una chiara necessità di trovare soluzioni facilmente adattabili sulla produzione in larga scala. In collaborazione con l'Istituto di Nutrizione dell'Università dello Stato di Rio de Janeiro, sono stati prodotti a livello laboratoriale *pão de queijo* sperimentali, sostituendo il formaggio con polpa di avocado ed emulsion-filled gel (EFG), prodotto con inulina e olio d'oliva. Questa ricerca, quindi, ha valutato gli effetti di ingredienti alternativi sulle proprietà fisico-chimiche, nutrizionali e reologiche dei pani al formaggio. Inoltre, è stato condotto un consumer test, per valutare la percezione sensoriale dei prodotti e l'accettabilità da parte dei consumatori. I risultati dell'analisi reologica hanno evidenziato che gli impasti presentavano un comportamento prevalentemente elastico e caratteristiche di shear-thinning, fondamentali sia per la percezione sensoriale, sia per le applicazioni nei processi alimentari. Il temperature sweep test ha mostrato che il campione controllo e la formulazione con sostituzione parziale del formaggio con polpa di avocado presentavano una riduzione del modulo elastico e del modulo viscoso, attribuibile alla fusione dei grassi. Il successivo aumento del modulo elastico e viscoso è stato associato alla gelatinizzazione dell'amido e alla denaturazione delle proteine. Come previsto, l'impiego di ingredienti di origine vegetale ha determinato un aumento del contenuto di fibre e una riduzione del contenuto lipidico. Di conseguenza, nei campioni sperimentali è stato osservato un miglioramento del profilo degli acidi grassi. In particolare, le formulazioni sperimentali hanno mostrato un indice aterogenico e trombogenico inferiore e un rapporto tra gli acidi grassi polinsaturi e saturi (PUFA/SFA) più elevato, evidenziando i potenziali effetti benefici sulla salute cardiovascolare. I campioni con sostituzione parziale del formaggio mediante polpa di avocado ed EFG hanno evidenziato valori più elevati di elasticità e più bassi di masticabilità, caratteristiche apprezzate dai consumatori. I risultati del test CATA ottenuti in questo studio, inoltre, hanno indicato che l'inclusione di avocado e di sistemi lipidici strutturati ha modificato le caratteristiche sensoriali del prodotto, conferendo alle formulazioni innovative specifici attributi di tipo vegetale. Nonostante la formulazione tradizionale continui a essere la più apprezzata, i risultati della preference map indicano che alcune formulazioni a base vegetale potrebbero incontrare il gradimento di specifici gruppi di consumatori, indicando opportunità per ulteriori ricerche e fornendo una solida base di partenza per il miglioramento del prodotto.

Il presente studio è stato condotto esclusivamente su scala di laboratorio; per un reale avanzamento saranno tuttavia necessari studi futuri orientati alla validazione del processo su scala pilota e industriale. Questi nuovi ingredienti, infatti, potrebbero essere facilmente adattati su una produzione in larga scala, e pertanto sviluppi successivi dovrebbero prevedere la produzione di queste formulazioni innovative mediante una macchina automatica per la formatura del *pão de queijo*, che comprende le fasi di impastamento, formatura e, eventualmente, farcitura, validando l'innovazione in un ambiente rilevante. Per la produzione su larga scala di emulsion-filled gel, inoltre, è fondamentale che le aziende effettuino prove utilizzando turboemulsori. Questi dispositivi impiegano pale controrotanti abbinate a turbine di omogeneizzazione per generare emulsioni stabili e di alta qualità. Tale apparecchiatura è particolarmente adatta all'impiego nel settore alimentare e, nello specifico, per la produzione di gel, creme e salse.

Il terzo capitolo della tesi ha affrontato il tema della sicurezza alimentare, che rappresenta una sfida globale per la salute pubblica, contribuendo a 600 milioni di casi di malattie trasmesse da alimenti e 420.000 decessi ogni anno. Le principali preoccupazioni sanitarie riguardano le intossicazioni alimentari causate da tossine naturali, come le ammine biogene (BAs), composti tossici prodotti principalmente dalla decarbossilazione di aminoacidi liberi. Questi composti possono causare disturbi digestivi, circolatori e respiratori, aggravati dall'assunzione di alcol. Queste premesse sottolineano la necessità di indagare sul contenuto di BAs nelle bevande alcoliche, come le birre artigianali, per proporre strategie, efficaci nella riduzione di queste molecole tossiche, da validare e dimostrare su scala industriale, in linea con il secondo obiettivo dell'Agenda 2030, il cui scopo è garantire a ogni individuo accesso universale a cibo sicuro, nutriente e sufficiente. L'attività di ricerca, in collaborazione con il Dipartimento di Scienze Agricole e Medicina Veterinaria di Cluj-Napoca, è iniziata con l'indagine delle BAs nelle birre prodotte da microbirrifici italiani e rumeni, con lo scopo di raccogliere dati ed identificare i punti critici nell'accumulo di BAs. Proponendo azioni correttive da applicare durante il processo di produzione, l'obiettivo era quello di produrre birre artigianali sicure per tutti i tipi di consumatori. Purtroppo, a causa del tempo ridotto, la fase di scale-up non è stata effettuata; tuttavia, l'attività di ricerca fornisce suggerimenti ed applicazioni pratiche, per produrre birre artigianali sicure. I risultati della ricerca, infatti, hanno mostrato che tiramina, triptamina, putrescina e cadaverina erano le principali BAs rilevate nelle birre artigianali. Sebbene l'istamina sia stata trovata a basse concentrazioni, l'86% dei campioni presentava concentrazioni superiori al limite di rilevabilità, rappresentando un rischio per persone con intolleranza all'istamina. Il consumo di birre artigianali in combinazione con altri alimenti fermentati dovrebbe essere valutato con attenzione dai consumatori sensibili, dato che il 35% dei campioni analizzati era caratterizzato da un contenuto totale di BAs superiore al limite suggerito in letteratura per questo tipo di consumatori (20 mg/L). Il rischio è inferiore per soggetti sani, poiché solo il 10% delle birre presentava un livello totale di BAs superiore a 50 mg/L, considerato dannoso per questo tipo di consumatori. I risultati, inoltre, hanno evidenziato che alcune fasi nella produzione di birre artigianali possono comportare un alto rischio per la sicurezza alimentare. La selezione delle materie prime, specialmente ingredienti non convenzionali, la tostatura del malto, l'ammostamento, il processo di fermentazione, la scelta dei microrganismi starter e le condizioni igieniche generali all'interno dei microbirrifici sono stati identificati come punti critici per l'accumulo di BAs nelle birre artigianali. Ricerche future dovrebbero testare, validare e dimostrare strategie di controllo e riduzione delle BAs, prima a livello laboratorio e poi su scala industriale.

In conclusione, questa tesi di dottorato dimostra il potenziale delle innovazioni alimentari nel passaggio dalla scala di laboratorio a quella industriale, attraverso modelli di collaborazione tra ricerca ed impresa e illustra prototipi innovativi alimentari facilmente trasferibili su larga scala. Affrontando i temi della sostenibilità, della nutrizione e della sicurezza alimentare, i casi di studio illustrano come la ricerca scientifica possa tradursi in risultati industriali concreti, contribuendo al progresso dell'innovazione nel settore alimentare, colmando il divario tra la ricerca di base e la validazione industriale e supportando gli obiettivi dell'Agenda 2030.

General introduction and thesis scope and aims

The food and drink industry represents the largest manufacturing sector globally (Alqudhaibi et al., 2024). Particularly, in the European Union, this sector creates more than 4.7 million jobs directly, producing a turnover of 1 billion dollars. It comprises more than 304.000 companies, many of which are small and medium enterprises. Over the last five years, investment by the food and drink industry in research and development has increased by over 10% (Fooddrinkeurope, 2025). The food sector, indeed, is under considerable pressure, as is facing global challenges, such as population growth, climate change, resource scarcity, pollution and waste, diet-related non-communicable diseases (NCDs) and food safety issues (European Commission, 2025; Lazaro-Mojica & Fernandez, 2021; McClements et al., 2021). The European Commission, through the Food 2030 Strategy, highlights the key role of research and innovation in building a sustainable, safe, secure and healthy food system (European Commision, 2025). Innovation could be defined as the development of new products, processes and services (Lazaro-Mojica & Fernandez, 2021). Particularly, Brody & Lord (2007) stated that the development of new prototypes keeps the food firms sustainable and competitive in a changing market, by filling gaps in marketplaces. These gaps represent combinations of benefits demanded by groups of consumers, who have not yet found an existing product or service that offers these benefits, and which can be identified and reached selectively (Brody & Lord, 2007). Thus, a gap emerges as a result of shifting consumer preferences, driven by demographic, socioeconomic, and lifestyle changes, which in turn transform the way people eat and the types of products that align with their consumption system (Brody & Lord, 2007).

Usually, universities and public institutions are the drivers of innovation, acting as generators of scientific breakthroughs and providing outputs that are often limited to laboratory scale and thus, making limited impact on the industrial food system (Pitt & Jones, 2016). The translation of these ideas into practical applications (scale-up), which is increasingly popular in the world of public research and development (Schut et al., 2020; Shilomboleni & De Plaen, 2019), is essential for achieving innovation within the sector (Gachanja, 2023). Recently, Sartas et al. (2020) reported that scaling refers to the increased use of innovations beyond the group involved in its initial design and testing. It is pivotal to address societal challenges related to health, food, agriculture, and environment, while also constituting a strategic process for organizations aiming to achieve the Sustainable Development Goals.

A convenient criterion to assess the maturity of a product or a technology versus its intended application is defined by the Technology Readiness Level (TRL) criterion, which describes typical products development. The metric defines nine levels of technology readiness, starting from basic scientific research (TRL 1) and progressing to fully developed systems that have been successfully validated during real-world missions (TRL 9). Often, universities and research centres play an important role in the advancement of a technology or a product from basic research (TRL 1) up to TRL 3 or 4 (validation at laboratory scale) (White et al., 2022). Companies typically pick up product or technology in TRL 4 and push it all the way to full scale-production and commercialization (TRL 9) (Shilomboleni & De Plaen, 2019). As a result, moving an innovation from a laboratory research to a pilot or industrial plant requires collaborative interaction among multiple stakeholders, including researchers and food enterprises (Shilomboleni & De Plaen, 2019); the latter, indeed, should cooperate to ensure that the food companies remain sustainable and able to address current global challenges (Lazaro-Mojica & Fernandez, 2021).

In this context, the aim of this PhD project, partially funded by the company Matarrese srl, sited in Alberobello (Bari, Apulia, Italy), was to upscale food innovative solutions proposed by research, towards a pilot or pre-industrial scale. The Department of Soil, Plant and Food Sciences (DiSSPA) of the University of Bari Aldo Moro, in collaboration with international research partners, carried out basic research (TRL 1-2), where basic principles were observed, potential applications were identified, and no experimental proof existed. Then, the PhD project progressed to applied research (TRL 3), where laboratory tests were carried out (TRL 4) and subsequently food prototypes were validated and demonstrated on a semi-industrial scale (TRL 5 and 6, validation and demonstration in a relevant environment). Although not all models have yet been validated in an industrial setting (TRL 5-6), these innovations are readily transferable to real-world production environments.

The project issues are in line with the Mission 4, Component 2 “M4C2” of the National Recovery and Resilience Plan, that provide to support investments in research and development, to promote innovations and their transfer to the food companies.

Accordingly, the thesis is divided into three chapters, each presenting one or more case studies focused on innovative food products that have either been successfully validated at industrial scale or demonstrate a strong potential for large scale application. These innovative food products are designed to support companies in addressing global challenges.

In the first chapter, work was carried out to assess the feasibility of developing on an industrial scale a fresh pasta with higher antioxidant activity and improved physiochemical and microbiological quality during storage, using olive oil by-products. Particularly, this research activity was designed to: i) reduce pollution, by upcycling a by-product; ii) reduce food waste, improving the quality of the product during storage; iii) potentially mitigate the risk of NCDs, increasing antioxidant activity of innovative food prototypes. These objectives are in line with the 12th Sustainable Development Goal of the 2030 Agenda.

In the second chapter, the aim was to identify new formulations, easy to be adopted on an industrial scale, for cheese bread production, aiming to improve its fatty acid profile, in order to support food companies in addressing the challenge of NCDs and promote healthy diets, in line with the 3rd Sustainable Development Goal of the 2030 Agenda.

Finally, the third chapter dealt with food safety issues, carrying out a survey on biogenic amines in craft beers, providing valuable suggestions and practical recommendations to craft brewers on how to improve safety of their products. This activity is in line with the 2nd goal of the 2030 Agenda, aimed at ensuring that all individuals have access to food that is safe, nutritious, and sufficient.

Although focused on different case studies, the three chapters are linked by a common Technology Readiness Level approach and describe a coherent pathway from basic research to industrial application, through technology transfer and scale-up.

To achieve the aims of the first chapter, basic research was conducted to explore the potential of natural antioxidants and, particularly those from olive pomace, in food functionalization and preservation. Then, an aqueous phenolic olive pomace extract (OPE) was obtained and applied in fresh pasta, in order to assess the optimal OPE concentration, which provides antioxidant and antimicrobial properties, without affecting the sensory profile. Following these laboratory trials, functional fresh pasta was produced on an industrial scale, validating the feasibility of producing a food prototype with higher antioxidant activity and improved microbiological and physicochemical quality during storage (**Publication no.1, Publication no. 2**).

Moving to the second chapter of this PhD thesis, in collaboration with the Nutrition Institute of Rio de Janeiro State University, we focused on a traditional Brazilian food product, *Pão de queijo* (cheese bread). Its traditional ingredients and nutritional profile were studied, in order to explore the possible areas for improvement and innovation. Following bibliographic research focused on the improvement of the fatty acid composition of the product, contributing to the reduction of NCDs, new ingredients (avocado pulp and emulsion-filled gel) were integrated into the production process on a small scale (**Publication no. 3**). However, these experimental breads could be readily transferred to pilot-scale production by using an automatic *pão de queijo* production machine, representing a logical next step to validate the proposed innovations in a relevant environment and to hereby facilitating the transition from laboratory-scale validation to TRL 5-6.

Finally, concerning chapter 3, basic research on the safety of fermented beverages was conducted in collaboration with the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca. A survey on the content of biogenic amines in craft beer was carried out, in order to: i) provide information to consumers and international organizations, to support the establishment of legal limits for these molecules in alcoholic beverages; ii) identify potential causes of biogenic amines accumulation in these matrices, in order to propose effective strategies to limit their formation. Although this research provides valuable suggestions and insights for brewers to improve the safety of their products (TRL 2-3) (**Publication no. 4**), unfortunately a craft beer with a reduced content of biogenic amines has not been produced on a laboratory level and subsequently validated on a pilot scale (TRL 5-6). However, the accurate selection of starter cultures and raw materials, the optimization of malt baking and mashing parameters could be suitable strategies to be validated on an industrial craft beer production, thereby supporting the assessment of the process scalability and the advancement of this process innovation toward higher TRL, with the aim of producing safer craft beers. Future steps toward higher TRL should include laboratory-scale investigations of both biogenic amine–degrading microorganisms and diamine oxidase enzyme to assess their effectiveness and compatibility with the beer matrix, followed by pilot- and industrial-scale validation (TRL 5-6) to support their practical application in craft beer production.

Together, these chapters cover different stages of Technology Readiness Levels, from fundamental research and proof-of-concept to laboratory-scale optimization (TRL 3-4), and in selected cases, industrial validation in relevant environments (TRL 5-6).

In conclusion, this thesis highlighted the strategic role of innovation as a driver of competitiveness and sustainability and illustrated that bridging the gap between laboratory-scale discoveries and industrial implementation is pivotal for addressing contemporary global challenges and Sustainable Development Goals.

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Chapter 1- Innovation in fresh pasta formulation and shelf-life extension: a strategy to promote healthier lifestyles and reduce food waste

Introduction

Chapter I presents case studies of scale-up within this doctoral project. They focus on the transfer of a laboratory-developed food innovation (fresh pasta fortified with bioactive compounds from olive pomace) to the industrial level. The objective was to demonstrate the feasibility of producing a fresh pasta with enhanced antioxidant properties and improved quality during storage on a large scale, thereby supporting food companies in reducing food waste and promoting healthier diets.

In the last decade, food companies have become increasingly aware of the environmental impacts of food waste and by-products (Rațu et al., 2023; Tavill, 2020). It is estimated that around 88 million tons of food are wasted annually in the EU, from primary production up to consumption (Socas-Rodríguez et al., 2021), causing environmental, economic and social negative implications. Because of their low biological stability, high concentration of organic components, large nutritional value, high water activity, poor oxidative stability, and optimal enzymatic activity, these food industry waste should not be disposed of in the environment; the decomposition of food waste in landfills, indeed, produces methane and carbon dioxide, two greenhouse gases that contribute to climate change (Borah et al., 2023). In terms of the economy, food waste have a direct, negative affect on the income of both farmers and consumers, as the amount of waste produced each year corresponds to a financial loss of €132 billion (Dobrucka et al., 2025). As the issue of food security is becoming increasingly important globally, the reduction of food losses and waste is considered as a first step towards achieving food security (Lu et al., 2024).

The development of a sustainable and secure food system requires the effective management of food waste and the valorisation of by-products, as these practices have a pivotal role for the sustainable and competitive growth of food companies (Borah et al., 2023).

The United States Environmental Protection Agency (US EPA) proposed strategies for food waste management (Tavill, 2020), which are reported in Figure 1. First of all, an effective food waste management strategy includes the avoidance of food waste generation, producing only the necessary amount of food to cover global nutritional needs, safeguarding food security. In retail and the consumption stages, such as the food service sector and households, food surplus prevention includes the supply of only what is required and correct portion sizing. For the surplus food that has not been consumed, the option proposed is to redistribute it to groups affected by poverty, assuming that food safety can be ensured. Moreover, food waste could be recycled into animal feed, and via composting. Once recycling efforts are exhausted, food waste may be directed towards energy recovery processes, through anaerobic digestion. Finally, disposal in landfills is the least favourable option for managing the remaining fraction of food waste, once all the other options are exhausted (Papargyropoulou et al., 2014).

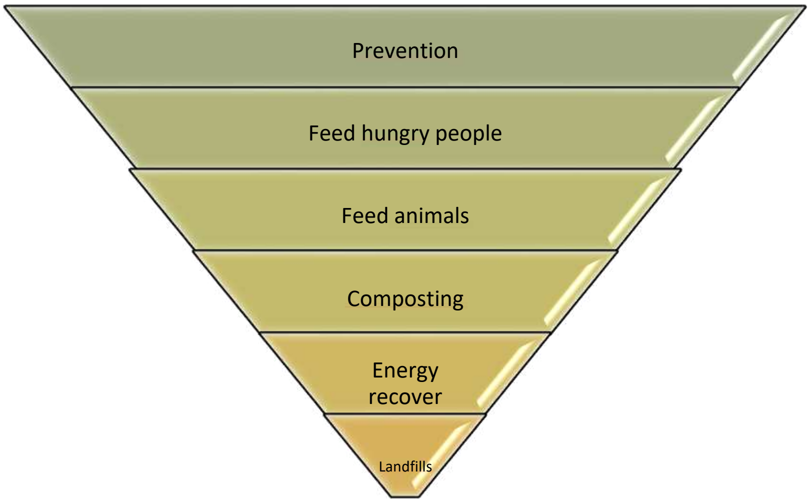

Figure 1. Food waste management hierarchy proposed by The United States Environmental Protection Agency.

Consumers are responsible for 60% of waste along the food cycle in industrialized nations, as a consequence of changes in consumer habits (Mesías et al., 2021). The increased participation of the female population in the labour market and the rising number of families in which both partners work (Eldesouky & Mesias, 2014), indeed, led to a gradual abandonment of the daily food purchasing habit, resulting in less frequent but larger shopping trips, during which greater quantities of food are purchased and stored. This transition in food purchasing habits increases the risk that some products will expire or deteriorate before being consumed, thereby contributing to household food waste. In line with this statement, Falasconi et al. (2019) showed that in Italy buying and preparing too much food cause the major food waste in households (Tavill, 2020). However, also factors associated with expiration dates contribute significantly to food waste generation (Falasconi et al., 2019). Therefore, food companies have a pivotal role to prevent food waste, employing product, process and packaging design innovation, which could deliver great food and fulfils consumer needs, while helping consumers to avoid waste generation through several features, such as portion control and longer shelf-life (Tavill, 2020). Designing foods with longer shelf-life can give consumers more time to plan to consume them, avoiding product return and food waste (Tavill, 2020).

In line with this statement, Spada et al. (2018) stated that strategic efforts in terms of shelf-life prolongation could help to face the food waste issue, in particular for products that reach the "best before date" between 30 and 50 days. Given that the household storage contributes to over 12% of total pasta losses (Principato et al., 2019) and that fresh pasta generally has a shelf-life of only 30 to 60 days (Manthey et al., 2008), it is essential to develop effective strategies to extend it, to mitigate food waste. Thus, innovations in formulation, processing technologies, and packaging of fresh pasta are pivotal for food companies (Tavill, 2020), in order to improve the quality of products during storage, while pursuing the 12th Sustainable Development Goal of the Agenda 2030, whose purpose is to reduce food waste by 50% by 2030 (Principato et al., 2019).

Historically, chemical additives have been a valuable strategy to extend the shelf-life of perishable products. Nevertheless, consumers are concerned about the potential health hazards linked to chemical preservatives and are becoming increasingly interested in organic alternatives and nutritious food with a longer shelf-life (El Alami El Hassani et al., 2025). Consequently, the food industry has introduced 'clean-label' preservatives, molecules, from natural sources such as plants, animals, or microorganism, that serve as effective alternatives to traditional chemical preservatives (Balasubramaniam et al., 2023). Focusing on plant source, secondary metabolites like polyphenols, terpenes, and alkaloids, could be recovered from vegetable by-products and used to improve the physicochemical and microbiological stability of food matrices (El Alami El Hassani et al., 2025). In line with this statement, Angiolillo et al. (2019), Lacivita et al. (2022) and Lordi et al. (2023) obtained promising results on the shelf-life extension of fresh pasta, produced at laboratory scale, employing several vegetable by-products.

Moreover, it is worth noting that enabling healthier and longer lives, through food and nutrition is an imperative for all society (Ruthsatz & Candeias, 2020), given that non-communicable diseases (NCDs) (chronic diseases of long duration characterized by slow development or progression) account for approximately 38 million deaths globally each year (Wagner & Brath, 2012). However, the approach for promoting healthy diets through the consumption of functional foods, characterized by formulations far from those the consumers are used to, has barely succeeded, due to the difficulty in changing consumer preferences (Bianchi et al., 2021). Conversely, fortifying staple foods, such as pasta, with bioactive compounds offers a promising strategy to encourage healthier diets, without changing consumers' eating habits (Carpentieri et al., 2022), thereby contributing to the 3th Goal of the 2030 Agenda, of reducing premature mortality caused by non-communicable diseases by one-third by 2030.

The use of vegetable by-products could be suitable not only for extending the shelf-life, but also for improving the nutritional profile of foods, as authors showed that compounds recovered from by-products could have beneficial biological effects (antioxidant, anticancer, antidiabetic and anti-hypocholesterolemic activity), mainly due to their richness in phenolic compounds (Lau et al., 2021). At the laboratory level, promising results have been obtained by Bianchi et al. (2024), Gumul et al. (2023), Kaur et al. (2022), Marinelli et al. (2015) and Panza et al. (2022), incorporating apple peel or pomace, fig peels, grape extract or skin, orange pomace, red chicory by-products and other ingredients into fresh pasta formulations, resulting in an increased levels of phenolic compounds, flavonoids, and antioxidant activity of pasta.

Among vegetable by-products, olive pomace is the major output of olive oil processing (Nunes et al., 2021). It is a solid by-product, as it is a combination of olive pulp, bark, stone fragments and moisture. It has been reported that one ton of olives produces about 400 kg of olive pomace (Schmidt et al., 2023), with a moisture content of 50–65%, depending on the type of decanter used (Difonzo et al., 2021). Particularly, as reported by Difonzo et al. (2021), phenolic compounds (hydroxytyrosol, tyrosol, cinnamic acid, caffeic acid, ferulic acid, rutin, luteolin, verbascoside and secoiridoids and its derivates), tocopherols (α-ocopherol, α-tocotrienol, β-tocopherol, γ-tocopherol), sterols and hydrocarbons (squalene, β-sitosterol, campesterol, stigmasterol, brassicasterol) are the main bioactive compounds of olive pomace. In addition, it contains minerals, dietary fibre (hemicellulose and lignina), *β*-carotene, polyunsaturated fatty acids and monounsaturated fatty acids.

Among all phenolic compounds, oleuropein, hydroxytyrosol and tyrosol are known for their bioactivity (Figure 2) (Schmidt et al., 2023). Moreover, several authors have reported that bioavailable phenolic compounds from olive pomace also have antimicrobial properties (Figure 2), highlighting their role as substitutes for chemical additives, in improving the physicochemical and microbiological stability of foods (Zhao et al., 2023).

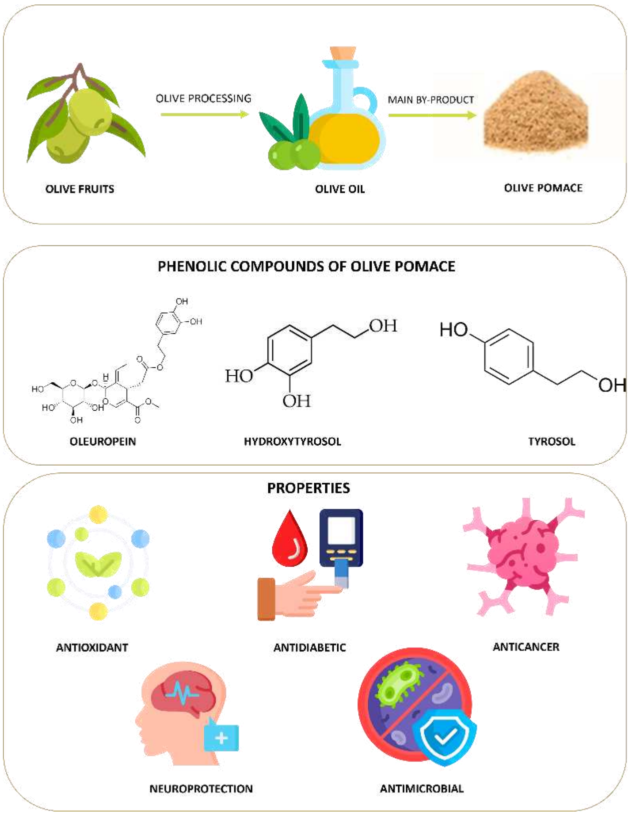

Figure 2. Phenolic compounds of olive pomace and their properties.

Thus, transforming olive pomace into a valuable resource reduces the environmental footprint of olive oil production and creates economic opportunities, as high added value foods can be designed at laboratory scale (TRL 4) and validated on a relevant environment (TRL 5-6), showing that innovation depends on the collaboration among researchers and enterprises.
As several authors tested olive pomace as natural preservatives and for food functionalization at laboratory scale (Balli et al., 2021; Padalino et al., 2018; Panza et al., 2020; Simonato et al., 2019), the aim of these activities was to validate and demonstrate at industrial level the production of fresh pasta with higher antioxidant activity and improved microbiological and physiochemical quality during storage. These case studies support the transition from research to industrial practice, providing a model of technological transfer, to help food companies in the reduction of waste and pollution, while promoting healthier lifestyles.
Therefore, first of all, the phenolic olive pomace extract was produced and applied in fresh pasta samples produced at laboratory scale, to assess its optimal concentration. Subsequently, the industrial production (scale-up) of the optimized samples was performed at Maffei company (Cutrofiano, Lecce, Italy) and the results obtained from industrial production are presented in Publication no. 1 and Publication no. 2. The aims of these activities were to: i) evaluate the effect of olive pomace extract on the nutritional, sensory and physicochemical characteristics of fresh pasta samples, in order to promote healthier food (Publication no.1); ii) assess the effects of olive pomace extract on the microbiological and physicochemical properties on fresh pasta during storage, in order to avoid food waste generation (Publication no. 2).

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Publication no.1:

Physicochemical and Nutritional Properties of Functional Fresh Pasta with Free and Microencapsulated Olive Pomace Extract

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Physicochemical and Nutritional Properties of Functional Fresh Pasta With Free and Microencapsulated Olive Pomace Extract

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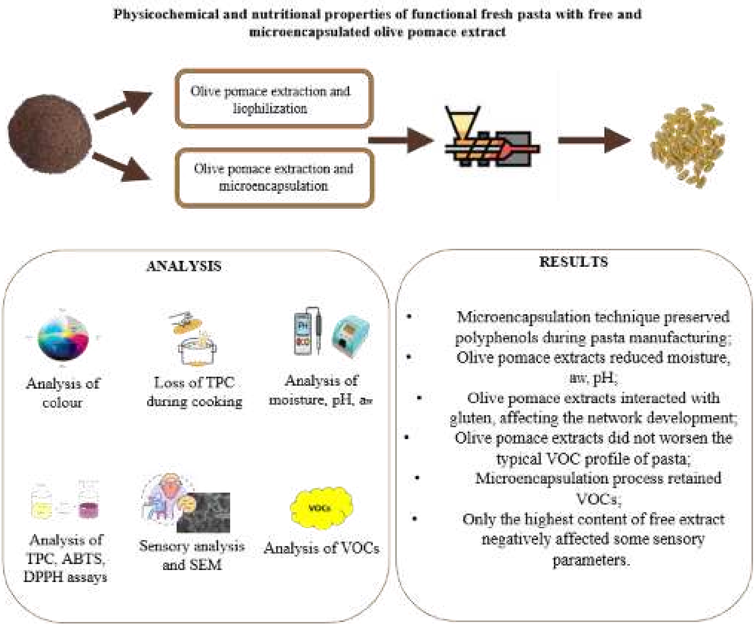
Graphical Abstract



Olive pomace (OP) polyphenols have been exploited to produce fresh pasta. The effect of OP extract addition, both as a free lyophilised extract (F-OPE) and as a microencapsulated extract (M-OPE) on the physicochemical, nutritional, and sensory properties of fresh pasta was studied.

Abstract

The increased demand of consumers for healthy foods has generated interest among researchers and food producers in developing functional foods. Olive pomace (OP) can be used to obtain high-added value products, being a valuable source of phenols. The aim of the study was to assess the influence of incorporating OP extract, as free lyophilized (F-OPE) and as microencapsulated (M-OPE), on the physicochemical, nutritional, and sensory properties of fresh pasta "cavatelli." Pasta samples with M-OPE were characterized by a higher yellow colour and had higher total phenolic compounds content compared to samples with F-OPE (on average 0.43 vs. 0.39 mg gallic acid equivalents (GAE) g^{-1} of fresh sample), showing that the microencapsulation technique preserved polyphenols during pasta manufacturing. The experimental samples had lower moisture (on average 29% vs. 31.14%) and a_w compared to the control pasta (CP), but still complied with Italian law, according to which fresh pasta must have at least 24% moisture and a_w not higher than 0.97. Microencapsulation proved to be useful in retaining volatile organic compounds, as well as enhancing antioxidant activity. Scanning electron microscopy (SEM) analysis showed that samples with F-OPE had more porous and spongy structures. On the other hand, the gluten network was lost in pasta sample with the highest content of M-OPE. The intensity of odour and taste linked with raw cereal was more intense in CP, than in experimental ones. Bitterness and astringency were perceived most in samples with F-OPE, especially at high concentration. Indeed, considering the overall acceptability, all the samples gained a positive score, except for the sample with the highest F-OPE content.

Keywords: olive pomace extracts; microencapsulation; functional foods; sensory properties; polyphenols; fresh pasta.

1. Introduction

Pasta is one of the most consumed products in the world, as it is the main constituent of the Mediterranean diet (Carpentieri et al., 2022; Catzeddu et al., 2023). According to Italian law, pasta is the result of a dough of durum wheat semolina and water, shaped in various forms (Italian Republic, 2001); it is considered a rich source of carbohydrates, with low content of proteins, fibres, and bioactive compounds (Sissons 2022). Being pasta a highly consumed product, easy to prepare, and cheap, the Food and Drug Administration (FDA) recognized it as an excellent vehicle to be fortified with functional compounds with demonstrated biological activity (Bianchi et al., 2021; Carpentieri et al., 2022). In addition, the increasing awareness of diet–disease consequences, together with consumers' preference for functional foods, encouraged food scientists to research new formulations for functional pasta manufacturing (Romano et al., 2021). These products are usually obtained by the addition of ingredients (as is or extracts) containing bioactive compounds (i.e., dietary fibres or phenolic compounds such as flavonoids, phenolic acids, tannins, and others) (Bianchi et al., 2024; Kabir et al., 2024). Agriculture and food industrial by-products are an attractive source of bioactive compounds, which can be exploited as functional ingredients and natural preservatives (Maddaloni et al., 2025). Several authors employed agri-food by-products for pasta functionalization, promoting healthier lifestyles without changing consumer habits (Carpentieri et al., 2022; Cuomo et al., 2024; Difonzo, De Gennaro et al., 2022; Lordi et al., 2023). During the last few years, the valorisation of olive oil by-products, produced in large quantities in a short period of time, has gained great attention (Balli et al., 2021; Difonzo et al., 2021). Olive pomace (OP) and its functional compounds (phenolic compounds, sterols, and tocopherols) have been exploited in cereal-based products (Cedola et al., 2019; Conterno et al., 2019), vegetable oils (Sánchez De Medina et al., 2011), fermented milk (Aliakbarian et al., 2015), and fish burgers (Cedola et al., 2017) to improve their phenolic content and antioxidant activity (Difonzo et al., 2021). These authors usually employed the functional ingredients as such or as an aqueous extract; furthermore, several authors tried to exploit the microencapsulation technique to enrich the final product with bioactive compounds. Actually, several functional cereal-based products were obtained by applying encapsulated vegetable extracts (Kamali Rousta et al., 2021). As regards pasta, many authors focused on improving its nutritional profile with encapsulated vegetable extracts (Elsebaie et al., 2022; González et al., 2021; Kamali Rousta et al., 2021; Pillai et al., 2012; Šeregelj et al., 2022). The encapsulation technique was considered by researchers for many reasons, as it also allowed them to overcome some sensorial issues (i.e., masking the bitter taste and astringency associated with polyphenols) (Barrientos et al., 2024; Bodbodak et al., 2024) and increase the functional ingredient thermal stability, especially for foods that require heat for preparation, such as fresh pasta (Zen et al., 2020). At the moment, literature has scarce information about the fortification of fresh pasta with OP extract (OPE), as recently Cecchi et al. (2019), Padalino et al. (2018), and Simonato et al. (2019) evaluated only the effect of olive paste as such on the technological, nutritional, and sensory profiles of pasta samples. To the best of our knowledge, no information is present concerning the use of OPE as such and as microencapsulated in fresh pasta. Therefore, the present investigation aimed to compare the physicochemical, nutritional, and sensory profile of functional fresh pasta enriched with OPE in two different ways: (i) addition of free lyophilized OPE (F-OPE) into the dough; (ii) addition of microencapsulated OPE (M-OPE) (by using inulin recovered by artichoke roots as carrier/wall material) into the dough.

2. Materials and Methods

2.1 Materials and Chemicals

Folin–Ciocalteu reagent; ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt); DPPH (2,2-diphenyl-1-picrylhydrazyl); dihydrorhodamine-123; Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Sigma-Aldrich (Milan, Italy). Milli-Q water was purchased from Merck (Darmstadt, Germany); sodium carbonate from Honeywell (Seelze, Germany). Whereas, nylon filters (pore size 0.45 µm) and plastic syringes were purchased from Chemic ALS s.r.l. (Bari, Italy).

2.2 OPE Preparation

Phenolic extract from OP (OPE) was obtained as described by De Gennaro et al. (2022), with some modifications. Briefly, the freeze-dried OP was added to ethanol: water (10:90 v/v) in a 1:5 (w/v) ratio. The mixture was homogenized for 10 min and subsequently treated with ultrasound for 15 min in an ultrasound bath (CEIA International S.A., 115/230 Vac 1- 50/60 Hz–400VA max, Viciomaggio, Italy), and then a final homogenization for 10 min was performed. Finally, the mixture was centrifuged at 15°C at 12,000 g min^{-1} for 10 min (Thermo Fisher Scientific, Osterode am Harz, Germany), and the supernatants were collected and filtered with nylon filters (pore size 0.45 µm). The obtained extract was divided into two aliquots; one was freeze-dried, obtaining the F-OPE (about 10 g of F-OPE were obtained from 100 g of OP), and the other one was used to produce the M-OPE, as described in Section 2.3. The extraction was performed in duplicate.

2.3 Microencapsulation of the OP Extract

At first, the inulin (with a high degree of polymerization) used as a carrier/wall material for the encapsulation process was recovered from artichoke roots, as described by Difonzo, De Gennaro et al. (2022). Then, the inulin for microencapsulation was prepared as follows: a 20% (w/w) inulin in ultrapure water was solubilized at 80°C under constant stirring until the powder was completely dissolved. Then, the spraying solution was prepared by mixing the phenolic olive pomace extract with the inulin solution, in order to get a final inulin concentration in the mixture of 5% (w/w), for a final dispersion amount of 2.7 kg. The mixture was stirred, in order to disperse the two phases and kept stirring throughout the spray-drying process. The encapsulation process was performed with a Mini Spray Dryer B-290 (Büchi Technology, Switzerland) with the following operating conditions: inlet air temperature 150°C, outlet air temperature 85–100°C, feed rate of 30%, and aspiration of 100%, which in the adopted operating conditions corresponded to an air flow of around 25–30 m^3 h^{-1}. The total phenolic content of the microencapsulated extract was evaluated by means of the Folin–Ciocalteu method as described in Section 2.4.

2.4 Characterization of OPE: Total Phenolic Content (TPC) and Antioxidant Activity

TPC extraction from OPE was performed as described by Troilo et al. (2022), with minor modifications. First of all, OPE was mixed with methanol: water (80:20) in a 1:10 (w/v) ratio. The mixture was homogenized for 10 min with a laboratory vortex and then subjected to ultrasound treatment for 15 min in an ultrasound bath. Before centrifugation (12,000 *g* min^{-1}, for 10 min at 15°C), the mixture was homogenized again for 15 min. Finally, the supernatants were collected and filtered with nylon filters (pore size 0.45 µm) and used for TPC, DPPH, and ABTS assays. TPC was determined according to the Folin–Ciocalteu method, following the procedure of Difonzo, Crescenzi et al. (2022). The results were expressed as mg of gallic acid equivalents (GAE) g^{-1} of extract. The evaluation of antioxidant activity was performed with DPPH and ABTS assays, as described by Caponio et al. (2022) and Difonzo, Crescenzi et al. (2022), respectively. The results were expressed in µmol Trolox equivalents (TE) g^{-1} of extract. The analyses were performed in triplicate.

2.5 Fresh Pasta Samples Preparation

Fresh pasta samples were produced at semi-industrial level in a factory located in Southern Italy (Pastificio Maffei, Cutrofiano, Lecce, Italy). The general manufacturing process was performed following the recipe used by Maffei company: 1000 g of durum wheat semolina (Casillo, Corato, Italy) were kneaded with 330 g of tap water for 15 min with a mixer (Kenwood Cooking Chef XL KCL95.424SI, United Kingdom). Doughs were extruded in order to obtain the typical "cavatelli" form, which were pasteurized at 100°C for a minute and a half. As regards experimental samples, M-OPE and F-OPE were first solubilized in tap water before being added to the dough. Seven types of fresh pasta samples were prepared: one control (CP, control pasta made with durum wheat semolina), three experimental samples added with M-OPE (MP-1, MP-2, and MP-3), and three experimental samples added with F-OPE (FP-1, FP-2, and FP-3). The experimental products were manufactured with different concentrations of OPE, obtaining pasta samples with increasing total phenolic concentrations: 0.17 mg GAE g^{-1} (MP-FP-1), 0.35 mg GAE g^{-1} (MP-FP-2), and 0.53 mg GAE g^{-1} (MP-FP-3), as reported in Figure 1. The OPE concentration to be added to fresh pasta samples was chosen on the basis of the data reported in the literature (Akbarbaglu et al., 2024; Conte et al., 2021; Difonzo et al., 2018; Difonzo, Totaro et al., 2022; Natrella, Difonzo et al., 2020) and derived from sensory preliminary trials (in which the highest concentration without negative effect on the sensorial characteristics of the product was considered). Two batches of each "cavatelli" type were produced and analysed.

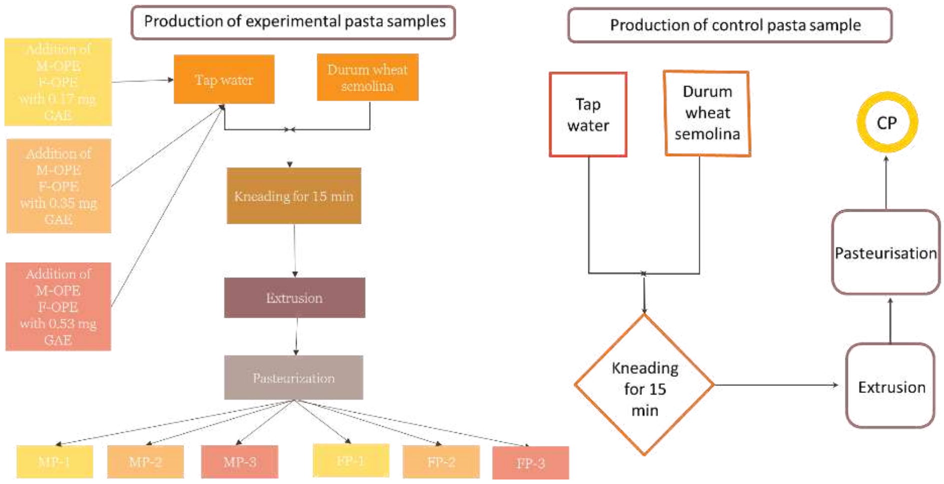


Figure 1. Fresh pasta production process.

Abbreviations: OPE, olive pomace extract; CP, control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE (M-OPE) at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE (F-OPE) at three different concentrations (FP-1, FP-2, FP-3).

2.6 Fresh Pasta Analyses

The following analyses were carried out on fresh pasta.

2.6.1 Physicochemical Analyses

The determination of moisture content was carried out according to AOAC International methods 950.46. The water activity (a_w) of the fresh pasta samples was evaluated using an electronic hygrometer (Aqua-Lab 100–240 VAC, Pullman, WA, USA) (Carini et al., 2012). The pH value was evaluated on the homogenized product (5 g of pasta sample + 50 mL of distilled water) by using a pH-meter (Hanna edge HI2020, Hanna Instruments, Villafranca Padovana, Italy) as described by Rainero et al. (2022). The colour stability of ground fresh pasta was analysed according to the International Lighting Commission (CIE) system, using the parameters L^* (lightness), a^* (redness/greenness), and b^* (yellowness/ blueness) with a colorimeter CM-600d (Konica Minolta, Tokyo, Japan) (Simonato et al., 2019). Colour data were collected with the software SpectraMagic NX (Konica Minolta, Tokyo, Japan). Each analysis was performed in triplicate.

2.6.2 TPC and Antioxidant Evaluation

TPC extraction from raw and cooked pasta samples was performed as described by De Gennaro et al. (2022), with some modifications. Fresh pasta samples were cooked in boiling distilled water for 6 min (time recommended by manufacturer), at a 1:10 (w/v) pasta-to-water ratio, as described by Filipčev et al. (2023). Both uncooked and cooked samples were freeze-dried, shaken for 15 min with a laboratory vortex, with 80% methanol (ratio 1/2, w/v), and treated with ultrasound for 20 min in an ultrasound bath. Then, the mixture was centrifuged at 5°C at 7500 g min^{-1} for 15 min, the supernatants were filtered with nylon filters of 0.45 μm (Sigma-Aldrich) and used for TPC, DPPH, and ABTS assays, performed as described above for OPE (Section 2.4). Before the absorbance reading, the mixture was filtrated with nylon filters. Each assay was performed in triplicate.

2.7 Volatile Organic Compounds (VOCs)

VOCs were determined through headspace solid-phase micro-extraction (SPME) coupled to gas chromatography/mass spectrometry (GC/MS), as reported by Caponio et al. (2022). In particular, 0.50 g of each sample was added with 4 mL of NaCl (20% w/v) aqueous solution and 150 μL of internal standard (1-propanol, 160 ppm). The volatile compounds were quantified by standardizing the peak areas of compounds of interest with the peak area of the internal standard. VOCs were expressed in μg g^{-1} of pasta sample. The analysis was performed in triplicate.

2.8 Sensory Analysis

Sensory analysis was carried out in order to have information about the effect of OPE on the sensory properties of cooked fresh pasta. Fresh pasta samples were cooked in boiling distilled water for 6 min, without the addition of salt, as described above in Section 2.6.2. The selected panel was composed of eight members (three males and five females, age range 25–56) belonging to the trained panel of the Food Science and Technology section of the University of Bari. Prior to the analysis, panellists were selected and trained following the international standard (ISO, 1993), as already reported by Natrella, Faccia, et al. (2020). A quantitative test was performed, as panellists were provided with a sheet containing the attributes that best represent the product and were asked to rate each of them on a scale from 0 to 9 (Table 1). The attributes were selected during a preliminary meeting of the selected judges. As usually polyphenols provide bitterness and astringency, panellists had to evaluate the effect of F-OPE and M-OPE on these sensory attributes compared to CP. Furthermore, panellists were asked to rate the typical taste and odour of cooked pasta, to assess if OPE extracts could worsen these attributes. Cooked fresh pasta samples were presented in white dishes, coded using a three-digit number and were distributed simultaneously to all panel members in a random order presentation.

Table 1. Sensory sheet provided to panellists for the quantitative sensory analysis.

Descriptor	Definition score
Colour	0- Yellow colour 9- Light-brown colour
Odour	0- Characteristic odour of cooked pasta not perceived 9- Characteristic odour of cooked pasta highly perceived
Hardness	0- Too hard or extremely soft texture 9- Expected hardness
Taste	0- No characteristic taste of cooked pasta 9- Characteristic taste of cooked pasta
Bitterness	0- Bitter taste not perceived 9- Bitter taste highly perceived
Astringency	0- Sensation of astringency not perceived 9- Sensation of astringency highly perceived
Overall acceptability	0- Fresh pasta not appreciated at all 9- Fresh pasta highly appreciated

2.9 Microstructure Analysis

Pasta microstructure was analysed by field emission scanning electron microscopy (FE SEM) LEO 1525 (Carl ZEISS, Oberkochen, Germany). The freeze-dried samples were deposited on an aluminium support using conductive carbon cement (Leit-C). Before the analysis, the samples were metalized with a thin layer of chromium (8 nm). Measurements were carried out using an in-lens detector at 5 kV.

2.10 Statistical Analysis

The data were statistically processed by XLSTAT software (Addinsoft SARL, New York, NY, USA) and OriginPro (OriginLab, New Hampton, MA, USA). The results of the physiochemical analysis were subjected to one-way and two-way analyses of variance (ANOVA) followed by post-hoc Tukey's HSD (honestly significant differences) test, considering the type and the concentration of OPE added as independent variables. The differences were considered significant at 95% probability level ($p < 0.05$). A generalized Procrustes analysis (GPA) was performed on the sensory analysis results, to obtain a spatial configuration of the samples through the judges' descriptions of the products, whereas the Pearson correlation coefficient was computed to extrapolate the correlations among the type of extract and the volatile compounds found.

3. Results and Discussion

3.1 Total Polyphenols and Antioxidant Activity of OPE

OP is considered a rich source of phenolic compounds with a wide array of biological activity, as polyphenols in olive oil comprise about 1% of total fruit phenols, whereas the remaining part is lost with wastewater and pomace (Morsi et al., 2016). Indeed, several biophenols have been identified in OP, as secoiridoids, simple phenols, flavonoids, phenolic acids, and lignans because of their antioxidant activity (Alimenti et al., 2023). For this reason, a preliminary characterization of the extracts was performed (in both free and microencapsulated form) to evaluate the TPC and the antioxidant activity (Figure 2). The results showed a higher content of TPC in F-OPE compared to M-OPE (110 vs. 71 mg GAE g^{-1} extract). The TPC results of F-OPE were in good agreement with those reported by Alimenti et al. (2023), who characterized aqueous extracts of OP. Mesquita et al. (2022) attributed the loss of TPC after microencapsulation of vegetable extracts to the dilution of the compounds present in the extract caused by the wall material. On the other hand, TPC losses could reasonably due to their thermal degradation during spray-drying treatment (Chen et al., 2021). Our results are consistent with Fuentes et al. (2023) and Sharayei et al. (2020), who reported a lower content of total polyphenols in microencapsulated extract of pomegranate peel and papaya, respectively, compared to free extracts. Moreover, Andrade et al. (2023) and Stojanovic et al. (2012) detected lower TPC in microencapsulated extracts of thyme and cashew apple, respectively, using inulin as wall material, with respect to extracts as such, corroborating our results. A loss of TPC of around 40%, similar to our outcomes, was observed by Nguyen et al. (2021) on cocoa extract following the microencapsulation technique. Concerning the antioxidant activity, both DPPH and ABTS assays showed F-OPE having higher values than M-OPE (88.63 vs. 71.60 and 24.50 vs. 22.31 μmol TE g^{-1} extract, respectively), matching TPC results. Indeed, when compared to F-OPE, OPE microcapsules exhibited a loss of 12% in antioxidant activity, being in line with results reported on free and microencapsulated azolla fern by Elsebaie et al. (2022). The ABTS assay values were in line with data reported by De Bruno et al. (2018) on the ethanol/water extract of OP. On the whole, these results corroborate the influence of the microencapsulation techniques on the characteristics of the extracts, as shown by the literature (Awolu et al., 2022; Fuentes et al., 2023; Mesquita et al., 2022; Nguyen et al., 2021). Indeed, Soottitantawat et al. (2005) reported that the type of wall and core compounds can significantly affect the chemical characteristics of microcapsules. The higher the volatility of the components of the core material, the higher the amount of their excretion from inside the microcapsules during the drying process.

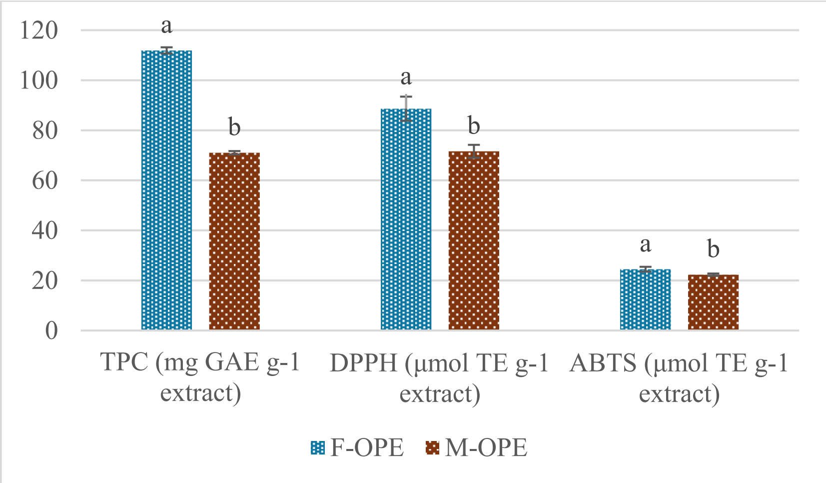


Figure 2. Total polyphenol content and antioxidant activity of OPE. The values represent means of triplicates ± standard deviation; [a,b] different letters mean significant statistical differences ($p < 0.05$) to one-way ANOVA followed by Tukey's HSD test.

Abbreviations: M-OPE, microencapsulated olive pomace extract; F-OPE, free lyophilised olive pomace extract; TPC, total phenolic compounds.

*$p < 0.05$.

3.2 Results of Physicochemical Analysis on Fresh Pasta Samples

Table 2 reports the results of a two-way ANOVA with first-order interaction performed on the experimental dataset. It is possible to observe that the ANOVA model was found to be significant in all the parameters considered. Moreover, one of the two variables explored, concentration of OPE (mg kg^{-1}), resulted in having a significant effect on all the indices, whereas the type of OPE added resulted in having a significant effect on all the indices, except for a_w. Concerning the first-order interaction of the two variables (type *X* concentration), both moisture and pH parameters were not significantly affected.

Table 2. Results of the analysis of variance (two-way ANOVA) performed on the analytical dataset.

Determinations	**Model**		**OPE concentration (mg/kg)**		**Type of OPE**		**OPE concentration (mg/kg) *X* type**	
	Average	p	Average	p	Average	p	Average	p
Moisture (%)	44.68	**	77.06	**	30.58	**	3.17	NS
a_w	20.46	**	32.20	**	0.73	NS	12.71	*
pH	9.48	*	15.55	**	8.84	*	0.69	NS
TPC (mg GAE g^{-1} of fresh sample)	126.61	**	221.20	**	63.25	**	16.40	**
DPPH (µmol TE g^{-1} of fresh sample)	764.56	**	1455.94	**	89.0	**	65.28	**
ABTS (µmol TE g^{-1} of fresh sample)	1790.73	**	3173.37	**	818.56	**	202.87	**
L*	166553	**	310194	**	36989	**	15873	**
a*	1963.05	**	3133.01	**	1979.21	**	200.25	**
b*	975.02	**	1072.36	**	2309.38	**	161.84	**

Abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GAE, gallic acid equivalents; TE, Trolox equivalents; TPC, total phenolic content; NS, not significant; OPE, olive pomace extract.

Statistical significance at *$p < 0.05$, **$p < 0.01$.

In Table 3 can be observed the influence of using F-OPE or M-OPE on chemical results compared to CP. The data obtained showed that the addition of OPE significantly influenced the moisture of the product, as CP showed the highest moisture value (31.14%) versus about 29% of the experimental samples. It could be hypothesized that the increased dry matter in experimental samples led to a water content reduction (O'Brien et al., 2003). In fact, Ashfaq et al. (2022) found that the addition of vegetable wastes in experimental samples led to a decrease in moisture with respect to control pasta. Moreover, Šeregelj et al. (2022) observed the lowest moisture content in pasta samples with encapsulated carrot waste extract, due to a greater protein–polysaccharide interaction. However, lower moisture in experimental samples could be a benefit for shelf-life purposes (Manurung et al. 2024), as water content is critical for microbial growth and degradation of physicochemical and enzymatic reactions (Zheng et al., 2019). The addition of OPE did not influence the a_w of the pasta samples, in agreement with the outcomes of Batista et al. (2021). Concerning pH, the experimental samples showed significantly lower values than CP ($p < 0.05$), with FP having the lowest values, followed by MP samples. Difonzo et al. (2021) reported that OP is rich in acid compounds, such as phenolic acids (cinnamic, *p*-coumaric, caffeic, ferulic, and vanillic). The latter statement could justify pH reduction in experimental samples. Furthermore, the highest TPC in F-OPE (Figure 2) could justify its lower pH value compared to CP and MP samples. Our results are consistent with those obtained by Zamankhani et al. (2024), who reported a lower pH in instant noodles enriched with vegetable extracts. Fortified pasta samples showed significantly higher values of TPC and antioxidant properties than CP, as reported by Ombra et al. (2023). Moreover, Merendino et al. (2014) observed a TPC below 0.300 mg GAE g^{-1} in control spaghetti, corroborating our results. Conversely, FP and MP samples showed average values of 0.390 and 0.428 mg GAE g^{-1}, respectively. Concerning antioxidant properties, MP and FP samples showed about 3-fold (DPPH) and about 2-fold (ABTS) higher values than CP. According to ABTS assay, MP samples had higher antioxidant activity compared to samples with F-OPE. Similar results were found in literature, where the use of microencapsulated bioactive compounds improves the antioxidant activity more than free molecules (Kamali Rousta et al., 2021; Pillai et al., 2012). Colour is one of the most important quality parameters, which influences consumers' buying decisions (Axentii et al., 2023), and it greatly depends on the characteristics of the raw materials (Gupta and Sharma 2023). The lightness (L^*) ranges from 0, which corresponds to black, to 100, which corresponds to white, and it measures the amount of light reflected from one colour. The lightness of the experimental samples was significantly lower than CP. This could be reasonably due to the dark colour of OPE, according to other studies showing the same trend. Particularly, Zen et al. (2020) showed that control fresh pasta had the highest L^* value compared to free spirulina and spirulina microsphere-enriched pasta samples. The redness index (a^*) was significantly influenced by the type of OPE added; it could range from negative to positive values. In fact, CP exhibited a green hue tendency, having negative a^* value, whereas MP and FP samples had statistically higher a^* values because of the brown colour of the extracts. This is probably due to the anthocyanins, responsible for the red colour, present in OP (Criado et al., 2008). Our trend agrees with other studies; Durante et al. (2019) observed an increase of a^* with the addition of free and α-cyclodextrin enclosing pumpkin oil in spaghetti pasta. Moreover, Simonato et al. (2019) reported higher a^* values for those pasta samples in which durum wheat semolina was substituted with freeze-dried OP. Notably, experimental samples with OPE microcapsules showed higher a^* values than those with F-OPE. It could be assumed that this outcome is related to the presence of inulin (used as wall material) in the M-OPE (Difonzo, De Gennaro et al., 2022), as reported by Garbetta et al. (2020) on durum wheat spaghetti with inulin. Concerning the *b** parameter, positive values indicate a yellow colour tendency; otherwise, negative values indicate blue colour. Particularly, MP and CP samples had the highest *b** index, significantly higher than FP samples. Our trend appears in line with a study of Czubaszek et al. (2021), who observed, in enriched breads, an increase in the yellow colour with the addition of microencapsulated extracts. On the whole, the encapsulation of OPE was responsible for the decreasing of *L** value and for the increasing of *a** and *b** values, resulting in the darkening of MP samples compared to the others, as also reported by Kabir et al. (2024).

Table 3. Effect of the olive pomace extract (OPE) type on physicochemical and quality indicators of fresh pasta samples.

Samples	Moisture (%)	a_w	pH	TPC (mg GAE g^{-1} of fresh sample)	DPPH (µmol TE g^{-1} of fresh sample)	ABTS (µmol TE g^{-1} of fresh sample)	*L**	*a**	*b**
CP	31.14±0.08 [a]	0.97±0.01 [a]	5.72±0.01 [a]	0.29±0.01 [b]	0.12±0 [b]	0.21±0 [c]	80.73±0.01 [a]	-0.60±0.01 [c]	22.20±0.01 [a]
MP	29.11±0.84 [b]	0.96±0.009 [a]	5.52±0.08 [b]	0.43±0.060 [a]	0.38±0.08 [a]	0.44±0.020 [a]	75.17±1.94 [b]	0.49±0.40 [a]	22.11±0.26 [a]
FP	29.78±0.70 [b]	0.96±0.0 [a]	5.44±0.005 [c]	0.39±0.03 [a]	0.35±0.12 [a]	0.39±0.05 [b]	76.20±1.91 [b]	0.09±0.22 [b]	21.47±0.47 [b]

Mean ± standard deviation. [a,c] Different superscript in the same column followed by different letters indicate significant differences ($p < 0.05$) by one-way ANOVA followed by Tukey's HSD test.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; CP, control fresh pasta; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPC, total phenolic content; GAE, gallic acid equivalents; TE, Trolox equivalents; FP, mean of three experimental fresh pasta samples with different concentrations of F-OPE (free lyophilized extract); MP, mean of three experimental fresh pasta samples with different concentrations of M-OPE (microencapsulated extract).

*$p < 0.05$.

Table 4 shows the physicochemical results of the interaction between the type of extract and its concentration. Moisture content ranged between 31.14% and 28.07%, complying with Italian law (Italian Republic, 2001), according to which fresh pasta must have at least 24% moisture. The addition of OPE significantly influenced moisture content, as already shown in the previous table; however, the higher the OPE concentration, the lower the moisture content of the product, in agreement with results of Šeregelj et al. (2022). In fact, MP-3 and FP-3 showed the lowest moisture values (28.07% and 28.96%, respectively), with the former showing significantly lower water content compared to the latter. This could be related to the water-binding activity of inulin, used as wall material, as reported by several researchers (Juszczak et al., 2012; Meyer and Blaauwhoed 2009). Moreover, Teodoro et al. (2014) observed a significantly higher moisture reduction of the dough with encapsulated rosemary oil compared to the dough with pure oil. Concerning a_w, no differences were observed among samples and CP, except for MP-3, which showed a significantly lower value compared to the control (0.95 vs. 0.97, respectively). Inulin, applied as wall material, contributes to greater binding with the water molecules, which may explain the reduction in a_w (Zen et al., 2020). However, fresh pasta samples still comply with D.P.R. February 9, 2001, No. 187, according to which the a_w of fresh pasta samples should not be lower than 0.92 and higher than 0.97. These results match those of Zen et al. (2020), in which samples of pasta added with microencapsulated spirulina presented lower a_w, as microencapsulated extracts were lyophilized, and the higher the amount used, the drier the dough compared to the control. In our study, the amount of lyophilized and microencapsulated extract added to the dough was different among MP and FP, because F-OPE contained higher TPC than M-OPE (as shown in Figure 2). Thus, to obtain the experimental samples with the same three levels of TPC, higher amounts of M-OPE were added to the dough. Concerning the pH, CP showed a significantly higher value than the experimental samples, among which no differences were found. As expected, the TPC increased with increasing concentrations of OPE added to fresh pasta samples. The MP-3 sample showed a significantly higher TPC value than FP-3 pasta sample. It was predictable, as the TPC deeply depends on the gross composition of the final product, which in turn is affected by the manufacturing process. In fact, it is known that the latter leads to a TPC loss in the final product, as reported by many authors (Choudhury et al., 2021; Kamali Rousta et al., 2021; Zhu et al., 2021). Considering the sum of the phenolic content found in CP and the theoretical phenolic concentration added with OPE, it is possible to estimate the TPC loss of each sample. FP samples showed a TPC percentage loss of 21%, 40%, and 47% for FP-1, FP-2, and FP-3, respectively; however, MP samples showed a reduced TPC percentage loss, with 21%, 32.8%, and 39% for MP-1, MP-2, and MP-3, respectively. These results suggested that processing procedures, as well as thermal treatment, could have reduced TPC, as previously reported by Dias et al. (2021) and Vuong et al. (2013). Notably, these outcomes suggest that microencapsulation better preserves

TPC from manufacturing process loss, as mentioned by several authors (Mehta et al., 2022; Pattnaik et al., 2021; Zabot et al., 2022). A similar kind of trend was observed by other researchers. Elsebaie et al. (2022) and Zen et al. (2020) observed a reduced antioxidant activity after heat treatment of Azolla fern and spirulina extract and its microcapsules, respectively; however, a smaller reduction was observed for the encapsulated extracts, corroborating our results. In fact, MP samples showed significantly higher TPC values than FP samples except for the lowest OPE concentration used. Concerning the antioxidant activity evaluation, it has been demonstrated that encapsulation could decrease the antioxidant capacity, underestimating the effective antioxidant activity (Dordoni et al. 2021). As expected, results showed lowest value for CP compared to MP and FP samples, which were characterized by high antioxidant activity in conjunction with the highest OPE concentration. Concerning colour parameters, the inclusion of both extracts in pasta samples caused significant differences in L^* compared to CP. In fact, the lightness decreased with increasing concentration of OPE added to fresh pasta samples. Similar conclusions were drawn by Elsebaie et al. (2022), who observed a significant decrease in lightness in macaroni samples with free and microencapsulated Azolla fern. Furthermore, the a^* value was also significantly influenced by the addition of OPE ($p < 0.05$), and for MP samples, it was directly proportional to M-OPE concentration. Contrary to FP-2, FP-1 and FP-3 showed higher ($p < 0.05$) a^* values compared to CP. Indeed, MP-3 and FP-3 samples demonstrated the highest a^* values because of the colour of raw materials. These results are in agreement with data reported by Ćetković et al. (2022), who observed an increase of a^* values by increasing the amount of encapsulated carrot waste extract to pasta samples. Interestingly, samples with M-OPE showed higher a^* values than FP samples when considering the same OPE concentration level added. Moreover, Zamankhani et al. (2024) reported lower a^* values for experimental noodles with free borage and black hollyhock extracts compared to the ones with microcapsules. In line with data reported by Simonato et al. (2019), yellowness showed a decreasing trend as the OPE concentration increased. It is important to mention that consumers greatly appreciate durum wheat pasta with a high yellowish colour (Troccoli et al., 2025). Among the experimental samples, MP samples were characterized by higher b^* values compared to pasta samples with F-OPE. Particularly, MP-1 had the highest yellowness among all pasta samples. These results match those obtained by Difonzo, De Gennaro et al. (2022), which showed that the higher the concentration of inulin, the lower the b^* values.

Table 4. Results of the interaction of the type of olive pomace extract (OPE) and its concentration on physicochemical and quality indicators of fresh pasta samples.

	CP	MP-1	MP-2	MP-3	FP-1	FP-2	FP-3
Moisture (%)	31.14±0.08 ^{a}	29.85± 0.04 ^{bc}	29.41±0.47 ^{cd}	28.07±0.15 ^{e}	30.09±0.02 ^{bc}	30.29±0.12 ^{b}	28.96±0.44 ^{d}
a_w	0.97±0.01 ^{a}	0.97±0.01 ^{a}	0.96±0.01 ^{ab}	0.95±0.01 ^{b}	0.96±0.01 ^{ab}	0.96±0.02 ^{ab}	0.96±0.01 ^{ab}
pH	5.72±0.01 ^{a}	5.48±0.10 ^{b}	5.53±0.04 ^{b}	5.55±0.10 ^{b}	5.45±0.07 ^{b}	5.44±0.04 ^{b}	5.44±0.50 ^{b}
TPC (mg GAE g^{-1} of fresh sample)	0.29±0.01 ^{d}	0.36±0.01 ^{c}	0.43±0.01 ^{b}	0.50±0.01 ^{a}	0.36±0.01 ^{c}	0.38±0.01 ^{c}	0.43±0.01 ^{b}
DPPH (µmol TE g^{-1} of fresh sample)	0.12±0.01 ^{g}	0.28±0.20 ^{e}	0.41±0.30 ^{c}	0.46±0.10 ^{b}	0.21±0.01 ^{f}	0.35±0.02 ^{d}	0.49±0.10 ^{a}
ABTS (µmol TE g^{-1} of fresh sample)	0.210±0 ^{e}	0.411±0 ^{c}	0.442±0 ^{b}	0.454±0 ^{a}	0.314±0.01 ^{d}	0.414±0 ^{c}	0.433±0 ^{b}
L*	80.73±0.01 ^{a}	77.60±0.02 ^{c}	74.74±0.01 ^{e}	73.17±0.01 ^{g}	77.81±0.01 ^{b}	77.11±0.02 ^{d}	73.68±0.01 ^{f}
a*	-0.60±0.01 ^{g}	0.20±0.02 ^{d}	0.25±0.01 ^{c}	1.03±0.04 ^{a}	-0.01±0.0 ^{e}	-0.11±0.0 ^{f}	0.37±0.03 ^{b}
b*	22.20±0.01 ^{b}	22.45±0.0 ^{a}	22.00±0.01 ^{d}	21.86±0.01 ^{e}	22.09±0.01 ^{c}	21.06±0.01 ^{g}	21.26±0.07 ^{f}

Means of triplicates ± standard deviation. ^{a,g} different superscript in the same raw followed by different letters indicate significant differences ($p < 0.05$) by two-way ANOVA followed by Tukey's HSD test. Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3). *$p < 0.05$.

Figure 3 shows the TPC values in raw and cooked fresh pasta samples. Cooked pasta results agree with those obtained by Simonato et al. (2019), who reported a loss of TPC because of the cooking process of pasta. The reduction observed could be attributed to the molecules' heat degradation during boiling treatment or their solubilization into the cooking water (Biernacka et al., 2022). Padalino et al. (2018) reported high levels of free phenolic compounds (e.g., luteolin and tyrosol) in OP, which are more water-soluble compared to the conjugated forms, corroborating our results. Despite the reduction, MP-3, MP-2, and FP-3 samples still had the highest polyphenol content. In general, MP samples showed a higher reduction percentage after the cooking process than FP samples. These outcomes could be accounted to the imperfect formation and low stability of the gluten matrix (especially for MP-3 sample, as reported in Section 3.5), which could promote losses during cooking (Vimercati et al., 2020). Nonetheless, MP-cooked samples had a final polyphenol concentration, on average, similar to or higher than that of FP samples. In fact, cooked MP-3 and FP-3 showed similar TPC values, and MP-1 and MP-2 had higher amounts of polyphenols than FP-1 and FP-2. In addition, despite the recorded losses, the experimental samples showed higher antioxidant activity than CP, according to the DPPH and ABTS assays (Table S1). These findings agree with data reported by Simonato et al. (2019) on cooked pasta enriched with OP.

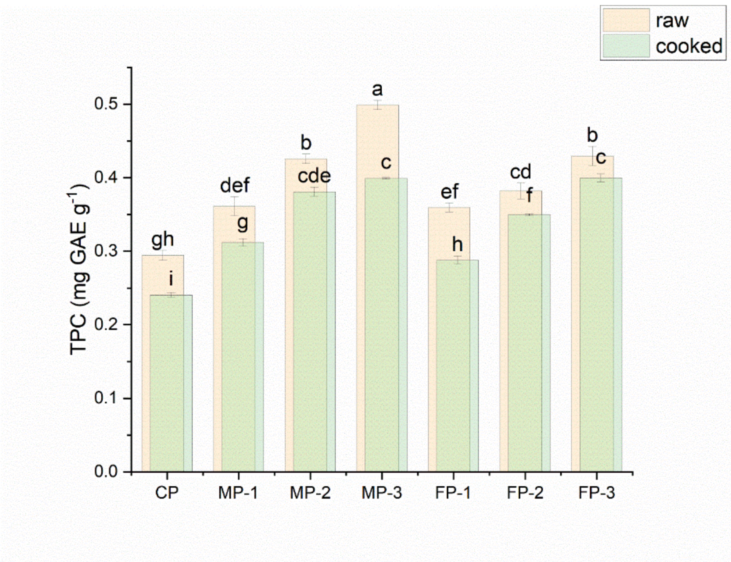


Figure 3. Total polyphenol content of raw and cooked fresh pasta samples. The values represent means of triplicates ± standard deviation; [a,h] different letters mean significant statistical differences ($p < 0.05$) to one-way ANOVA followed by Tukey's HSD test.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3); GAE, gallic acid equivalents; TPC, total phenolic content. *$p < 0.05$.

3.3 Volatile organic Compounds

Fresh pasta samples were subjected to the analysis of volatile compounds through SPME-GC/MS. Ten VOCs were identified, belonging to various chemical classes, such as aldehydes, alcohols, furans, and acids. The flavour of pasta products deeply depends on the ingredients and the manufacturing process (Santagata et al., 2021). Figure 4 shows the scatter plot of the volatile profile of all fresh pasta samples, where on the right sides are reported the distribution curves of the single VOC. The latter gives information about the variability of the single VOC concentration among the samples; thus, the wider the curve, the higher the variability among samples. Indeed, the hexanal showed a wide variability (it ranged from 10 to 24 $\mu g\ g^{-1}$ of fresh pasta), whereas other compounds showed a narrowed distribution range (as nonanal, 3.4–6.4 $\mu g\ g^{-1}$). Interestingly, it was observed that all the samples shared the same volatile compounds, which means that the addition of the extract did not enrich the product with new molecules that may worsen the typical VOC profile of fresh pasta. However, differences ($p < 0.05$) in single-volatile compounds were found and are shown in Table S2. The results highlighted that, for most of the single compounds, there is a slight uptrend in conjunction with the OPE concentration, as for pentanal, heptanal, and amyl alcohol ($p < 0.05$), whereas in some other cases, the increase was not significant (i.e., *trans*-2-heptenal, hexanoic acid, and nonanal). Cecchi et al. (2022) investigated the VOCs of OP and reported the presence of 20 aldehydes, most of which derive from the lipoxygenase pathway. Considering that 70% of the volatile compounds of our samples are represented by aldehydes, a great contribution to their upward trend may be due to the OPE addition. As reported by Caponio et al. (2022), the addition of olive oil by-products led to an increase in benzaldehyde, as it is a common volatile compound of *Drupacee*. Other pathways could lead to aldehyde and furan enrichment (i.e., nonanal, heptanal, hexanal, and 2-pentylfuran), as the Maillard reaction and oxidation of oleic and linoleic fatty acids (Caponio et al., 2022; Tomé-Rodríguez et al., 2021). On average, fresh pasta samples with F-OPE showed a higher VOC content than those added with M-OPE, meaning that microencapsulation limits the release of volatile compounds. On the other hand, F-OPE may have a greater influence on the sensory characteristics of the product by releasing molecules having low odour threshold (i.e., hexanal, benzaldehyde, and 2-pentylfuran) (Marzano et al., 2022).

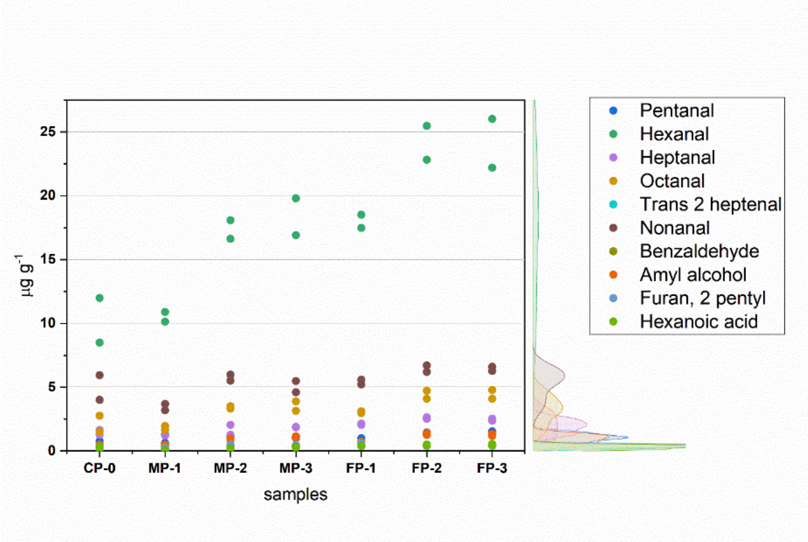


Figure 4. Scatter plot of volatile organic compounds of control pasta and experimental samples. Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3).

Figure 5A, B shows the correlation plot of the FP and MP samples, considering VOC and OPE concentrations. The upper part of the graph showed the positive (red) or negative (blue) correlation, the bigger the circle, the higher the correlation. Moreover, the numbers above the circles indicate a non-significant correlation ($p > 0.05$). However, the lower part of the graph reports the Pearson correlation coefficient (r). Almost all the volatile compounds of FP samples showed a strong positive correlation with OPE concentration, with an r value up to 0.92, even if not always significant ($p > 0.05$). A negative correlation was observed only for 2-pentylfuran; although no significant differences were observed according to Table S2, there was a slight decreasing trend from FP-1 to FP-3. Such molecule could be responsible for off-flavour, as it is the typical degradation product of the linoleic acid acyl group, and it is usually found in oxidized oil (Zhang et al., 2023). Concerning Figure 5B, all the volatile compounds showed a strong positive correlation with the OPE increasing concentrations, but only five of them were statistically significant (hexanal, octanal, *trans*-2-heptenal, amyl alcohol, and hexanoic acid), which means that the higher the OPE concentration, the higher the VOCs concentration. The r value resulted very high range, from 0.85 to 0.98 (for those with $p < 0.05$).

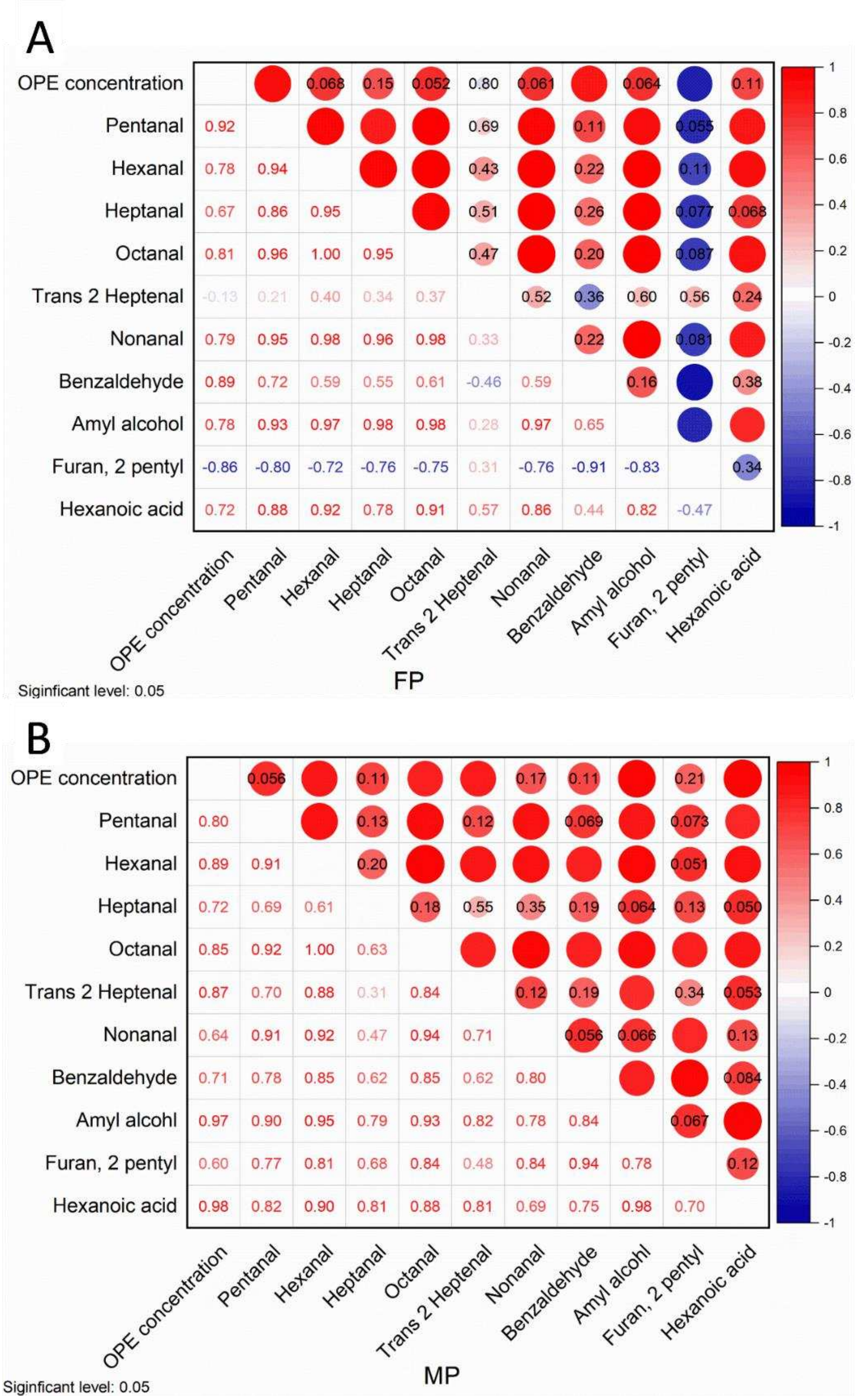


Figure 5. Correlation map between volatile organic compounds of the pasta samples and the concentration of free OPE (A) and microencapsulated OPE (B) used. *$p < 0.05$.

3.4 Sensory Analysis

Figure 6 shows the GPA of the sensory analysis performed on cooked fresh pasta samples. GPA is a multivariate exploratory technique useful for the comparison of panellists' results, obtaining a consensual configuration among the descriptors of the products. The analysis explained 89.02% of variance, 76.77% of which was along the first component (PC1) and 12.25% along the second component (PC2). The first component clearly showed the effect of the addition of different OPE concentrations. In fact, it is possible to observe a clear separation among samples within three different clusters. The one on the positive side of PC1 represents the samples with the mildest and most typical sensory characteristics, in fact, this area comprises CP and the experimental samples with the lowest OPE concentration, indicating that these samples resulted in very similar results for judges. Then, on the negative side of the PC1 clustered the other two groups, representing the samples at higher OPE concentration used. These two groups differed by considering the PC2, meaning that they were perceived differently by judges. Concerning the descriptors, the group that lay on the II quadrant was characterized by a characteristic odour and taste. As shown in Figure S1, the intensity of odour and taste linked with raw cereal was significantly more intense in CP in comparison to FP-3 and MP-3. Despite the typical odour of fresh pasta being less perceived in experimental samples, the addition of OPE did not impair the smell profile of samples, as judges did not perceive unpleasant odours. The latter outcome was expected, as the concentration of OPE to be added to the dough was chosen also on the basis of preliminary trials. Indeed, no significant differences in odour were found between CP and MP-1, demonstrating the capability of the encapsulation process to retain unpleasant odours, in line with the data obtained from VOCs analysis. Cecchi et al. (2019) reported a higher wheat odour and taste intensity in the control sample with respect to pasta sample added with freeze-dried pâté, corroborating our results. As regards hardness, in line with data reported by Zamankhani et al. (2024) on noodles with free and microencapsulated vegetable extracts, although no significant differences were shown among CP and experimental samples, CP and MP-1 were the ones with the most appreciated texture, followed by MP-3 and FP-2. On the other hand, as shown in Figure 6, the other two groups were characterized by colour, astringency, and bitterness, surely provided by the increasing OPE concentration. Indeed, in accordance with instrumental analysis, control fresh pasta was characterized by a lighter yellow intensity than other samples in which OPE was added, which showed a colour similar to light brown. This was particularly noted by panellists in MP-3 and FP-3, followed by MP-2 and FP-2. Nonetheless, bitterness was perceived mostly in FP-3 and FP-2, whereas FP-3 also showed the highest astringency. However, it has to be said that, when considering the overall acceptability (Figure S1), all the samples gained a positive score, except for FP-3, which gained the lowest final score. Zamankhani et al. (2024) reported that experimental samples with free black hollyhock extract gained a lower score for overall acceptability compared to the control and to experimental samples with microencapsulated extract. Furthermore, our outcome agrees with data reported by Cecchi et al. (2019) and Padalino et al. (2018), who reported reduced acceptability for spaghetti samples with dehydrated olive paste.

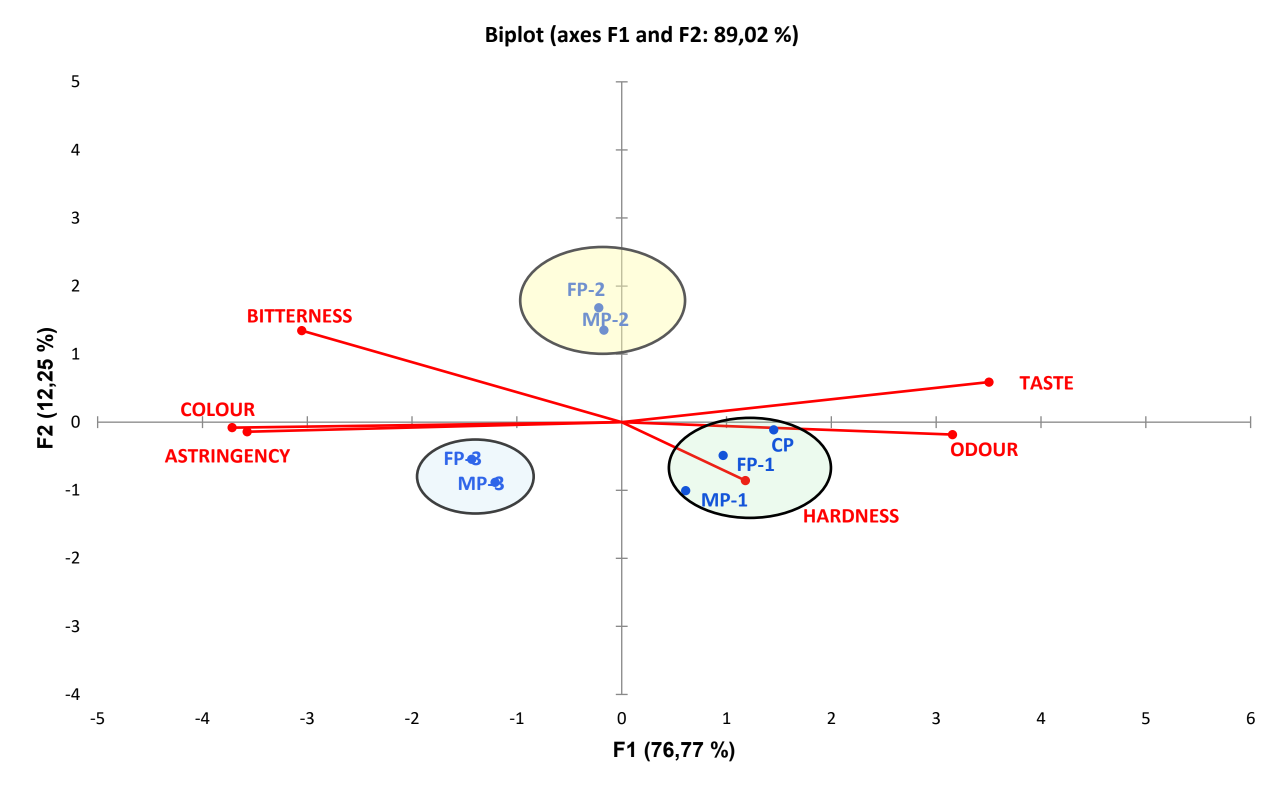


Figure 6. Generalized Procrustes Analysis (GPA) of the sensory analysis results.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3).

3.5 Microstructural Evaluation by SEM

The microstructural properties of cooked pasta samples as affected by OPE enrichment were investigated by using SEM (Figure 7). Cross-section images of the raw pasta samples are reported in Figure S2. Concerning CP, structural changes were revealed in both FP and MP samples, which were in turn affected by the amount of OPE added to the formulation. In line with results reported by Pillai et al. (2012), CP showed a complex, porous but uneven gluten network with filamentous regions that define the presence of honeycomb structures, which are the consequences of water penetration and diffusion gradients, as well as of protein coagulation and starch gelatinization. In FP-1, the addition of the freeze-dried extract determined the formation of a denser but discontinuous matrix, whereas further extract amounts resulted in more porous (FP-2) and spongy structures (FP-3), in which larger pores were found. In MP-1 sample, the protein network seemed denser and characterized by a finer and more regular porosity with respect to CP, whereas a further OPE addition revealed an irregular network with an alternation of compact/agglomerated and loose zones. Honeycomb structures were present in both MP-1 and MP-2. Unexpectedly, the addition of the highest M-OPE amount determined the formation of a much denser and agglomerated network in MP-3, where the typical gluten network development was lost. The addition of OPE clearly affected pasta microstructure, and the effect was different depending not only on the concentration added but also, concentration being equal, on its typology (free or microencapsulated), showing a direct involvement of the wall material used for the entrapment. Several works in the literature report the capacity of either phenolic compounds or inulin to interact with gluten and, in turn, affect the network development, as well as quality attributes of the derived pasta (Difonzo, De Gennaro et al., 2022; Padalino et al., 2018). Although the microstructural diversity among the pasta samples is evident, this had no impact on texture attributes, as no significant differences were perceived by the panellists (Figure S1).

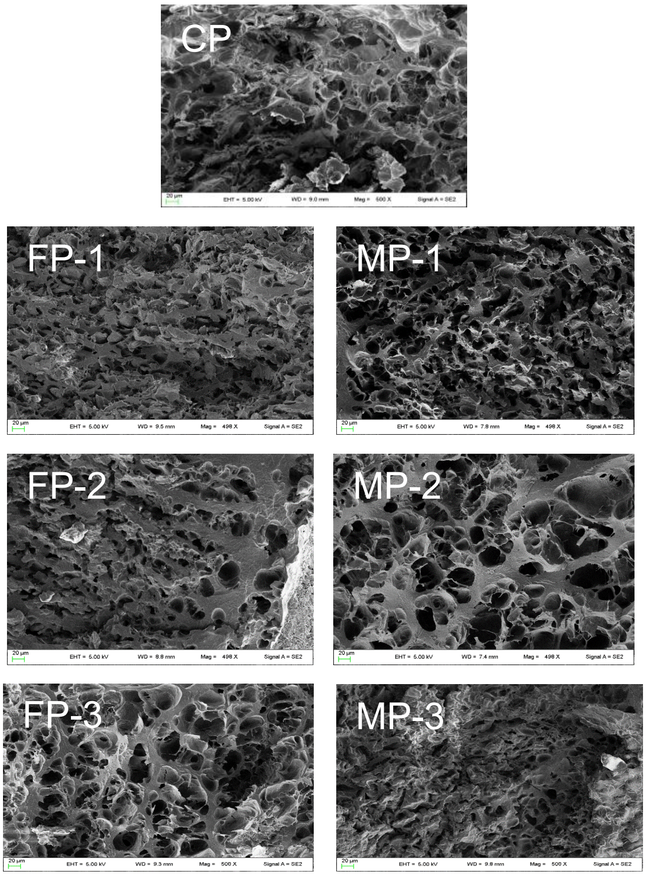


Figure 7. SEM cross-section micrographs of cooked pasta samples.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3).

4. Conclusions

This study explored the effect of the addition of OP extract as such and microencapsulated on the chemical, microstructural, and sensorial properties of enriched fresh pasta samples. Although the inclusion of M-OPE and F-OPE in fresh pasta formulations decreased the water content, all the experimental samples still complied with Italian Law (Italian Republic, 2001), according to which fresh pasta must have at least 24% moisture. This study proved that the microencapsulation process could be an effective way to preserve polyphenols during food production. Indeed, cooked samples with microencapsulated extract had, on average, similar or higher total polyphenol content than that of samples with OPE as such. Moreover, experimental samples with microencapsulated extracts had higher *b** values, greatly appreciated by consumers. The volatile organic profile was not worsened by the inclusion of OP in pasta formulations; however, the microencapsulation technique limited the release of volatile compounds. According to SEM analysis, the addition of OPEs clearly affected pasta microstructure; nevertheless, panellists did not perceive significant differences, as regards texture, among control and experimental samples. The latter were greatly appreciated by consumers, except for the experimental sample with the highest concentration of OPE as such. In conclusion, from a practical point of view, the use of OPE in the food sector allows the upcycling of the main olive oil by-product, improving at the same time the nutritional profile of the fortified products and obtaining fresh pasta samples with high added value. On the basis of the promising results, especially from a nutritional point of view, further investigation should focus on the stability of experimental products during storage, evaluating the ability of such functional ingredients to extend the shelf-life of the final product.

Supplementary Table 1. Antioxidant activity of cooked fresh pasta samples.

	DPPH (μmol TE g^{-1} fresh sample)	ABTS (μmol TE g^{-1} fresh sample)
CP cooked	0.02 ± 0.01^{f}	0.02 ± 0.01^{g}
MP-1 cooked	0.07 ± 0.01^{e}	0.19 ± 0.01^{e}
MP-2 cooked	0.23 ± 0.01^{b}	0.22 ± 0.01^{c}
MP-3 cooked	0.31 ± 0.01^{a}	0.28 ± 0.01^{a}
FP-1 cooked	0.11 ± 0.02^{d}	0.21 ± 0.01^{d}
FP-2 cooked	0.15 ± 0.02^{c}	0.16 ± 0.01^{f}
FP-3 cooked	0.29 ± 0.01^{a}	0.27 ± 0.01^{b}

Means of triplicates ± standard deviation. ^{a,g} Different superscript letter in same column followed by different letters indicate significant differences in the function of the treatment ($p < 0.05$) by one-way ANOVA followed by Tukey's HSD test.

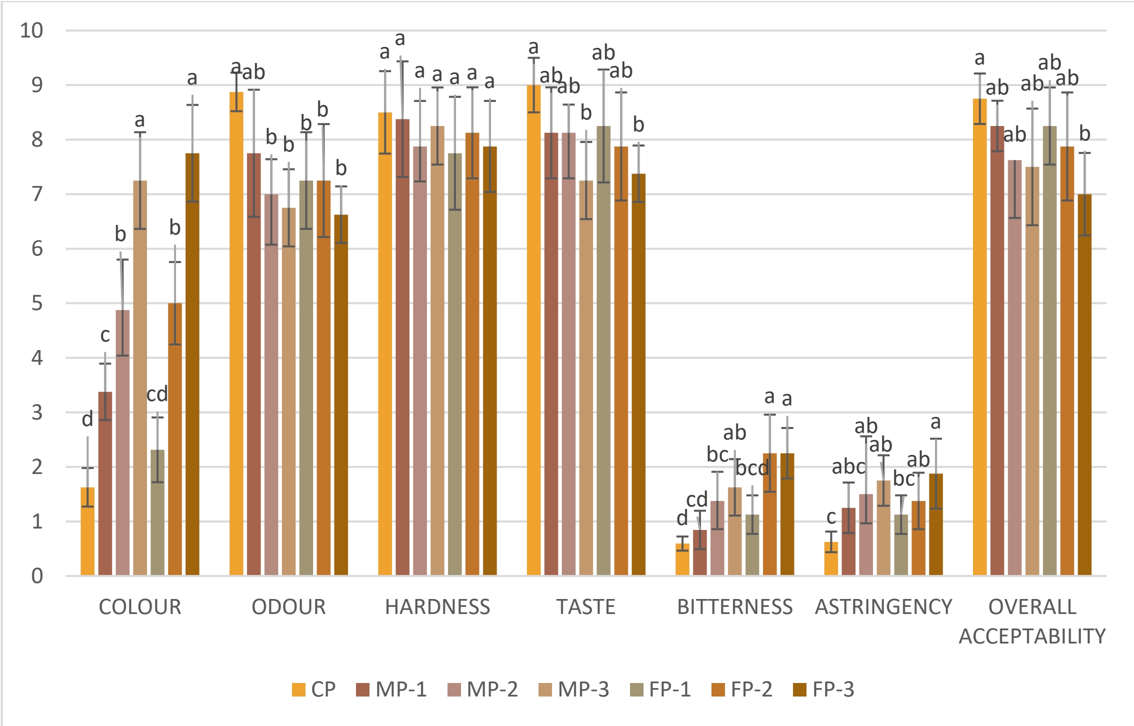
Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3). *$p < 0.05$.

Supplementary Table 2. Volatile organic compounds of experimental and control pasta samples.

	Pentanal	Hexanal	Heptanal	Octanal	Trans-2 - heptenal	Nonanal	Benzaldehyde	Amyl alcohol	Furan, 2-pentyl	Hexanoic acid
CP	0.678±0.15 de	10.249±2.47 b	1.471±0.26 b	2.144±0.88 b	0.259±0.23 a	4.982±1.38 ab	0.325±0.03 b	0.425±0.11 d	0.228±0.08 b	0.254±0.16 abc
MP-1	0.581±0.07 e	10.523±0.54 b	1.290±0.10 b	1.811±0.22 b	0.244±0.0 a	3.446±0.36 b	0.319±0.04 b	0.507±0.01 d	0.230±0.03 b	0.079±0.02 c
MP-2	1.108±0.03 bc	17.359±1.02 ab	1.668±0.55 ab	3.422±0.14 ab	0.345±0.10 a	5.750±0.33 ab	0.411±0.20 ab	0.937±0.06 bc	0.445±0.09 ab	0.198±0.03 bc
MP-3	1.050±0.05 bc	18.357±2.04 a	1.883±0.020 ab	3.524±0.52 ab	0.423±0.03 a	5.050±0.62 ab	0.404±0.04 ab	1.120±0.05 ab	0.394±0.12 ab	0.297±0.02 abc
FP-1	1.010±0.003 cd	17.998±0.73 a	2.100±0.07 ab	3.042±0.10 ab	0.438±0.0 a	5.407±0.26 ab	0.408±0.00 ab	0.733±0.0 cd	0.607±0.0 a	0.392±0.0 ab
FP-2	1.371±0.13 ab	24.146±1.87 a	2.594±0.08 a	4.412±0.44 a	0.447±0.04 a	6.460±0.35 a	0.454±0.43 a	1.341±0.1 a	0.448±0.05 ab	0.469±0.06 ab
FP-3	1.498±0.07 a	24.108±2.70 a	2.457±0.11 a	4.442±0.49 a	0.431±0.03 a	6.436±0.23 a	0.493±0.00 a	1.270±0.12 a	0.432±0.04 ab	0.500±0.08 a

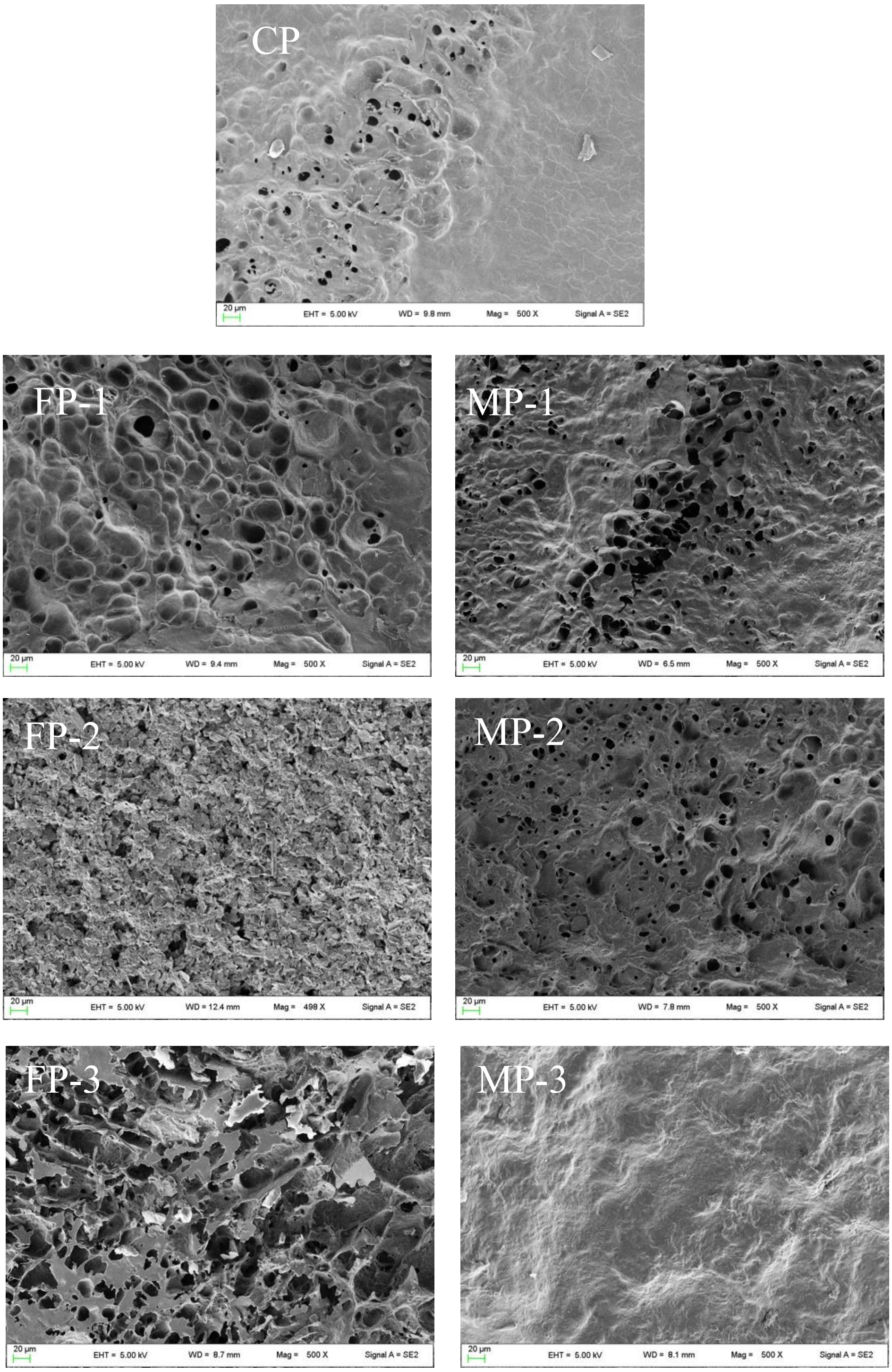
Means of duplicates ± standard deviation. [a,e] Different superscript letter in the same column followed by different letters indicate significant differences in the function of the treatment ($p < 0.05$) by two-way ANOVA followed by Tukey's HSD test. The results are expressed as μg g^{-1} of fresh pasta.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3). *$p < 0.05$.



Supplementary Figure 1. Results of sensory analysis of cooked fresh pasta samples. The error bars indicate the standard deviation. Different letters mean significant statistical differences ($p < 0.05$) to one-way ANOVA followed by Tukey's HSD test.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3). *$p < 0.05$.



Supplementary Figure 2. SEM surface micrographs of cooked pasta samples. Abbreviations: CP, Control fresh pasta; MP, fresh pasta sample added of microencapsulated OPE at three different concentrations (MP-1, MP-2, MP-3); FP, fresh pasta added of free OPE at three different concentrations (FP-1, FP-2, FP-3).

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Publication no. 2:

Impact of Free and Microencapsulated Olive Pomace Extract on the Quality Evolution of Fresh Pasta During Storage

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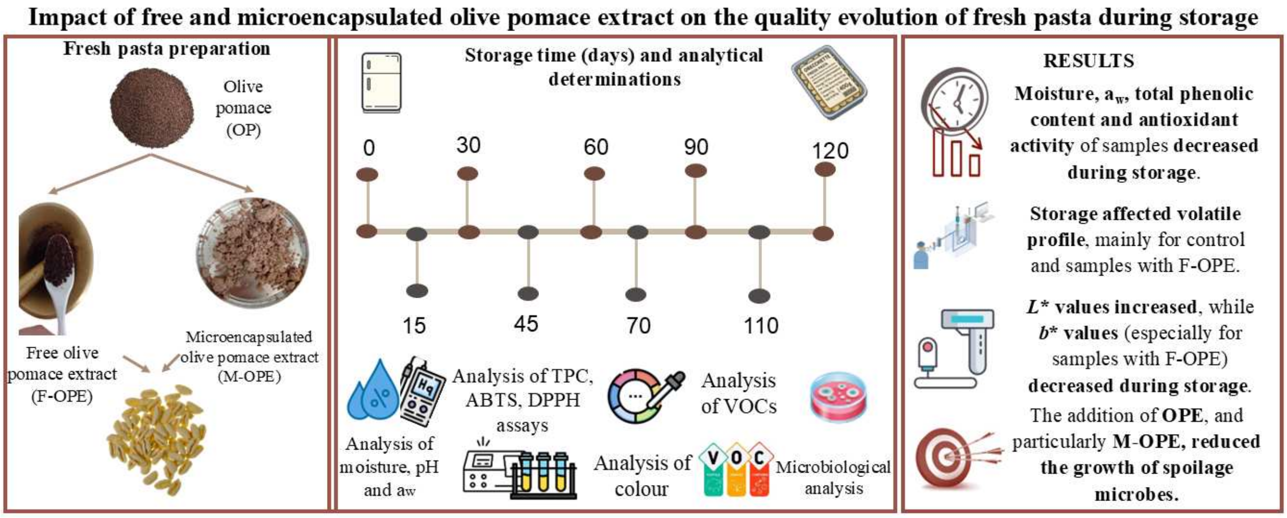
Graphical Abstract



Abstract

Microencapsulated and freeze-dried phenolic olive pomace extracts (M-OPE and F-OPE, respectively) were added to "cavatelli" fresh pasta samples, aiming to evaluate their effects on the physicochemical and microbiological quality of fresh pasta during refrigerated storage for 120 days. The results showed a reduction in moisture, a_w, total phenolic compounds and antioxidant activity throughout storage. However, at the end of storage, pasta samples had moisture and a_w higher than 24% and 0.92, respectively. Notably, experimental samples with M-OPE showed lower water content, and higher TPC, compared to the corresponding samples with F-OPE. Both M-OPE and F-OPE significantly reduced pH values, compared to control pasta (CP). *L** values decreased as the extracts increased, showing an uptrend from the time of production, until 120 days of storage. Samples with M-OPE had higher *a** values, compared to the corresponding samples with F-OPE. On the other hand, yellow index (*b**) showed a decreasing trend until end of storage time, more pronounced for samples with F-OPE. Just after production, CP and experimental samples showed low concentrations of volatile organic compounds. However, the oxidation process during storage affected volatile profile, particularly for CP and for the sample with the highest F-OPE content. The addition of the extracts, and particularly M-OPE, reduced the growth of spoilage microorganisms; experimental samples exhibited significantly lower counts for total aerobic mesophilic bacteria, moulds and yeast microbial groups, compared to CP, showing OPE potential to extend the shelf-life of fresh pasta.

Keywords: fresh pasta; storage; olive pomace extract; microencapsulation; microbiological quality.

1. Introduction

Pasta is a cereal-based food of the traditional Mediterranean diet, appreciated worldwide due to its convenience, palatability, and nutritional quality (Zardetto et al., 2021). According to the Italian legislation (Presidential Decree no. 187 of 9 February 2001), "pasta" is defined as the product obtained by extrusion or lamination and successive drying (to 12.50% maximum water content) of a dough made of durum wheat semolina and water, whereas the use of soft wheat flour is allowed for the fresh pasta production. The latter contains at least 24% of moisture, which makes it more susceptible to spoilage and alteration. For this reason, fresh pasta requires refrigerated storage temperatures and a thermal treatment at least equivalent to pasteurization (as its water activity ranges from 0.92 to 0.97) to ensure shelf-life periods ranging from 30 to 60 days (Marzano et al., 2022; Pagani et al., 2007). Fresh pasta spoilage is mainly due to the microbial activity of yeasts, bacteria and moulds, which can rapidly proliferate in this product (Angiolillo et al., 2017; Costa et al., 2010; Del Nobile et al., 2009b), compromising its safety and depleting the typical sensory characteristics (Marzano et al., 2022). In order to improve the microbial stability and extend the shelf-life of fresh pasta, different methods have been applied in literature: i) the modified atmosphere packaging (MAP) technique (Costa et al., 2010; Del Nobile et al., 2009a; Lee et al., 2001; Marzano et al., 2022; Sanguinetti et al., 2011; Sanguinetti et al., 2016; Zardetto, 2005); ii) the addition of chemical preservatives, such as calcium propionate, potassium sorbate, sodium benzoate and sodium dehydroacetate (Li et al., 2011; Marzano et al., 2022); iii) gamma irradiation and bio-preservation (Cassares et al., 2020; Duan et al., 2025; Marzano et al., 2022; Nie et al., 2023; Schettino et al., 2020). On the other hand, the use of natural ingredients with antimicrobial properties has gained considerable attention from researchers (Maddaloni et al., 2025), driven by the increasing consumer demand for more natural and high quality food. Such food is characterized by a well-balanced nutritional profile, that simultaneously supports a more favourable environment in the gut (Celano et al., 2024) and contains a low content or complete absence of synthetic additives ("clean label"). For this reason, flaxseed flour, lemon and grapefruit extracts, chitosan, and spice extracts have been investigated in fresh pasta, as natural preservatives (Costa et al., 2010; Del Nobile et al., 2009a; Manthey et al., 2008; Miceli et al., 2015; Schettino et al., 2020). Moreover, from a sustainability perspective, fruit and vegetable by-products could be used as a valuable source of phytochemicals, with recognized antimicrobial and antioxidant activities (Lacivita et al., 2022; Maddaloni et al., 2025; Panza et al., 2023; Pasqualone et al., 2017), reducing their environmental and economic impact. Based on these considerations, fig and pomegranate peels and broccoli by-products were used successfully to improve the microbiological and physiochemical quality of fresh pasta during storage (Angiolillo et al., 2019; Lacivita et al., 2022; Lordi et al., 2023; Panza et al., 2022). Olive oil chain plays an important role in the Mediterranean area (Simonato et al., 2019), but at the same time, it leads to the production of a huge quantity of waste and by-products (Dantas Palmeira et al., 2023), representing an important environmental issue. Olive pomace (OP), the major by-product from the olive oil industry (Difonzo et al., 2021; Zhao et al., 2023), is rich in functional compounds, such as polyphenols, tocopherols and sterols, with well-known antioxidants and anti-inflammatory properties (Difonzo et al., 2021; Simonato et al., 2019). In addition, authors stated that OP has antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi (Difonzo et al., 2021; Nunes et al., 2021). In line with these results, dried OP was used to improve the storage stability of cod sticks (Panza et al., 2020), mango (Motrih et al., 2025) and salamis (Jovanovichs et al., 2025). Phenolic compounds from OP were also applied in edible coatings, in order to reduce microbial proliferation (mesophilic aerobic bacteria, yeasts and moulds) of strawberries (Martins et al., 2024). Furthermore, Lordi et al. (2023) and Panza et al. (2023) used olive oil by-products as natural preservatives to maintain the quality of handmade fresh pasta for a longer period. However, food treatments and storage may reduce the antimicrobial efficacy of bioactive compounds (Castro-Rosas et al., 2017; Hosseini et al., 2019; Maddaloni et al., 2025). Indeed, microencapsulation of these natural preservatives can help to protect their activity and keep them stable, when added to food matrices (Castro-Rosas et al., 2017). Ganje et al. (2016) and Jafari et al. (2021) demonstrated that encapsulated phenols from olive leaf maintained the original quality and reduced the mould and fungal growth in tomato pastes. Jolayemi et al. (2021) used encapsulated olive leaf phenolic extract to protect salad dressings toward oxidation phenomena and physical instability during storage. Inulin, a soluble dietary fibre, has been successfully applied as wall material for the microencapsulation of essential oils, garlic extract and pineapple peel extract, through microencapsulation technique (Rodsuwan et al., 2024). Several authors, indeed, showed that inulin microcapsules were characterised by higher bulk density and wettability property (de Barros Fernandes et al., 2014; Fernandes et al., 2014), which are pivotal parameters for food production processes. Taking a sustainability-oriented approach, inulin could be recovered from artichoke roots, which are important agriculture waste (Difonzo et al., 2022b), especially in Southern Italy, which is the main globe artichoke–producing area in the country (Rana et al., 2023).

Recently, Natrella et al. (2025) focused on the physicochemical, nutritional and sensory properties of fresh pasta samples enriched with phenolic extract from olive pomace (OPE), obtaining samples with higher antioxidant activity, reduced moisture content, without compromising sensory acceptance. However, to the best of our knowledge, no studies have investigated the physicochemical and microbiological changes occurring in fresh pasta enriched with OPE during its shelf-life. In this context, the aim of this study was to comprehensively investigate the effects of the incorporation of this ingredient (in both its free and microencapsulated forms), on the physicochemical and microbiological quality of fresh pasta during storage.

2. Materials and methods

2.1 Production of olive pomace extract

Phenolic extract from olive pomace was obtained following the method proposed by De Gennaro et al. (2022), with some modifications. First of all, OP was freeze-dried (Büchi Technology, Flawil, Switzerland, LyovaporTM L-200) and mixed with a solution of ethanol: water (10:90 v/v) in a 1:5 (w/v) ratio. The mixture was subjected to ultrasound treatment for 15 min in an ultrasound bath, after being homogenized for 10 min. The mixture was homogenized again for 10 min and finally centrifugated at 15 °C and 12,000 × g for 10 min (Thermo Fisher Scientific, Osterode am Harz, Germany). The supernatants were collected, filtered with nylon filters (pore size 0.45 µm) and split in two aliquots; one was freeze-dried to obtain free lyophilised olive pomace extract (F-OPE) and the other part was used to produce microencapsulated olive pomace extract (M-OPE), produced as described below.

2.2 Microencapsulation of olive pomace extract

As first, inulin – to be used as wall material for the encapsulation process – was recovered from artichoke roots, as described by Difonzo et al. (2022b). Then, the inulin for microencapsulation was prepared as follows: a 20% (w/w) inulin in ultrapure water was solubilized at 80 °C under constant stirring, until the powder was completely dissolved. Then, the spraying solution was prepared by mixing the phenolic olive pomace extract with the inulin solution, in order to get a final inulin concentration in the mixture of 5% (w/w). The mixture was stirred in order to disperse the two phases and kept stirring throughout the spray-drying process. The encapsulation process was performed with a Mini Spray Dryer B-290 (Büchi Technology, Flawil, Switzerland) with the following operating conditions: inlet air temperature 150 °C, outlet air temperature 85-100 °C, feed rate of 30%, and aspiration of 100%, which in the adopted operating conditions corresponded to an air flow of around 25–30 m^3 h^{-1}; the encapsulation yield was 66%.

2.3 Fresh pasta samples preparation

Fresh pasta samples were produced on a semi-industrial plant in a factory located in Southern Italy (Pastificio Maffei, Cutrofiano, LE) according to the company's standard manufacturing procedure. Seven types of fresh pasta samples were prepared: one control (CP, control pasta made with durum wheat semolina), three experimental samples added with M-OPE (MP-1, MP-2, and MP-3), and three samples added with F-OPE (FP-1, FP-2, and FP-3). The experimental products were manufactured with different concentrations of OPE, obtaining dough samples with increasing total phenolic concentrations: 170 mg gallic acid equivalents (GAE) kg^{-1} of dough (MP-FP-1), 350 mg GAE kg^{-1} of dough (MP-FP-2) and 530 mg GAE kg^{-1} of dough (MP-FP-3), as reported in Figure 1. As M-OPE was characterised by lower total phenolic compounds, comparted to F-OPE (Natrella et al., 2025), a higher amount of M-OPE by weight was added to the samples than F-OPE to ensure equal phenolic compound concentration in the samples (MP-1, MP-2, MP-3 added of 2500, 5000 and 7500 ppm of M-OPE, respectively; FP-1, FP-2, FP-3, added of 1560, 3150 and 4730 ppm of F-OPE). Tap water and durum wheat semolina (Casillo, Corato, Italy) (33% v/w) were kneaded for 15 min with mixer (Kenwood Cooking Chef XL KCL95.424SI, United Kingdom) to prepare pasta dough, as previously reported by Natrella et al. (2025) and Carini et al. (2010). Before mixing F-OPE and M-OPE to pasta doughs, they were first solubilised in tap water. Subsequently, the pasta was shaped into "cavatelli", through a semi-industrial extrusion line and were pasteurized at 100 °C for a minute and a half, following the protocol of Marzano et al. (2022). Packaging was carried out with a semi-automatic packaging machine, under modified atmosphere (MAP) composed of 70:30 N_2/CO_2 using polypropylene/polyamide multilayered film bags (Bropack Gmbh, Villach, Austria). The film oxygen transmission rate (OTR) was ≤ 18 cc m^{-2} day^{-1} and the water vapor transmission rate (WVTR) was ≤ 2 g m^{-2} day^{-1}. Samples were stored at 4 °C and were analysed immediately after packaging (D0) and then after 15, 30, 45, 60, 70, 90, 110 and 120 days of storage. For each sampling time, three batches of each pasta type were analysed.

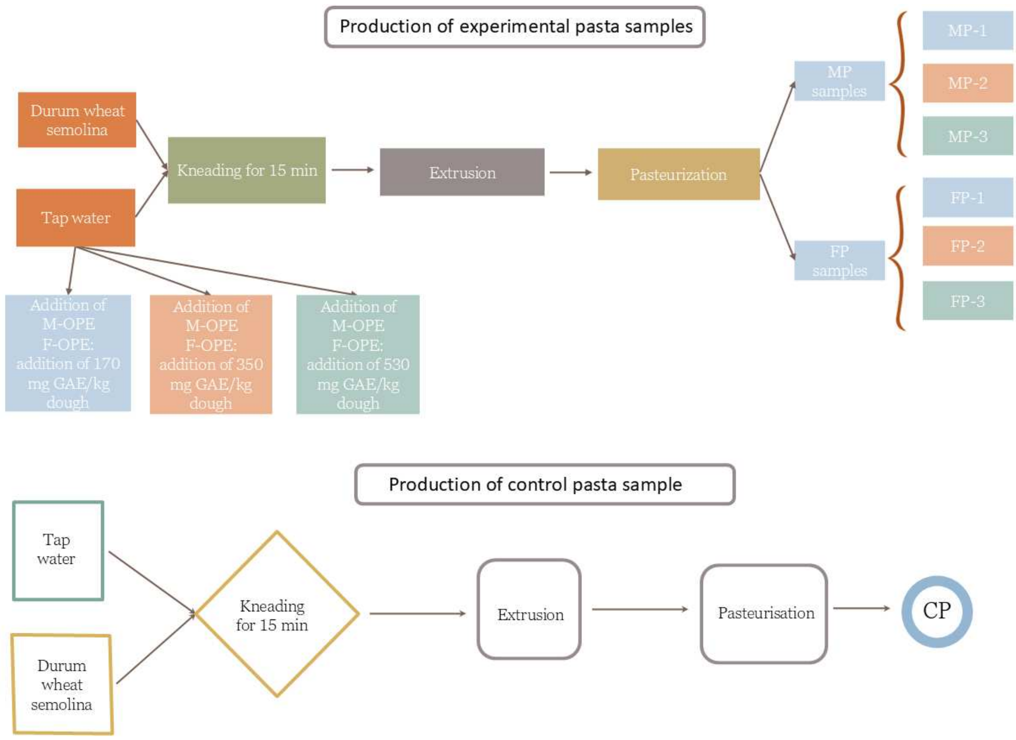


Figure 1. Fresh pasta production process. Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated OP extract); FP, fresh pasta samples with F-OPE (free lyophilised OP extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used.

2.4 Gas analysis

For each pasta sample, the gas composition (percentages of O_2 and CO_2) of three packages was determined using a gas analyser (CheckMate 3, DanSensor, Ametek, Milan). Gas analysis was performed immediately after packaging and at each subsequent inspection time.

2.5 Microbiological analysis

Microbiological analyses were carried out on the seven types of fresh pasta samples: control (CP), experimental samples added with M-OPE (MP-1, MP-2, and MP-3), and samples added with F-OPE (FP-1, FP-2, and FP-3) at different times: immediately after packaging (D0) and after 15, 30, 45, 60, 70, 90, 110 and 120 days of refrigerated storage at 4 °C (D15, D30, D45, D60, D70, D90, D110, D120). Twenty five grams of samples were aseptically removed from each package and homogenized with 225 mL of sterile buffered peptone water (0.10% w/v) in a stomacher (Bag Mixer, Interscience International, Roubaix, France) for 2 min, as described previously (Marzano et al., 2022). A tenfold dilution series of these suspensions were made and plated to a suitable agar media, in duplicate, for detection and enumeration of microorganisms as follows: total aerobic mesophilic bacteria (TAMB) by using Plate count agar (PCA) (Oxoid, Thermo Fisher Scientific Inc.) medium and incubation of the plates at 30 °C for 48 h; mesophilic lactic acid bacteria (LAB) by de Man–Rogosa–Sharpe (MRS) agar medium added of cycloheximide (100 mg L^{-1} , w/v) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germania) and incubation at 30 °C for 48 h under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid); *Enterobacteriaceae* by Violet Red Bile Glucose Agar (VRBGA) (Oxoid, Thermo Fisher Scientific Inc.) medium and incubation at 37 °C for 24 h; coagulase-positive *Staphylococcus* using Baird Parker medium (Oxoid, Thermo Fisher Scientific Inc.) supplemented with Egg Yolk Tellurite (Oxoid, Thermo Fisher Scientific Inc.) incubated at 37 °C for 48 h; spore-forming bacteria (SFB) on plate count agar (PCA) after heat-treatment at 10 min at 90 °C of the microbial suspension and incubation for 24 h at 30 °C; *Clostridium perfringens* in sulphite polymyxin sulfadiazine (Biolife, Milan, Italy) agar medium incubated in anaerobic condition at 37 °C for 24 h; yeasts and moulds by using Wort agar (WA) (Oxoid) supplemented with chloramphenicol (100 mg L^{-1}) (Sigma Aldrich) incubated at 25 °C for 48 h; and moulds also using Potato Dextrose Agar (PDA) (Oxoid) supplemented with chloramphenicol (100 mg L^{-1}) and incubated at 25 °C for 96 h. Furthermore, all pasta samples, were investigated for detection and enumeration of coliform bacteria (according to ISO 4832:2006, 2015), β-glucuronidase-positive *Escherichia coli* (according to ISO 16649-1:2001, 2013), presumptive *Bacillus cereus* counts (according to ISO 7932:2004, 2004), Salmonella spp. (according to ISO 6579-1:2017, 2017) and *Listeria monocytogenes* (according to ISO 11290-1:2017, 2018). Results were expressed as Log CFU g^{-1} of sample.

2.6 Physicochemical analysis

Fresh pasta samples were analysed for moisture (AOAC 950.46), according to the Official Methods of Analysis of AOAC (AOAC, 2011). Water activity (a_w) of raw pasta samples during storage was determined using an electronic hygrometer (Aqua Lab 100-240 V AC, Pullman, WA, USA). The measurement of pH on the homogenised product was performed by using a pH-meter (Hanna edge® HI2020, Hanna Instruments, Villafranca Padovana, Italy), following the method proposed by Rainero et al. (2022). Colour was evaluated instrumentally on ground pasta samples, using the CIELAB system (L*: lightness, a*: redness, b*: yellowness), with a colorimeter CM-600d (Konica Minolta, Tokyo, Japan). Colour data were collected with the software Spectra Magic NX (Konica Minolta, Tokyo, Japan).

2.7 *Total phenolic compounds and antioxidant evaluation*

Total phenolic compounds (TPC) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) and DPPH (2,2-diphenyl-1-picrylhydrazyl); dihydrorhodamine-123; Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) assays for antioxidant activity were measured on pasta samples during storage time. For these analyses the following chemicals were adopted: Folin-Ciocalteu reagent; ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) and DPPH (2,2-diphenyl-1-picrylhydrazyl); dihydrorhodamine-123; Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), supplied from Sigma-Aldrich (Milan, Italy). Phenolic extracts from pasta samples for TPC, ABTS and DPPH assays were obtained following the procedure reported by De Gennaro et al. (2022). TPC were determined by Folin-Ciocalteu method, as reported by Difonzo et al. (2022a) and expressed as mg of gallic acid equivalents (GAE) kg-1 of fresh pasta. The evaluation of antioxidant activity was performed by DPPH and ABTS assays, as described by Caponio et al. (2022) and Difonzo et al. (2022a), respectively. The results were expressed in μmol Trolox equivalents (TE) kg^{-1} of fresh pasta samples.

2.8 Analysis of volatile organic compounds

Volatile organic compounds (VOCs) of pasta samples immediately after production and after 120 days of storage were identified and quantified through headspace solid phase micro-extraction (SPME), coupled to gas-chromatography/mass spectrometry (GC/MS), as reported by Caponio et al. (2022). Particularly, 0.50 g of each sample was added with 4 mL of NaCl (20% w/v) aqueous solution and 150 μL of internal standard (1-propanol, 160 ppm). The extraction of volatile compounds was performed by exposing a 75 μm Carboxen/polydimethylsiloxane (CAR/PDMS) SPME fibre (Supelco, Bellefonte, PA, USA) in the headspace of the vials containing the samples at 50°C for 40 min. The fibre was desorbed for 6 min in the injection port of the gas-chromatography operating a split-less mode at 230°C for 3.5 min. The determination was carried out using an Agilent 6580 gas-chromatography equipped with an Agilent 5975 mass-spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). Volatile compounds were separated with a capillary column HP-Innowax (60 m length × 0.25 mm i.d. × 0.25 μm film thickness) under the following conditions: injector temperature of 250°C, oven temperature was held at 35°C for 5 min, then increased to 210°C at 5.5°C min^{-1}. This temperature was kept constant for 5 min. Mass detector was set at the following conditions: interface temperature of 230°C, source temperature of 230°C, ionization energy of 70 eV, scan range of 33–260 amu. Helium was used as a carrier with a pressure 30 kPa and a flow of 1 ml min^{-1}. The volatile compounds were quantified by standardizing the peak areas of compounds of interest with the peak area of the internal standard. VOCs were identified by matching each experimental spectra with the NIST library; then, their quantification was expressed in μg g^{-1} of pasta sample.

2.9 Statistical analysis

Physicochemical data were statistically processed by one-way and two-way ANOVA followed by post-hoc Tukey's HSD (honestly significant difference) procedure (XLSTAT software, Addinsoft, NY, USA). The differences were considered significant at 95% probability level ($p < 0.05$). Furthermore, the same data were submitted to the principal component analysis (PCA) considering storage time, type of OPE and its concentration. The Pearson correlation coefficient was computed to extrapolate the correlations among O_2 and CO_2 concentrations and microbial growth and among pH values and microbial growth ($p < 0.05$). The sPLS-DA and relevance network plot of volatile organic compounds was performed by using R studio and mixOmics v6.23.4 R package. As reported by De Flaviis et al. (2024), sparse PLS was developed to perform simultaneous variable selection, by including Least Absolute Shrinkage and Selection Operator (LASSO) penalisations in PLS to minimize the mean squared error (MSE) of the model. Irrelevant variables from each loading vector are assigned a zero weight, and latent components are calculated only on a subset of "relevant" variables. The zero weights are assigned optimally with LASSO, whilst ensuring that the covariance between datasets is maximised (Lê Cao et al., 2008). Microbiological evaluations were carried out in triplicate for each batch of samples. Data were subjected to one-way ANOVA; pair comparison of reatment means was achieved by post-hoc Tukey's HSD (honestly significant difference) procedure at $p < 0.05$, using the statistical software Statistica 12.5 (StatSoft Inc., Tulsa, USA). The graphs for the physicochemical and microbiological analyses were generated using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), while the graphs for the volatile compounds were produced using OriginPro 2023 (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1 Gas analysis

The O_2 concentration inside packages was close to 0% just after packaging and evolved to a maximum of 6.46 ± 1.97 % at the last sampling time (Figure 2A). Marzano et al. (2022) packed fresh pasta using a film with a OTR similar to ours (20 cc m^{-2} day^{-1}), obtaining a significant increase of O_2 concentration inside packages during 90 days of storage, corroborating our results. No significant differences were observed among samples, except for trays of FP samples after 30 and 45 days of storage. Specifically, after 30 and 45 days of storage, trays of FP-1 and FP-2, respectively, showed a significantly higher O_2 concentration (data not shown), compared to other samples, which could be related to limited growth of total aerobic mesophilic bacteria (TAMB) (Figure 7A). Significant O_2 consumption generally coincides with the onset of microbial growth (Samapundo et al., 2010); indeed, a strong negative Pearson correlation was observed between O_2 concentration and TAMB growth, with r = -0.60 for FP-1 and r = -0.99 for FP-2. Moreover, O_2 concentration was negatively correlated with yeast growth (Figure 7C) in trays of FP-3 after 45 days of storage (r = -0.50), since the higher microbial activity led to greater O_2 consumption.

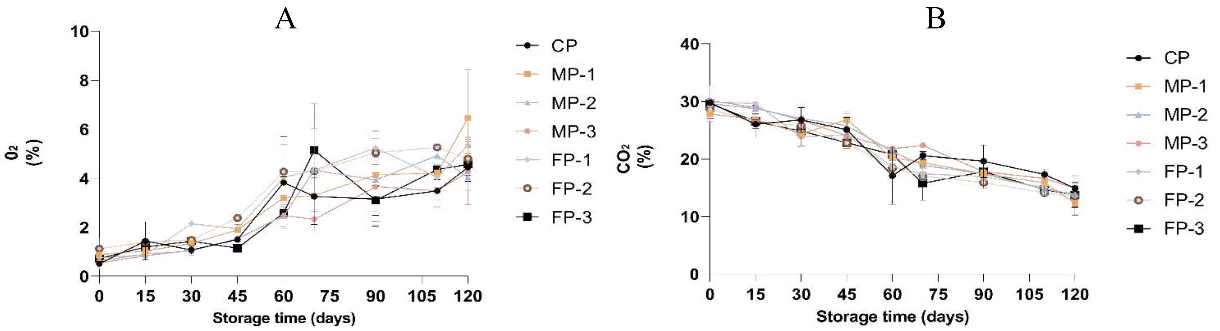


Figure 2. Evolution of O_2 (A) and CO_2 (B) concentrations (%) in the headspace of fresh pasta trays during 120 days of storage. Values are expressed as mean. The error bars indicate the standard deviation. Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated OP extract); FP, fresh pasta samples with F-OPE (free lyophilised OP extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used.

On the other hand, as already reported by Sanguinetti et al. (2016), the content of CO_2 decreased during storage, reaching a minimum of 12.3 ± 2.12%, after 120 days from the production (Figure 2B). This phenomenon could be ascribed to the absorption of this gas by fresh pasta samples (Bai et al., 2017; Sanguinetti et al., 2016; Zardetto et al., 2022). Also, Lee et al. (2001) reported a constant decrease of CO_2 in the MAP packaging of Korean fresh pasta, reaching a minimum value of 17% after 60 days of storage. After 70 days of storage, FP-3 trays showed a higher CO_2 content ($p < 0.05$)

(data not shown). It could be hypothesized that this result could be due to the greater growth of TAMB, as reported in Figure 7A, showing strong positive correlation with higher CO_2 content (r = 0.86). Putnik et al. (2016) reported a positive correlation among CO_2 production and TAMB growth, corroborating our results. No packaging collapse was observed in any of the samples during storage, due to the presence of nitrogen.

3.2 Moisture content, water activity and pH

Moisture and a_w of CP and experimental samples (MP and FP samples) immediately after production and after 120 days of storage are reported in Figure 3. Moisture and a_w are important factors for the processing and preservation of fresh food products, as have a great influence on appearance and preservation stability (Kamble et al., 2020). Indeed, high water content and a_w in food products may lead to deteriorative reactions, growth of microorganisms and physicochemical changes, limiting their shelf-life (Tapia et al., 2020; Wang et al., 2023; Yang et al., 2021; Zheng et al., 2019). Moisture showed a constant decreasing trend from 0 to 120 days of storage, due to water release into the surrounding atmosphere, resulting in the condensation within the packaging (Sanguinetti et al., 2011). This outcome is consistent with the model proposed by Zardetto et al. (2025), which simulate changing in water content of packed fresh pasta during refrigerated storage, confirming that moisture diffuses from the product to the pack headspace, leading to water loss. Moisture content of CP decreased from 31.10% to 28.18% ($p < 0.05$) (Table S1), corroborating results of Lordi et al. (2023) and Marzano et al. (2022), who reported a percentage reduction in water content similar to ours during refrigerated storage of control fresh pasta. On the other hand, moisture in MP and FP samples declined on average from 29.10% to 25.87% and from 29.78% to 27.08%, respectively ($p < 0.05$) (Table S1). These results are similar to those reported by Li et al. (2022) and Manthey et al. (2008) on refrigerated storage of noodles and fresh pasta, respectively. Particularly, until 30 days of storage, MP and FP samples were characterized by a lower water content, compared to CP ($p < 0.05$) (Table S1). O'Brien et al. (2003) reported that the overall increase in dry matter could account for the moisture reduction in samples with OPE. After 30 days of storage, samples with lower concentrations of F-OPE showed moisture values comparable to CP. Notably, MP samples showed significantly lower moisture content compared to CP and to the corresponding samples with F-OPE, throughout storage time (Table S1). This could be probably related to the presence of inulin, used as wall material. In line with our results, Difonzo et al. (2022b) found lower water content in fresh tagliatelle produced by substituting 5% and 10% of durum wheat semolina, with inulin from global artichokes. Overall, moisture results of MP and FP samples, agree with those obtained by Šeregelj et al. (2022), who reported that as concentration of carrot waste in durum wheat pasta increased, water content decreased. a_w showed a significant decrease for all the samples, during storage (Figure 3), as previously reported by Zheng et al. (2019). This phenomenon may result from the water migration from samples to atmosphere inside the packaging (Gao et al., 2022; Wang et al., 2023), as confirmed by the results on moisture. The addition of OPE caused a decrease of a_w (with some exceptions), which could be related to its water-binding properties, as previously reported by Elsebaie et al. (2022) on fresh macaroni with free and microencapsulated Azolla fern powder. This reduction was starker in samples with M-OPE (Table S1). In fact, after 120 days of storage, CP had the highest a_w (0.945), followed by FP samples (0.943) and MP samples (0.939). It could be hypothesized that the wall material, used to encapsulate the vegetable extract, had the ability to bind water molecules, as confirmed by Elsebaie et al. (2022) and Zen et al. (2020). Indeed, these authors reported that fresh pasta with microencapsulated vegetable extracts presented lower a_w when compared to the control, due to calcium chloride and alginate, used as encapsulating agents. However, throughout storage time, all pasta samples still complied with Italian law (Presidential Decree no. 187 of 9 February 2001), according to which fresh pasta must have at least 24% of moisture and a_w ranging from 0.92 to 0.97.

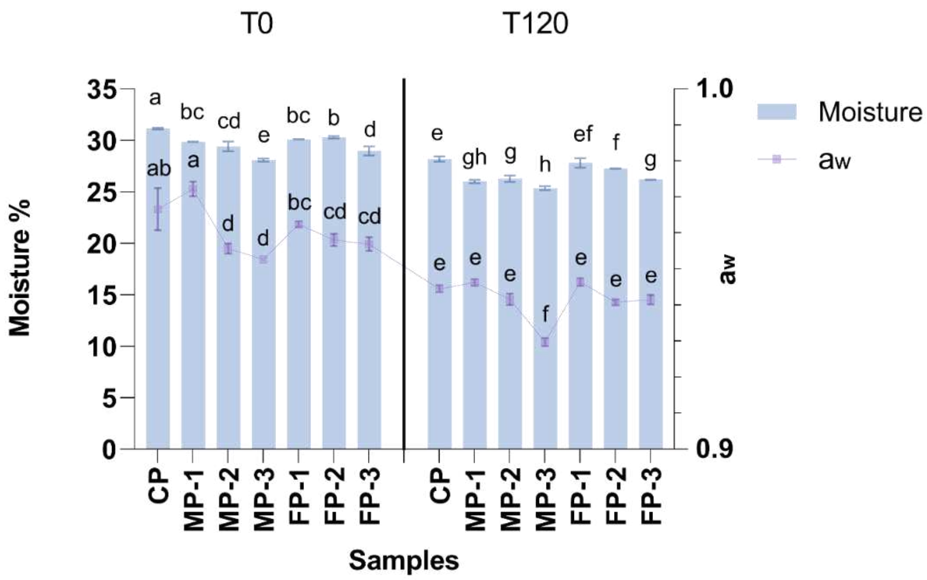


Figure 3. Evolution of moisture (%) and a_w of fresh pasta samples at the time of production (T0) and after 120 days of storage (T120). Values are expressed as mean. The error bars indicate the standard deviation. [a,h] Different letters mean significant statistical differences ($p < 0.05$) to two-way ANOVA followed by Tukey's HSD test.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated OP extract); FP, fresh pasta samples with F-OPE (free lyophilised OP extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used. * $p < 0.05$.

Immediately after production, the addition of OPE had a significant effect on pH ($p < 0.05$) (Table S1). All experimental samples showed lower pH values, compared to CP. This trend remained stable after 15, 60 and 90 days of storage. Acid compounds, such as phenolic acids in OPE extracts, could account for pH reduction (Zamankhani et al., 2024). Indeed, Difonzo et al. (2021) reported that among phenolic compounds, cinnamic acid, *p*-coumaric acid, caffeic acid and ferulic acid could be found in OP. A decrease in the pH of noodles and pasta, due to the increase of by-product extracts was also reported by Kazemi et al. (2017), Lordi et al. (2023) and Panza et al. (2023). After 30, 45 and 70 days from the time of production, samples with the highest OPE content showed lower pH, compared to CP (Table S1). Differences among samples flattened after 110 and 120 days of storage. Thus, no significant differences were observed among samples, at the last sampling time, except for MP-3 (5.52 ± 0.11), which showed the lowest pH. Changes of pH can occur in food products during storage as a result of chemical reactions and activities of microorganisms (Gökmen, 2025). According to the data reported in Table S1, pH showed a slight but significant increase, except for MP-3, from day 0 up to 120 days after production. Several authors (Li et al., 2017; Manthey et al., 2008; Yang et al., 2021; Yeoh et al., 2023) reported that proteins in fresh pasta could be decomposed by some proteolytic bacteria and moulds, releasing some basic compounds, such as amine and ammonia, which can increase pH values. Our results are consistent with those observed by Gökmen (2025), on storage of stuffed pasta. Specifically, despite the CO_2 reduction inside the packaging (Section 3.1), a process that may reduce pH values (Sanguinetti et al., 2011), most of the samples did not show significant differences up to 30 days of storage. From that moment on, pH increased and then showed a decreasing trend for the last sampling times. Particularly, CP and FP-2 showed a decreasing trend starting from 110 days of storage. The same trend can be observed after 90 and 60 days after the production for MP-2 and FP-1, respectively. Conversely, MP-1 and FP-3 did not show significant differences starting from 45 and 60 days of storage, respectively, up to 120 days (Table S1). Although the correlations analysis (Pearson) between the pH values of the analysed samples and microbiological data were found to be not statistically significant (data not shown), the pH reduction could be related to microbial activities. Indeed, the latter are common in foods rich in carbohydrates, which can be utilized by microorganisms to produce acids, which can decrease pH (Li et al., 2017; Yang et al., 2022). Yeoh et al. (2023) reported a similar trend for pH of alkaline noodles during storage, observing an increase of pH for the first 15 days of storage and a subsequent decrease until 29 days of storage, attributed to microorganisms' proliferation. Altough food microbial communities are susceptible to changes in environmental conditions, such as decreases and further increases in pH, that occur during the fresh pasta production process. Furthermore, the relationships and interactions among community members can also influence the community structure itself. The growth, survival, and activity of desirable, spoilage or pathogenic organisms are determined by other species or strains.

3.3 Results of TPC and antioxidant activity

TPC and antioxidant activity of samples at T0 and T120 are presented in Figure 4A and 4B, respectively. The lowest content of phenols (294.00 ± 6.65 mg GAE kg^{-1} fresh pasta) and antioxidant activity was recorded for pasta without by-product extracts. It is noteworthy that higher OPE concentrations significantly increased TPC and antioxidant activity ($p < 0.05$; Figure 4A and 4B), consistent with findings reported in the literature (Kabir et al., 2024). In fact, a significant increase of phenols content and antioxidant activity was already observed by adding apple flour and olive pomace in pasta (Espinosa-Solis et al., 2019; Simonato et al., 2019), mango peel powder in macaroni and noodles (Kabir et al., 2024), fig peels in tagliatelle (Panza et al., 2022) and broccoli (Angiolillo et al., 2019), pomegranate (Lacivita et al., 2022) and olive oil by-products in fresh pasta (Lordi et al., 2023). Particularly, from Table S2, it can be observed that as regards TPC, FP-1 samples starting from 15 days of storage did not show significant differences with CP, whereas its experimental counterpart (MP-1) had TPC values comparable to CP only after 90 days of storage (62.42 ± 1.21 mg GAE kg^{-1} fresh pasta). According to Kabir et al. (2024), encapsulation of natural ingredients enhances the stability and functionality of valued-added food products; this finding clearly suits with our results. Actually, samples with M-OPE showed, on average, a lower TPC loss (84.46 %) from the time of production to 120 days of storage, compared to FP samples (86.33%). In line with these findings, at the end of storage time, fresh pasta sample with different F-OPE concentration showed statistically lower TPC content than M-OPE (Table S2). Phenolic compounds are generally associated to antioxidant activity of food matrix (Swallah et al., 2020). The OPE addition resulted in a significative increment of the antioxidant activity in the pasta samples, and the assessed antioxidant activity resulted to be strictly correlated to the amount of OPE added to the semolina, according to both DPPH and ABTS assays (Figure 4B). In line with TPC results, FP-1 at the end of storage, showed antioxidant activity, according to DPPH assay, similar to CP. On the contrary, MP samples recorded higher antioxidant activity (for both DPPH and ABTS assays), confirming results of TPC (Figure 4B). Comparing data at T0 to data at T120 for both TPC and antioxidant activity assays, a general decrease has been observed. Specifically, our results corroborate the findings of Panza et al. (2022) and Mohan et al. (2025), who observed a significant decrease of TPC and antioxidant activity from the time of production, to end of storage of fresh pasta enriched with fig peels and seaweed, respectively. The phenomena occurring during storage, such as hydrolytic and oxidative reactions, resulting from O_2 increase in the packaging (Section 3.1), could have promoted the recorded losses and consequently compromise the availability of phytochemicals (Panza et al., 2022). Indeed, Maddaloni et al. (2025) reported that changes in polyphenols during food preservation are related to physiochemical factors (pH, light, oxygen, presence of endogenous enzymes in the food matrix). However, the main reaction reducing polyphenols and antioxidant activity during storage is oxidation (Maddaloni et al., 2025; Mohan et al., 2025).

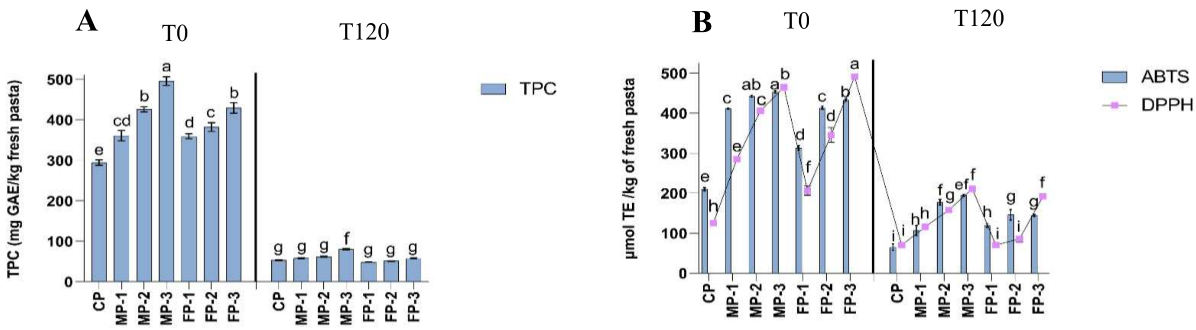


Figure 4. Evolution of TPC (A) and antioxidant activity according to ABTS and DPPH assays (B) of fresh pasta samples at the time of production (T0) and after 120 days of storage (T120). Values are expressed as mean. The error bars indicate the standard deviation.

[a,g] Different letters mean significant statistical differences ($p < 0.05$) to two-way ANOVA followed by Tukey's HSD test.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated extract); FP, fresh pasta samples with F-OPE (free lyophilised extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used. * $p < 0.05$.

3.4 Changes of colour

Colour is an important parameter of food products acceptability for consumers, as refers to their freshness and quality (Wang et al., 2023; Zamankhani et al., 2024). It is based on the properties of raw ingredients, as well as on the production processes and storage conditions (Elsebaie et al., 2022). Colour changes have been associated with various factors, such as heat treatment, enzymatic browning or nonenzymatic browning and oxidation of food commodities (Kamble et al., 2020). According to International Commission on Illumination (CIE), L^*, a^*, b^* are the three-color coordinates. L^* value, being a function of diffuse light reflectance (Zheng et al., 2019), represents the lightness and ranges from 0 (absolute black), to 100 (diffuse light). According to Table S3, L^* values declined significantly during storage, until 70 days after production (for CP and FP-3), 90 (for all MP samples) and 60 days of storage (for FP-1 and FP-2). Subsequently, L^* showed an increasing trend. Wang et al. (2023) stated that the decreasing lightness could be attributed to the oxidation of phenols (corroborating results reported in the Section 3.3) and browning reaction. In line with these statement, Yang et al. (2022) reported that polyphenol oxidase enzyme can interact with polyphenols in fresh noodles, causing darkening of the products. On the other hand, increase of L^* values could be due to the wet surfaces of samples, which consequently caused an increase of the light scatter, as confirmed by water releases in the packaging atmosphere (Section 3.2). Our findings agree with data reported by Šaponjac et al. (2016), who observed a significant increase of *L** values during 4 months of storage of cookies with encapsulated cherry pomace extract. Progressive decrease in lightness value was also observed, increasing level of M-OPE and F-OPE. This could be due to the dark colour of the by-products added to the pasta formulations, according to other studies showing the same trend (Bekiroglu et al., 2025; Simonato et al., 2019; Zen et al., 2020). On average, for each sampling time, samples with M-OPE showed lower *L** values, compared to corresponding samples with F-OPE ($p < 0.05$). The same trend was observed by Jeyakumari et al. (2016) in cookies fortified with fish oil encapsulates, with respect to cookies fortified with fish oil as such. On the other hand, these results are not consistent with those reported by Ćetković et al. (2022) and Zamankhani et al. (2024), who observed that the effect of free extracts on changing *L** values of pasta and noodles, respectively, was greater than that of microencapsulated extracts. According to Table S3, *a** values of all samples, except for MP-2 and FP-3, decreased during storage. Our trend agrees with data reported by De Almeida et al. (2018), Šaponjac et al. (2016), and Mohan et al. (2025) on *a** values of cookies and fresh pasta, respectively. Anthocyanins degradation or their polymerization with other phenolic compounds may be correlated to *a** reduction of processed foods (Šaponjac et al., 2016). In fact, during oil extraction, anthocyanins are retained in the OP, because of their hydrophilic nature (Criado et al., 2008). Furthermore, authors stated that aqueous ethanol solutions, which we used to obtain OPE, are suitable for anthocyanins extraction (Cacace & Mazza, 2003). On the contrary, *a** values of MP-2 and FP-3 increased. The same trend was already observed by Wang et al. (2023). This phenomenon might be the result of the formation of nonenzymatic browning components at the time of storage, as also observed by Kumar et al. (2017), in case of storage of cereal-based food products. As expected, the addition of OPE extracts to the dough, significantly increased *a** values. This is probably due to the anthocyanins, responsible for red colour, present in OPE (Criado et al., 2008). Our trend agrees with other studies; a significant change of *a** values from negative green to positive red, was previously reported by Simonato et al. (2019) on pasta with freeze-dried OP. Durante et al. (2019) observed an increase of redness index in spaghetti pasta with the addition of free and α-cyclodextrin encapsulated pumpkin oil. On average, cavatelli samples with M-OPE had higher *a** values, compared to FP samples ($p < 0.05$). It could be assumed that inulin used as wall material for the microencapsulation could increase redness index, as confirmed by Difonzo et al. (2022b) on fresh tagliatelle with inulin. Moreover, these results are in agreement with data reported by other authors (Ćetković et al., 2022; Elsebaie et al., 2022; Zamankhani et al., 2024), who observed an increase of redness values by increasing the amount of encapsulated vegetable extract to pasta samples. Concerning *b** parameter, when it is positive indicates a yellow colour tendency, otherwise, negative values indicate blue colour. Consumers greatly appreciate durum wheat pasta with high yellowish colour (Bekiroglu et al., 2025; Zingale et al., 2023). On the whole, all pasta samples were characterized by a decreasing trend of *b** values throughout the storage time (Table S3). Also, Gökmen, (2025) and Zheng et al. (2019) showed that *b** values of stuffed pasta and of fresh barley noodles, respectively, decreased significantly during storage. On average, for every sampling time, especially for samples with F-OPE, the greater the addition of OPE, the lower the yellow index. The same finding was also observed by Simonato et al. (2019) on pasta with dried OP. Furthermore, as reported by other authors (Elsebaie et al., 2022; Zen et al., 2020), samples with microencapsulated extracts were characterized by higher *b** value, compared to the corresponding samples with extract as such. Actually, after 120 days of refrigerated storage, MP samples still had on average higher *b** values (19.63), than CP (18.96) and FP samples (18.38).

3.5 Determination of volatile organic compounds of fresh pasta samples

Concerning the VOC results, a supervised multivariate analysis was performed considering the type of sample and the storage time (Figure 5A, B, C). A sPLS-DA analysis was performed, in which the reported compounds are those that best characterize pasta samples. The relevance network of the pasta samples at day 0 (Figure 5A) showed that CP and MP-1 had a negative correlation with the selected VOCs, as indicated by the edges connecting the samples to the nodes (representing volatile compounds), meaning that their profile was relatively poor in molecules; MP-2 and FP-1 showed weak correlation with volatile compounds (the edges were mostly black); MP-3 showed a strong positive correlation with hexanal, pentanal, amyl alcohol and octanal, whereas, FP-2 and FP-3 showed a high positive correlation (red edges) with all the VOCs selected, meaning that were found at high concentrations in these samples. The VOC profile of samples was more complex after 120 days of storage (Figure 5B), a strong positive correlation was observed between CP and most of the selected compounds, except for few molecules (trans-2-hexenal, pentanal, 1-hexanol and hexanal); MP-1 and MP-2 showed no strong correlation with volatile compounds, while MP-3 exhibited low positive correlations with hexanal, *trans*-2-hexenal, 1-hexanol and pentanal. Among FP samples, FP-1 showed positive correlation only with amyl alcohol and 1-hexanol; FP-2 samples had positive correlations with pentanal, hexanal, 1-hexanol, trans-2-hexenal; then, FP-3 showed positive correlation with all VOC except for octanoic acid, ethyl ester and amyl alcohol. Finally, Figure 5C clearly highlights the evolution of volatile profiles during storage, regardless of the specific type of pasta. Fresh pasta only had positive correlation with nonanal and octanal, whereas samples at 120 days of storage showed a strong positive correlation for all the VOCs selected, except those reported in fresh samples (nonanal and octanal). As previously reported, the oxidation process affected TCP and antioxidant activity (Section 3.3.), as well as volatile profile. In general, the presence of aldehydes in pasta are possibly linked to autoxidation, thermal oxidation, photo-oxidation processes or lipoxygenase-assisted oxidation as reported by literature (Borrelli et al., 1999; Gaggiotti et al., 2019; Neff et al., 1983; Palombi et al., 2024; Rodríguez-Bernaldo De Quirós et al., 2000; Yang et al., 1993). Some aldehydes were already present at day 0 (hexanal and nonanal), probably resulting from Maillard reaction during pasta preparation (Hussein et al., 2021). In accordance with literature, hexanal was the most abundant compound found in pasta samples at day 0 (Beleggia et al., 2009; Gerardi et al., 2023; Pasqualone et al., 2014). However, its concentration increased over time, highlighting some oxidative process during storage, with CP showing not the highest concentration, but the highest increasing rate. Other aldehydes, as pentanal, originates from the degradation of oleic fatty acids (Smit et al., 2009). Lipid oxidation led also to the production of alcohols (i.e. 1-hexanol), which showed similar content among experimental samples and lower amount in CP (Pasqualone et al., 2014). Esters as ethyl esters of octanoic, butanedioic and decanoic acid were absent in fresh pasta samples, but at the end of storage were present in all samples, with the highest abundance in CP sample. These compounds likely arise due to microbial activity during storage (Cecchi et al., 2021). Overall, the evolution of the volatile profile of samples showed low VOC concentrations for CP and most of the experimental samples (except those added of high amounts of OPE) at day 0, then, a significant volatile compounds production uptrend was observed for all samples up to 120 days, particularly for CP and FP-3.

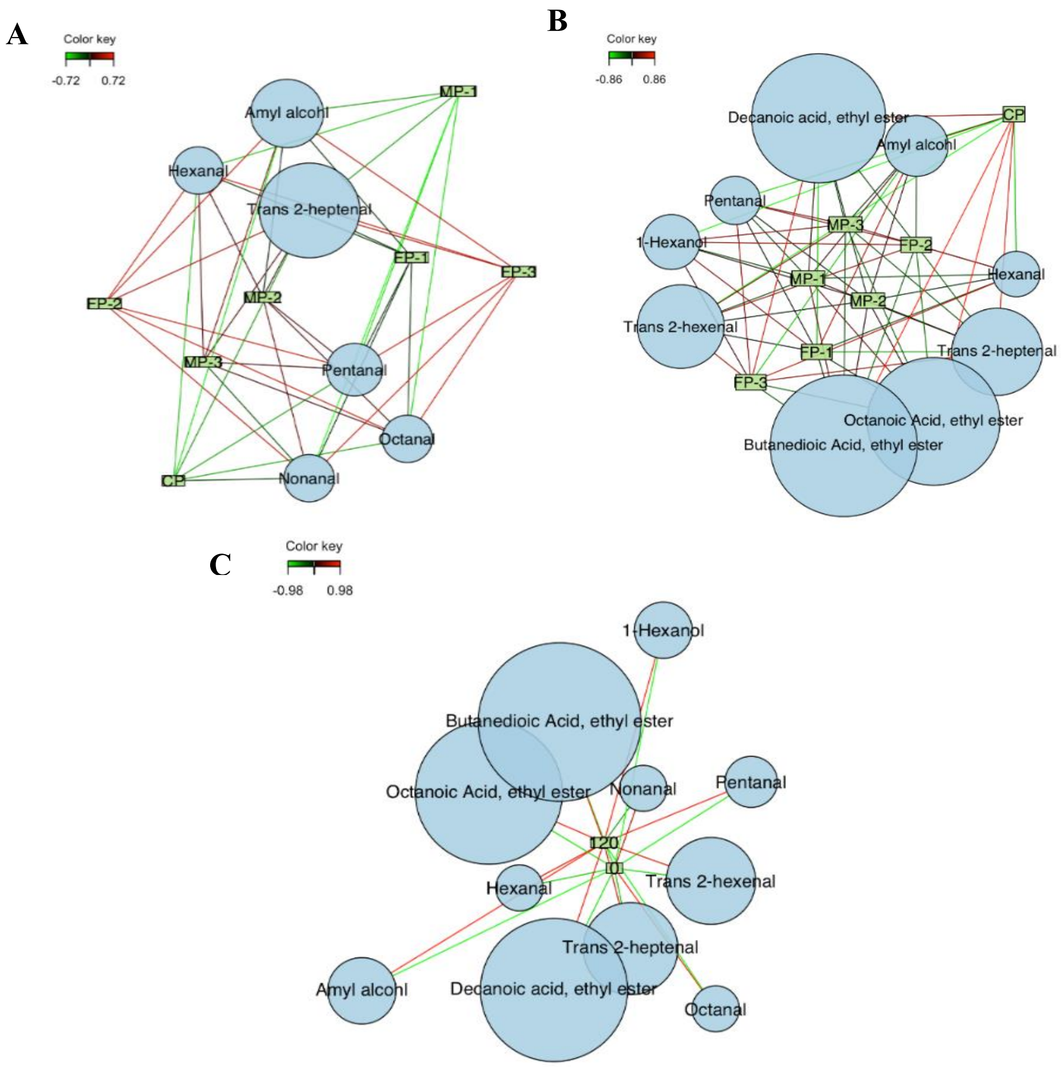


Figure 5. Relevance network of volatile compounds in different type of pasta samples at day 0 (A) and 120 days of storage (B); relevance network of volatile profile evolution between fresh and stored samples regardless the type of pasta (C).

Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated extract); FP, fresh pasta samples with F-OPE (free lyophilised extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used.

3.6 Principal component analysis of physicochemical determinations during storage time

In order to have an overview of the quality changes of fresh pasta samples throughout the storage time, a principal component analysis (PCA) was carried out as a comprehensive multivariate study for a better interpretation of the results. Thus, Figure 6 shows the biplot of pasta samples and the independent variables evaluated (moisture, a_w, pH, colour, TPC and antioxidant activity according to both ABTS and DPPH assays) throughout storage time. The PCA explained 82.21% of variance, 50.22% of which was along the first component and 31.99% along the second component. The plot clearly showed two interpretative ways, both considering the storage time and the addition of OPE. Considering the latter, samples were positioned following a logical pattern (from top left to bottom right) in which CP and samples with low OPE concentration placed on top, while those enriched with higher OPE concentration laid at the bottom. The grouping pattern also reflected the storage time, showing a clear logical separation among samples (top right-bottom left). On the whole, the first component effectively captured the influence of time, while the second component accounted for the effect of OPE on the studied parameters. In summary, the exploratory multivariate analysis condenses the observations discussed above into a single plot. Fresh samples (top-right, green dots) were characterized by high moisture, a_w and *b**, moreover, TPC, ABTS and DPPH independent variables extends towards OPE enriched samples. As the storage proceed, samples moved away from the positive side of PC1 towards its negative side, meaning that there is an impoverishment of the variables cited above. Regarding the PC2, samples were placed according to their amount of OPE added. The CP samples always laid on top of the positive side of PC2, as no OPE has been added, followed by FP-1, MP-1, FP-2, MP-2, FP-3 and MP-3 (mostly at the bottom, negative PC2). Thus, observing Figure 6, it is possible to conclude that CP and experimental samples with intermediate and high OPE concentration were quite different to each other. Obviously, most of the samples with high OPE concentration (especially the fresh ones) were highly correlated with TPC, ABTS, DPPH and *a** active variable, as reported previously.

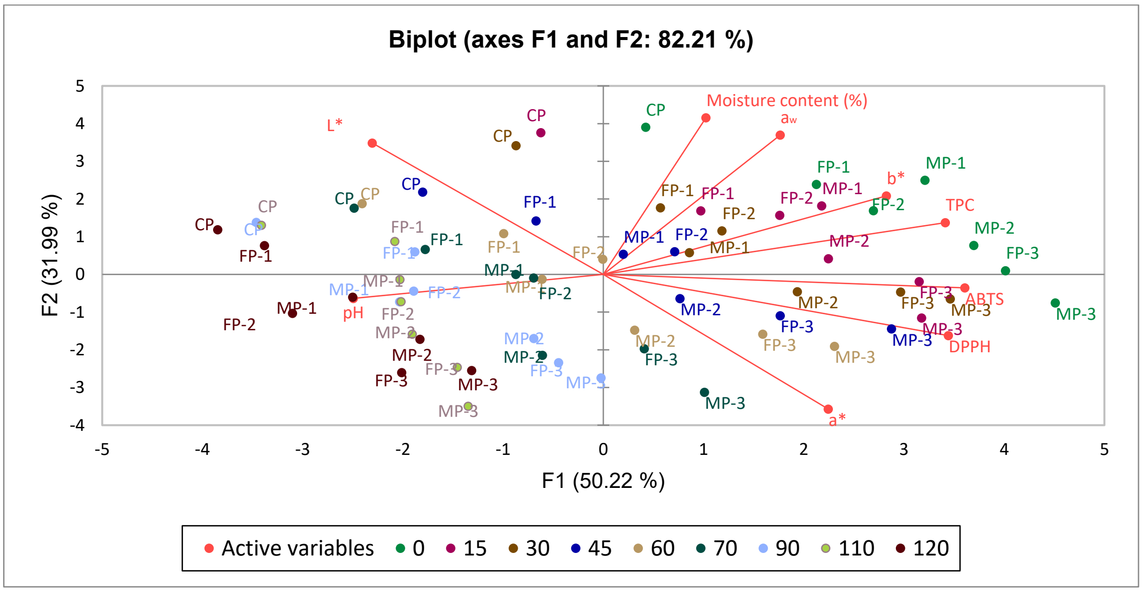


Figure 6. Principal component analysis of physicochemical parameters during storage. Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated extract); FP, fresh pasta samples with F-OPE (free lyophilised extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used. TPC are expressed as mg of gallic acid equivalents (GAE) kg^{-1} of fresh pasta; ABTS and DPPH are expressed in µmol Trolox equivalents (TE) kg^{-1} of fresh pasta samples.

3.7 *Microbiota of fresh pasta*

Fresh pasta products are particularly vulnerable from microbiological point of view, according with their water content, some stage in processing and, when filled, for some ingredients. Microorganisms play a priority role by contributing to the quality and desirable properties of the products (Angiolillo et al., 2017; Marzano et al., 2022). Culture dependent analyses were performed on microorganisms that naturally occur and that are known to produce alterations in fresh pasta during refrigerated storage (Angiolillo et al., 2017; Costa et al., 2010; Del Nobile et al., 2009b). Based on the use of raw materials with good quality, suitable hygienic practices during the manufacturing, as well as effective thermal solution and MAP, a microbial inhibition was observed in all samples at beginning (D0) of refrigerated storage (data not shown), in line with previous results (Marzano et al., 2022; Ricci et al., 2017). All pasta types were appropriate under the hygienic profile, as highlighted by the absence of pathogenic microorganisms (*Salmonella* spp., *L. monocytogenes*) and presumptive *Bacillus cereus* and the assessment of beta glucuronidase-positive *E. coli*, *Clostridium* spp, coagulase-positive *Staphylococcus* and coliforms cell densities below the detection limit (<1 log CFU g^{-1}) in all samples up to 120 days (data not shown). However, the efficacy of thermal approach in terms of shelf-life cannot be absolute during storage but affected by both the density of surviving microorganisms and the nature of heat treatments (Lopez et al., 1998). In fact, in our study microbial profiles slightly changed because of olive pomace addition and/or storage. The addition of OPE reduced the growth of spoilage microbes. Mesophilic aerobic, moulds and yeast microbial groups (Figure 7A,B,C) investigated registered a significant reduction ($p < 0.05$) of ca. 0.5–1 logarithmic cycles in samples added with extract in free or encapsulated form, compared to CP. Total mesophilic aerobic cell (Figure 7A) densities maintain values near the accepted value by the reference standard (< 6 log CFU g^{-1}) (Circular of the Italian Ministry of Health August 3, 1985, n°32) for up to 120 days in all pasta samples, although CP pasta exhibits ca 0.5 - 1 Log CFU g^{-1} higher at days 110 and 120, compared to experimental ones. Significantly ($p < 0.05$) lower counts were recorded for mould (Figure 7B) and yeast (Figure 7C) in F-OPE and M-OPE fresh pasta, compared to CP, especially at 110 days of refrigerated storage. The significant reduction observed can be due to the presence of the phenolic extracts, both in free and encapsulated form. As reviewed by Thielmann et al. (2017), the concentration of the phenolic compounds in the extracts from olive fruit or leaves may have a direct inhibitory antimicrobial action against a wide variety of bacteria and fungi so they are suitable to act as food preservative, as already demonstrated when added to vegetable oils, fish burgers, fermented milk, and in the edible coating of fruit (Difonzo et al., 2021; Jaiswal et al., 2012). In comparison to F-OPE, M-OPE delayed microbial growth during 120 days of storage at 4 °C and this outcome represents a key factor for the feasibility of the use of OPE for improved stability of fresh pasta during storage. Lactic acid bacteria (LAB) did not reach a value greater than 1 Log CFU g^{-1} at the final collection time (120 days of refrigerated storage) in samples, without significant differences for addition of OPE (Figure 7D). In general, LAB are more resistant to phenolic compounds than other bacteria, for their abilities to develop structural or metabolic resistance towards these compounds and their derivatives, but in food this capacity depends on the compound's structure and appears to be dose-dependent (Caponio et al., 2019). However, it is important to consider that antimicrobial activity of phenolic compounds could increase because of synergic effects between them or with other antimicrobial strategies, such as thermic and packaging treatments applied at the pasta product. However, it is also important to realize that the polyphenols may have metabolized and the metabolites that result from metabolic activity may differ from the native substances in terms of biological activity. This is a metabolic detoxication process common to many xenobiotics that restricts their potential toxic effects and may explain their reduction of activity by increasing their hydrophilicity resulting in a community protection (Liu et al., 2007; Manach et al., 2004). Enterobacteriaceae (ENT) cell density (Figure 7E) did not change among samples, reaching values < 2 Log CFU g^{-1} after 120 days of refrigerated storage.

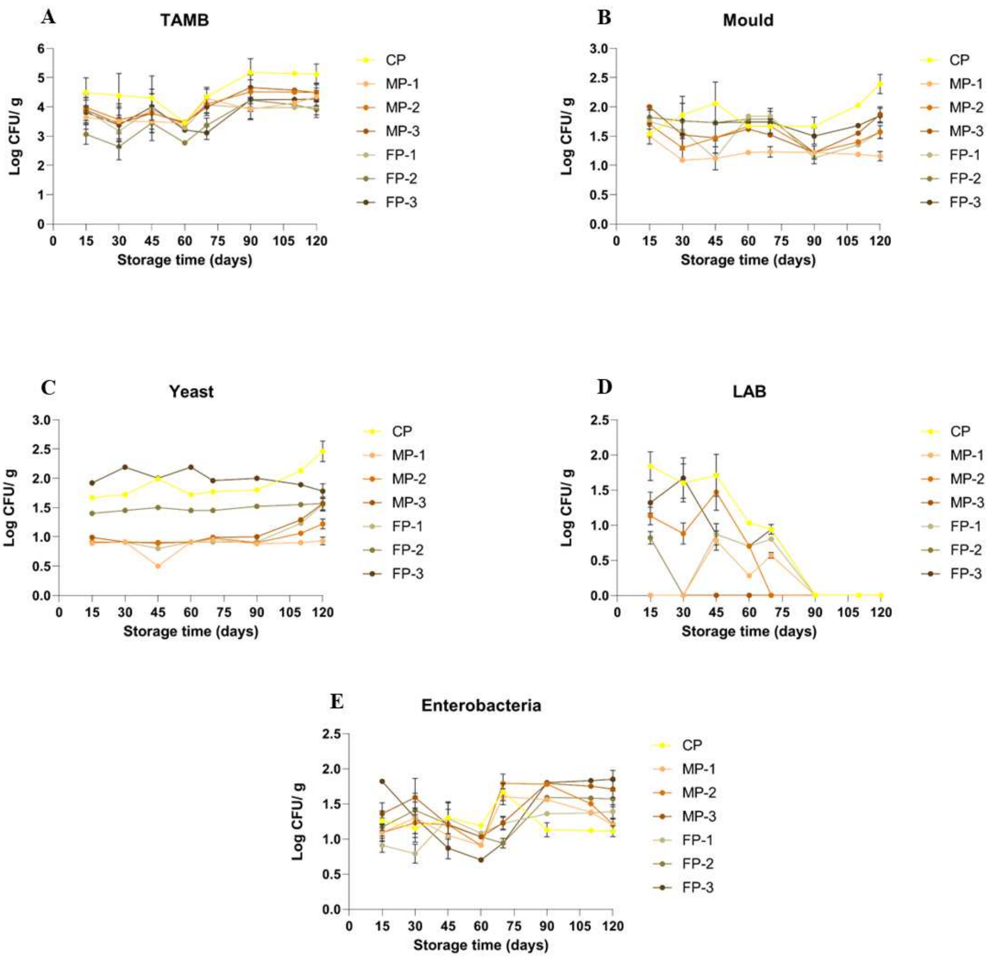


Figure 7. Line chart concerning the growth of microorganisms (expressed as Log CFU/g) in fresh pasta samples analysed after 15, 30, 45, 60, 70, 90, 110 and 120 days (D) of refrigerated storage. Values are expressed as mean. The bars indicate the standard deviation. Panel A: total aerobic mesophilic bacteria (TAMB); Panel B: Moulds (MO); Panel C: Yeast (YE); Panel D: Lactic acid bacteria (LAB); Panel E: Enterobacteriaceae (ENB).

Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated OP extract); FP, fresh pasta samples with F-OPE (free lyophilised OP extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used.

4. Conclusions

The increasing consumer demand for functional foods with low content of synthetic additives led researchers to focus on natural compounds with antimicrobial and antioxidant properties. Vegetable waste and by-products are interesting sources of phytochemicals, successfully applied to extend the shelf-life of food matrices. Particularly, olive pomace (OP) is the major by-product of olive-oil chain, rich in sterols and phenolic compounds. As fresh pasta is a perishable food product, in this study, phenolic extracts of olive pomace (OPE) in free (F-OPE) and microencapsulated (M-OPE) form were applied in “cavatelli” fresh pasta samples to assess their effect on the physicochemical and microbiological quality during refrigerated storage, from day 0, up to 120 days. Results of early days of storage showed that both M-OPE and F-OPE reduced moisture, a_w and pH, compared to control pasta sample (CP). Particularly, lower values of these parameters were observed for fresh pasta samples with M-OPE, compared to the corresponding samples with F-OPE (as regards moisture, on average 25.87% *vs* 27.07%, after 120 days of storage). Just after production, the OPE addition did not impair volatile profile, as control and experimental samples had a low content of volatile organic compounds (VOCs). However, a significant VOCs production uptrend was observed for all samples up to 120 days of storage, particularly for CP and for the samples with the highest F-OPE content. Microencapsulation process was useful to protect bioactive compounds during storage, as samples with M-OPE showed greater values of TPC and antioxidant activity, than samples added of F-OPE (as regards TPC, on average 66.93 *vs* 52.49 mg GAE/kg fresh pasta, at the last sampling time). As expected, TPC, antioxidant activity, moisture and a_w decreased during storage. Also, yellow index showed a decreasing trend; notably MP samples at the end of storage had higher *b** values (19.60) compared to the control and to samples with F-OPE (18.39). Moreover, the addition of OPE, and particularly M-OPE reduced the growth of spoilage microorganism of one logarithmic cycle; experimental samples had significantly lower counts for total aerobic mesophilic bacteria, mould and yeast, compared to CP. Thus, delaying microbial growth, OPE showed its potential in extending the shelf-life of fresh pasta sustainably. Overall, OPE demonstrated significant potential as a sustainable functional ingredient for food applications, providing high levels of phenolic compounds, along with marked antioxidant and antimicrobial activities, aligning with sustainability and circular economy principles. In particular, microencapsulated OPE showed greater technological performance, allowing better preservation of total phenolic content, antioxidant capacity, yellow colour index, and retention of VOCs during storage. On the whole, MP-2 may represent the optimal concentration, as it combines, antimicrobial effects with high phenolic content and significant antioxidant activity, thus indicating its suitability for future commercial development. Despite these promising results, several limitations should be acknowledged. Sensory analysis was not included in the present study and should be addressed in future research to evaluate the impact of OPE and M-OPE on consumer acceptance during storage. Moreover, a consumer test would be useful to assess consumer behaviour towards a functional pasta produced with a by-product and their willingness to pay. From an industrial perspective, cost-effectiveness requires further evaluation; experimental samples, indeed, may potentially be more expensive than CP; the major costs may arise from the extraction of phenolic compounds, as well as from drying and microencapsulation processes. These experimental products may be of particular interest to sustainability- and functionality-oriented consumers.

Supplementary Table 1. Moisture, a_w and pH of fresh pasta samples during storage.

	STORAGE TIME (DAYS)								
SAMPLES	0	15	30	45	60	70	90	110	120
					Moisture (g/100 g)				
CP	31.10 ± 0.08 ^{A,a}	31.08 ± 0.11 $^{A, a}$	30.85 ± 0.1 $^{A, a}$	29.13 ± 0.02 $^{B, a}$	29.09 ± 0.10 $^{B, a}$	29.05 ± 0.08 $^{B, a}$	28.78 ± 0.02 $^{B, a}$	28.11 ± 1.17 $^{B, a}$	28.18 ± 0.26 $^{B, a}$
MP-1	29.89 ± 0.01 $^{A, bc}$	28.25 ± 0.37 $^{B, c}$	27.47 ± 0.34 $^{BCD, d}$	27.88 ± 0.37 $^{BC, bc}$	27.04 ± 0.13 $^{CD, bc}$	26.94 ± 0.63 $^{DE, bcd}$	26.71 ± 0.04 $^{DEF, d}$	26.1 ± 0.11 $^{EF, bc}$	26 ± 0.17 $^{F, cd}$
MP-2	29.39 ± 0.47 $^{A, cd}$	28.4 ± 0.06 $^{B, c}$	27.53 ± 0.3 $^{BC, d}$	27.76 ± 0.01 $^{BC, bc}$	26.98 ± 0.71 $^{CD, bc}$	26.32 ± 0.05 $^{DE, cd}$	26.43 ± 0.01 $^{DE, d}$	26.03 ± 0.21 $^{E, bc}$	26.28 ± 0.31 $^{DE, c}$
MP-3	28.03 ± 0.15 $^{A, e}$	27.88 ± 0.13 $^{AB, c}$	27.59 ± 0.03 $^{AB, d}$	27.46 ± 0.16 $^{BC, c}$	26.88 ± 0.37 $^{C, c}$	26.09 ± 0.01 $^{D, d}$	25.88 ± 0.37 $^{DE, e}$	25.38 ± 0.12 $^{E, c}$	25.35 ± 0.20 $^{E, d}$
FP-1	30.08 ± 0.02 $^{A, bc}$	29.74 ± 0.01 $^{AB, b}$	29.71 ± 0.07 $^{AB, b}$	29.49 ± 0.28 $^{AB, a}$	29.14 ± 0.14 $^{BC, a}$	28.43 ± 0.3 $^{CD, ab}$	28.29 ± 0.20 $^{D, ab}$	27.94 ± 0.42 $^{D, a}$	27.8 ± 0.45 $^{D, ab}$
FP-2	30.33 ± 0.12 $^{A, b}$	30.23 ± 0.34 $^{A, b}$	29.86 ± 0.1 $^{AB, b}$	29.17 ± 0.10 $^{ABC, a}$	29.02 ± 0.09 $^{BC, a}$	28.25 ± 0.05 $^{CD, ab}$	28.12 ± 0.03 $^{CD, bc}$	27.68 ± 1.14 $^{D, ab}$	27.25 ± 0.04 $^{D, b}$
FP-3	28.94 ± 0.44 $^{A, d}$	28.47 ± 0.17 $^{AB, c}$	28.36 ± 0.14 $^{AB, c}$	28.13 ± 0.04 $^{AB, b}$	27.83 ± 0.15 $^{AB, b}$	27.7 ± 1.28 $^{AB, abc}$	27.6 ± 0.28 $^{B, c}$	26.11 ± 0.03 $^{C, bc}$	26.18 ± 0.01 $^{C, c}$
					a_w				
CP	0.968 ± 0.01 $^{A, ab}$	0.971 ± 0.01 $^{A, b}$	0.966 ± 0.01 $^{A, a}$	0.959 ± 0.01 $^{B, ab}$	0.956 ± 0.01 $^{B, ab}$	0.956 ± 0.01 $^{B, a}$	0.947 ± 0.01 $^{C, b}$	0.946 ± 0.01 $^{C, b}$	0.945 ± 0.01 $^{C, ab}$
MP-1	0.973 ± 0.01 $^{A, a}$	0.974 ± 0.01 $^{A, a}$	0.956 ± 0.01 $^{BC, bc}$	0.956 ± 0.01 $^{BC, bc}$	0.954 ± 0.01 $^{BC, ab}$	0.957 ± 0.01 $^{B, a}$	0.952 ± 0.01 $^{C, a}$	0.953 ± 0.01 $^{BC, a}$	0.946 ± 0.01 $^{D, a}$
MP-2	0.957 ± 0.01 $^{A, cd}$	0.956 ± 0.01 $^{A, e}$	0.951 ± 0.01 $^{B, c}$	0.951 ± 0.01 $^{B, e}$	0.945 ± 0.01 $^{CD, c}$	0.937 ± 0.01 $^{E, d}$	0.948 ± 0.01 $^{BC, ab}$	0.943 ± 0.01 $^{D, b}$	0.942 ± 0.01 $^{D, bc}$
MP-3	0.952 ± 0.01 $^{BC, d}$	0.95 ± 0.01 $^{C, f}$	0.962 ± 0.01 $^{A, ab}$	0.955 ± 0.01 $^{B, cd}$	0.955 ± 0.01 $^{B, ab}$	0.939 ± 0.01 $^{D, d}$	0.942 ± 0.01 $^{D, c}$	0.927 ± 0.01 $^{E, c}$	0.93 ± 0.01 $^{E, d}$
FP-1	0.963 ± 0.01 $^{A, bc}$	0.959 ± 0.01 ^{AB,de}	0.959 ± 0.96 $^{AB, abc}$	0.96 ± 0.01 $^{AB, a}$	0.956 ± 0.01 $^{BC, ab}$	0.95 ± 0.01 $^{DE, bc}$	0.952 ± 0.01 $^{CD, a}$	0.952 ± 0.01 $^{CD, a}$	0.946 ± 0.01 $^{E, a}$
FP-2	0.958 ± 0.01 $^{B, cd}$	0.962 ± 0.01 $^{A, c}$	0.96 ± 0.01 $^{AB, ab}$	0.96 ± 0.01 $^{AB, a}$	0.96 ± 0.01 $^{AB, a}$	0.952 ± 0.01 $^{C, b}$	0.942 ± 0.01 $^{D, c}$	0.942 ± 0.01 $^{D, b}$	0.941 ± 0.01 $^{D, c}$
FP-3	0.956 ± 0.01 $^{AB, cd}$	0.961 ± 0.01 $^{A, cd}$	0.959 ± 0.01 $^{AB, abc}$	0.953 ± 0.01 $^{ABC, de}$	0.952 ± 0.01 $^{BCD, b}$	0.948 ± 0.01 $^{CDE, c}$	0.939 ± 0.01 $^{F, c}$	0.944 ± 0.01 $^{DEF, b}$	0.941 ± 0.01 $^{EF, bc}$
					pH				
CP	5.71 ± 0.01 $^{H, a}$	5.91 ± 0.01 $^{DE, a}$	5.74 ± 0.02 $^{GH, a}$	6.05 ± 0.03 $^{C, ab}$	6.13 ± 0.03 $^{B, a}$	5.99 ± 0.02 $^{CD, b}$	6.25 ± 0.06 $^{A, a}$	5.81 ± 0.02 $^{FG, a}$	5.87 ± 0.02 $^{EF, a}$
MP-1	5.50 ± 0.1 $^{D, b}$	5.46 ± 0.11 $^{D, b}$	5.69 ± 0.06 $^{C, a}$	6.01 ± 0.02 $^{AB, bc}$	6.03 ± 0.02 $^{A, b}$	5.95 ± 0.02 $^{AB, bc}$	5.87 ± 0.03 $^{ABC, b}$	5.86 ± 0.10 $^{ABC, a}$	5.84 ± 0.01 $^{BC, a}$
MP-2	5.56 ± 0.04 $^{D, b}$	5.47 ± 0.06 $^{D, b}$	5.5 ± 0.05 $^{D, b}$	5.97 ± 0.04 $^{BC, bc}$	6.01 ± 0.01 $^{AB, bc}$	6.11 ± 0.04 $^{A, a}$	5.94 ± 0.03 $^{BC, b}$	5.89 ± 0.04 $^{C, a}$	5.89 ± 0.02 $^{C, a}$
MP-3	5.54 ± 0.1 $^{DE, b}$	5.6 ± 0.10 CD $^{E, b}$	5.64 ± 0.04 $^{BCDE, a}$	5.79 ± 0.15 $^{ABC, d}$	5.89 ± 0.03 $^{A, de}$	5.90 ± 0.02 $^{A, c}$	5.85 ± 0.05 $^{AB, b}$	5.77 ± 0.02 $^{ABCD, a}$	5.52 ± 0.11 $^{E, b}$
FP-1	5.44 ± 0.01 $^{E, b}$	5.61 ± 0.06 $^{D, b}$	5.63 ± 0.03 $^{D, a}$	6.21 ± 0.01 $^{A, a}$	5.94 ± 0.03 $^{B, cd}$	5.98 ± 0.03 $^{B, b}$	5.92 ± 0.06 $^{BC, b}$	5.83 ± 0.03 $^{C, a}$	5.83 ± 0.03 $^{C, a}$
FP-2	5.44 ± 0.01 $^{E, b}$	5.62 ± 0.05 $^{D, b}$	5.71 ± 0.03 $^{D, a}$	5.84 ± 0.02 $^{C, cd}$	5.98 ± 0.04 $^{B, bc}$	6.00 ± 0.04 $^{B, b}$	6.21 ± 0.04 $^{A, a}$	5.82 ± 0.02 $^{C, a}$	5.82 ± 0.07 $^{C, a}$
FP-3	5.45 ± 0.01 $^{D, b}$	5.49 ± 0.02 $^{D, b}$	5.43 ± 0.04 $^{D, b}$	5.99 ± 0.02 $^{A, bc}$	5.83 ± 0.03 $^{C, e}$	5.89 ± 0.04 $^{BC, c}$	5.94 ± 0.04 $^{AB, b}$	5.82 ± 0.03 $^{C, a}$	5.89 ± 0.05 $^{BC, a}$

Values shown are mean ± standard deviation. [a,f] Different superscript letters from the same time period (column) followed by different letters indicate significant differences in the function of the treatment ($p < 0.05$) by two-way ANOVA followed by Tukey's HSD test. * $p < 0.05$.

[A,F] Different superscript letters from the same treatment (row) followed by different letters indicate significant differences in the function of time ($p < 0.05$) by one-way ANOVA followed by Tukey's HSD test. * $p < 0.05$.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated extract); FP, fresh pasta samples with F-OPE (free lyophilised extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used.

Supplementary Table 2. Total phenolic compound (TPC) and antioxidant activity of fresh pasta samples during storage.

	STORAGE TIME (DAYS)								
SAMPLES	0	15	30	45	60	70	90	110	120
					TPC (mg GAE/kg fresh pasta)				
CP	294.00 ± 6.65 $^{A, d}$	195.98 ± 4.29 $^{B, e}$	185.72 ± 5.78 $^{B, d}$	150.04 ± 2.12 $^{C, c}$	112.63 ± 6.21 $^{D, d}$	65.19 ± 2.77 $^{E, e}$	62.42 ± 1.21 ^{EF,c}	52.03 ± 1.99 $^{F, c}$	53.12 ± 0.54 $^{F, d}$
MP-1	361.00 ± 12.89 $^{A, c}$	216.83 ± 6.51 $^{B, d}$	216.94 ± 10.00 $^{B, c}$	208.43 ± 3.66 $^{B, b}$	129.54 ± 5.93 $^{C, c}$	89.46 ± 3.25 $^{D, d}$	62.37 ± 0.21 $^{E, c}$	67.00 ± 3.45 $^{E, ab}$	58.15 ± 1.13 $^{E, c}$
MP-2	425.92 ± 6.34 $^{A, b}$	227.44 ± 1.80 $^{B, d}$	224.60 ± 6.90 $^{B, c}$	220.93 ± 3.45 $^{B, b}$	140.34 ± 4.20 $^{C, c}$	142.58 ± 7.92 $^{C, b}$	70.35 ± 2.63 $^{D, b}$	62.64 ± 3.01 $^{D, b}$	62.34 ± 1.16 $^{D, b}$
MP-3	495.72 ± 10.75 $^{A, a}$	288.21 ± 8.61 $^{B, a}$	279.87 ± 1.63 $^{B, a}$	249.72 ± 8.52 $^{C, a}$	191.52 ± 1.65 $^{D, a}$	160.66 ± 4.16 $^{E, a}$	79.17 ± 0.55 $^{F, a}$	72.16 ± 1.89 $^{F, a}$	80.30 ± 1.76 $^{F, a}$
FP-1	359.50 ± 6.12 $^{A, c}$	202.44± 0.94 $^{B, e}$	184.85 ± 3.30 $^{C, d}$	162.70 ± 2.79 $^{D, c}$	115.75 ± 2.18 $^{E, d}$	67.30 ± 2.22 $^{F, e}$	63.93 ± 1.06 $^{F, c}$	70.37 ± 1.40 $^{F, a}$	48.59 ± 0.32 $^{G, e}$
FP-2	382.13 ± 10.65 $^{A, c}$	254.40 ± 5.90 $^{B, c}$	246.62 ± 5.04 $^{B, b}$	208.26 ± 4.69 $^{C, b}$	165.73 ± 6.61 $^{D, b}$	123.96 ± 1.46 $^{E, c}$	75.55 ± 1.48 $^{F, a}$	72.29 ± 3.26 $^{F, a}$	51.07 ± 0.66 $^{G, de}$
FP-3	429.56 ± 13.09 $^{A, b}$	270.72 ± 3.47 $^{B, b}$	261.23 ± 5.06 $^{B, b}$	256.33 ± 6.95 $^{B, a}$	180.23 ± 3.04 $^{C, a}$	135.91 ± 2.20 $^{D, b}$	76.64 ± 0.72 $^{E, a}$	70.61 ± 0.64 $^{EF, a}$	57.80 ± 1.32 $^{F, c}$
					DPPH (μmol TE/kg fresh pasta)				
CP	125.21 ± 2.06 $^{A, g}$	109.54 ± 6.02 $^{B, f}$	103.63 ± 3.11 $^{BC, g}$	97.96 ± 5.37 $^{CD, f}$	86.31 ± 4.80 $^{EF, f}$	85.64 ± 1.46 $^{EF, e}$	90.19 ± 1.68 $^{DE, e}$	79.51 ± 0.35 $^{FG, f}$	70.32 ± 3.85 $^{G, f}$
MP-1	289.97 ± 1.79 $^{A, e}$	286.44 ± 3.36 $^{A, d}$	267.99 ± 4.31 $^{B, e}$	244.78 ± 4.99 $^{C, d}$	231.67 ± 4.42 $^{D, d}$	221.77 ± 1.00 $^{E, c}$	147.57 ± 2.89 $^{F, d}$	128.92 ± 3.73 $^{G, d}$	116.20 ± 0.45 $^{H, d}$
MP-2	406.25 ± 0.38 $^{A, c}$	297.97 ± 5.05 $^{CD, cd}$	317.05 ± 10.26 $^{B, c}$	306.58 ± 4.30 $^{BC, c}$	289.91 ± 3.43 $^{D, c}$	265.94 ± 1.56 $^{E, b}$	247.89 ± 4.88 $^{F, b}$	162.40 ± 4.44 $^{G, c}$	158.15 ± 0.25 $^{G, c}$
MP-3	465.01 ± 3.19 $^{A, b}$	416.56 ± 0.37 $^{B, b}$	443.86 ± 1.65 $^{A, a}$	401.80 ± 6.88 $^{BC, a}$	394.03 ± 2.29 $^{C, a}$	386.58 ± 6.44 $^{C, a}$	231.25 ± 20.56 $^{D, b}$	211.86 ± 1.49 $^{D, a}$	210.6 ± 4.40 $^{D, a}$
FP-1	206.84 ± 11.75 $^{A, f}$	200.87 ± 4.36 $^{A, e}$	197.84 ± 6.70 $^{A, f}$	187.33 ± 16.48 $^{AB, e}$	168.58 ± 4.99 $^{B, e}$	125.14 ± 2.71 ^{CD,d}	141.67 ± 3.60 $^{C, d}$	106.58 ± 6.04 $^{D, e}$	70.66 ± 3.23 $^{E, f}$
FP-2	345.56 ± 18.46 $^{A, d}$	301.66 ± 3.47 $^{B, c}$	296.74 ± 7.36 $^{B, d}$	244.57 ± 11.15 $^{C, d}$	230.07 ± 0.49 $^{C, d}$	231.46 ± 2.16 $^{C, c}$	183.46 ± 9.18 $^{D, c}$	168.85 ± 5.35 $^{D, c}$	85.36 ± 7.36 $^{E, e}$
FP-3	491.02 ± 2.04 $^{A, a}$	456.30 ± 4.13 $^{B, a}$	401.93 ± 11.15 $^{C, b}$	360.27 ± 11.45 $^{D, b}$	343.94 ± 6.17 $^{D, b}$	259.86 ± 17.31 $^{E, b}$	279.32 ± 4.39 $^{E, a}$	189.54 ± 8.16 $^{F, b}$	192.44 ± 6.26 $^{F, b}$
					ABTS (μmol TE/kg fresh pasta)				
CP	210.10 ± 4.21 $^{A, e}$	205.24 ± 1.69 $^{A, e}$	159.86 ± 4.94 $^{B, f}$	165.04 ± 2.27 $^{B, f}$	158.01 ± 3.34 $^{B, g}$	119.00 ± 0.66 $^{C, f}$	127.10 ± 3.18 $^{C, e}$	87.15 ± 2.00 $^{D, f}$	65.15 ± 7.92 $^{E, d}$
MP-1	411.33 ± 1.36 $^{A, c}$	400.97 ± 8.48 $^{A, c}$	334.62 ± 2.08 $^{B, d}$	335.44 ± 2.14 $^{B, d}$	253.69 ± 7.48 $^{C, e}$	187.64 ± 8.67 $^{D, d}$	147.49 ± 0.16 $^{E, d}$	144.13 ± 1.78 $^{E, e}$	106.93 ± 12.46 $^{F, c}$
MP-2	442.71 ± 2.51 $^{A, b}$	440.44 ± 4.20 $^{A, b}$	433.53 ± 2.86 $^{A, b}$	351.57 ± 0.93 $^{C, c}$	386.41 ± 6.09 $^{B, c}$	521.01 ± 6.36 $^{D, c}$	206.67 ± 5.77 $^{E, c}$	165.63 ± 4.09 $^{F, cd}$	177.40 ± 7.05 $^{F, a}$
MP-3	453.57 ± 2.81 $^{BC, a}$	465.06 ± 13.99 $^{AB, a}$	474.56 ± 4.03 $^{A, a}$	463.33 ± 5.26 $^{AB, a}$	446.22 ± 2.32 $^{C, a}$	285.74 ± 2.70 $^{D, a}$	230.36 ± 0.17 $^{E, b}$	183.10 ± 4.60 $^{F, ab}$	194.76 ± 7.05 $^{F, a}$
FP-1	313.53 ± 6.04 $^{A, d}$	327.25 ± 11.42 $^{A, d}$	272.25 ± 5.80 $^{B, e}$	223.62 ± 4.63 $^{C, e}$	200.60 ± 2.98 $^{D, f}$	153.19 ± 0.25 $^{E, e}$	159.39 ± 4.78 $^{E, d}$	160.57 ± 7.66 $^{E, d}$	119.42 ± 4.48 $^{F, c}$
FP-2	413.84 ± 3.78 $^{A, c}$	415.91 ± 4.16 $^{A, c}$	356.56 ± 2.02 $^{B, c}$	352.61 ± 9.24 $^{B, c}$	293.75 ± 0.41 $^{C, d}$	269.75 ± 0.77 $^{D, b}$	256.23 ± 8.52 $^{D, a}$	174.88 ± 0.44 $^{E, bc}$	146.02 ± 12.83 $^{F, b}$
FP-3	432.87 ± 2.91 $^{AB, b}$	441.52 ± 4.94 $^{A, b}$	443.52 ± 2.86 $^{A, b}$	421.90 ± 0.74 $^{BC, b}$	418.57 ± 2.69 $^{C, b}$	266.95 ± 6.01 $^{D, b}$	251.96 ± 8.79 $^{E, a}$	190.88 ± 7.92 $^{F, a}$	144.19 ± 3.07 $^{G, b}$

Values shown are mean ± standard deviation. [a,f] Different superscript letters from the same period (column) followed by different letters indicate significant differences in the function of the treatment ($p < 0.05$) by two-way ANOVA followed by Tukey's HSD test. * $p < 0.05$.

[A,F] Different superscript letters from the same treatment (row) followed by different letters indicate significant differences in the function of time ($p < 0.05$) by one-way ANOVA followed by Tukey's HSD test. * $p < 0.05$.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated extract); FP, fresh pasta samples with F-OPE (free lyophilised extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used.

Supplementary Table 3. *L**, *a**, *b** of fresh pasta samples during storage.

	STORAGE TIME (DAYS)								
SAMPLES	0	15	30	45	60	70	90	110	120
					L*				
CP	80.75 ± 0.01 $^{E, a}$	80.98 ± 0.05 $^{D, a}$	81.03 ± 0.08 $^{D, a}$	80.72 ± 0.05 $^{E, a}$	80.39 ± 0.06 $^{F, a}$	79.62 ± 0.01 $^{G, a}$	81.3 ± 0.03 $^{C, a}$	82.12 ± 0.04 $^{B, a}$	82.31 ± 0.08 $^{A, a}$
MP-1	77.63 ± 0.02 $^{BC, c}$	77.68 ± 0.16 $^{B, b}$	77.64 ± 0.2 $^{BC, b}$	77.31 ± 0.02 $^{C, b}$	76.47 ± 0.13 $^{D, c}$	76.33 ± 0.02 $^{D, c}$	76.36 ± 0.08 $^{D, d}$	78.3 ± 0.13 $^{A, c}$	78.36 ± 0.16 $^{A, c}$
MP-2	74.72 ± 0.01 $^{D, e}$	75.42 ± 0.04 $^{C, d}$	74.71 ± 0.05 $^{D, d}$	74.83 ± 0.16 $^{D, c}$	74.13 ± 0.04 $^{E, e}$	74.09 ± 0.07 $^{E, d}$	73.83 ± 0.23 $^{E, e}$	77.23 ± 0.04 $^{A, d}$	76.71 ± 0.20 $^{B, e}$
MP-3	73.16 ± 0.01 $^{C, g}$	73.7 ± 0.07 $^{B, e}$	73.73 ± 0.02 $^{B, e}$	72.7 ± 0.22 $^{D, e}$	72.32 ± 0.33 $^{D, g}$	71.89 ± 0.07 $^{E, e}$	71.34 ± 0.11 $^{F, f}$	73.53 ± 0.02 $^{BC, f}$	76.46 ± 0.04 $^{A, e}$
FP-1	77.84 ± 0.01 $^{D, b}$	77.81 ± 0.12 $^{D, b}$	77.76 ± 0.10 $^{D, b}$	77.03 ± 0.12 $^{E, b}$	77.62 ± 0.05 $^{D, b}$	78.24 ± 0.04 $^{C, b}$	78.49 ± 0.06 $^{C, b}$	79.73 ± 0.20 $^{B, b}$	81.57 ± 0.03 $^{A, b}$
FP-2	77.14 ± 0.02 $^{ABC, d}$	76.99 ± 0.03 $^{BC, c}$	76.61 ± 0.38 $^{CD, c}$	74.98 ± 0.01 $^{E, c}$	74.91 ± 0.03 $^{E, d}$	76.3 ± 0.18 $^{D, c}$	77.46 ± 0.33 $^{AB, c}$	77.17 ± 0.01 $^{AB, d}$	77.58 ± 0.23 $^{A, d}$
FP-3	73.67 ± 0.01 $^{B, f}$	73.75 ± 0.02 $^{B, e}$	73.47 ± 0.02 $^{BC, e}$	73.22 ± 0.15 $^{C, d}$	72.73 ± 0.04 $^{D, f}$	71.43 ± 0.30 $^{E, f}$	73.69 ± 0.18 $^{B, e}$	75.28 ± 0.22 $^{A, e}$	75.66 ± 0.19 $^{A, f}$
					a*				
CP	-0.62 ± 0.01 $^{A, g}$	-0.7 ± 0.01 $^{A, e}$	-0.68 ± 0.05 $^{A, f}$	-0.67 ± 0.04 $^{A, g}$	-0.81 ± 0.04 $^{B, g}$	-0.82 ± 0.04 $^{B, f}$	-0.99 ± 0.02 $^{C, e}$	-1.05 ± 0.05 $^{C, g}$	-1.07 ± 0.06 $^{C, e}$
MP-1	0.21 ± 0.02 $^{A, d}$	-0.05 ± 0.04 $^{B, d}$	-0.13 ± 0.01 $^{C, d}$	-0.17 ± 0.01 $^{CD, e}$	-0.18 ± 0.01 $^{CD, e}$	-0.23 ± 0.01 $^{DE, e}$	-0.30 ± 0.02 $^{F, d}$	-0.32 ± 0.05 $^{F, e}$	-0.27 ± 0.02 $^{EF, c}$
MP-2	0.25 ± 0.01 $^{C, c}$	0.14 ± 0.04 $^{D, c}$	0.14 ± 0.01 $^{D, c}$	0.2 ± 0.01 $^{CD, c}$	0.23 ± 0.02 $^{C, c}$	0.34 ± 0.02 $^{B, c}$	0.35 ± 0.05 $^{B, b}$	0.33 ± 0.02 $^{B, c}$	0.42 ± 0.02 $^{A, b}$
MP-3	1.04 ± 0.04 $^{AB, a}$	0.93 ± 0.08 $^{BC, a}$	0.85 ± 0.03 $^{C, a}$	1.06 ± 0.04 $^{A, a}$	1.06 ± 0.05 $^{A, a}$	1.07 ± 0.01 $^{A, a}$	0.82 ± 0.01 $^{C, a}$	0.69 ± 0.05 $^{D, b}$	0.34 ± 0.03 $^{E, b}$
FP-1	-0.02 ± 0.01 $^{A, e}$	-0.08 ± 0.02 $^{A, d}$	-0.23 ± 0.04 $^{B, e}$	-0.32 ± 0.07 $^{BC, f}$	-0.32 ± 0.05 $^{BC, f}$	-0.29 ± 0.02 $^{BC, e}$	-0.35 ± 0.02 $^{C, d}$	-0.48 ± 0.02 $^{D, f}$	-0.77 ± 0.01 $^{E, d}$
FP-2	-0.10 ± 0.01 $^{AB, f}$	-0.12 ± 0.01 $^{B, d}$	-0.09 ± 0.02 $^{AB, d}$	-0.07 ± 0.02 $^{A, d}$	-0.07 ± 0.02 $^{AB, d}$	-0.09 ± 0.02 $^{AB, d}$	-0.07 ± 0.01 $^{A, c}$	-0.19 ± 0.01 $^{C, d}$	-0.25 ± 0.03 $^{D, c}$
FP-3	0.39 ± 0.03 $^{D, b}$	0.39 ± 0.01 $^{D, b}$	0.56 ± 0.01 $^{C, b}$	0.64 ± 0.01 $^{B, b}$	0.81 ± 0.02 $^{A, b}$	0.82 ± 0.01 $^{A, b}$	0.84 ± 0.02 $^{A, a}$	0.83 ± 0.05 $^{A, a}$	0.79 ± 0.02 $^{A, a}$
					b*				
CP	22.22 ± 0.01 $^{A, b}$	21.32 ± 0.04 $^{B, c}$	20.91 ± 0.02 $^{BC, d}$	20.77 ± 0.01 $^{CD, c}$	20.41 ± 0.05 $^{DE, cd}$	20.26 ± 0.04 $^{E, c}$	19.93 ± 0.04 $^{EF, cd}$	19.48 ± 0.50 $^{F, ab}$	18.96 ± 0.03 $^{G, c}$
MP-1	22.43 ± 0.01 $^{A, a}$	21.8 ± 0.15 $^{B, a}$	21.39 ± 0.03 $^{C, b}$	21.08 ± 0.01 $^{D, b}$	20.87 ± 0.22 $^{D, ab}$	20.91 ± 0.02 $^{D, a}$	19.77 ± 0.10 $^{F, d}$	20.16 ± 0.13 $^{E, a}$	19.83 ± 0.06 $^{F, a}$
MP-2	22.04 ± 0.01 $^{A, d}$	21.62 ± 0.10 $^{AB, ab}$	21.15 ± 0.04 $^{BC, c}$	20.73 ± 0.04 $^{CD, cd}$	20.68 ± 0.2 $^{CD, bc}$	20.64 ± 0.03 $^{D, b}$	20.37 ± 0.24 $^{D, b}$	19.61 ± 0.36 $^{E, ab}$	19.52 ± 0.11 $^{E, b}$
MP-3	21.90 ± 0.01 $^{A, e}$	21.51 ± 0.05 $^{BC, bc}$	21.55 ± 0.03 $^{B, a}$	21.37 ± 0.1 $^{C, a}$	21.18 ± 0.03 $^{D, a}$	20.72 ± 0.05 $^{E, b}$	20.84 ± 0.04 $^{E, a}$	19.48 ± 0.13 $^{F, ab}$	19.55 ± 0.03 $^{F, b}$
FP-1	22.07 ± 0.01 $^{A, c}$	21.58 ± 0.01 $^{B, ab}$	21.57 ± 0.03 $^{B, a}$	21.19 ± 0.09 $^{C, b}$	20.71 ± 0.04 $^{D, bc}$	20.88 ± 0.11 $^{D, a}$	20.2 ± 0.16 $^{E, bc}$	20.16 ± 0.04 $^{E, a}$	18.93 ± 0.05 $^{F, c}$
FP-2	21.10 ± 0.01 $^{A, g}$	20.79 ± 0.07 $^{B, d}$	20.34 ± 0.03 $^{C, f}$	20.26 ± 0.01 $^{C, e}$	20.13 ± 0.01 $^{D, d}$	20.06 ± 0.01 $^{D, d}$	19.39 ± 0.04 $^{E, e}$	19.07 ± 0.02 $^{F, bc}$	17.93 ± 0.05 $^{G, e}$
FP-3	21.29 ± 0.07 $^{A, f}$	20.92 ± 0.10 $^{B, d}$	20.69 ± 0.07 $^{C, e}$	20.59 ± 0.04 $^{C, d}$	20.27 ± 0.01 $^{D, d}$	20.09 ± 0.01 $^{D, d}$	19.76 ± 0.03 $^{E, d}$	18.62 ± 0.18 $^{F, c}$	18.3 ± 0.03 $^{G, d}$

Values shown are mean ± standard deviation. [a,f] Different superscript letters from the same period (column) followed by different letters indicate significant differences in the function of the treatment ($p < 0.05$) by two-way ANOVA followed by Tukey's HSD test. * $p < 0.05$.

[A,F] Different superscript letters from the same treatment (row) followed by different letters indicate significant differences in the function of time ($p < 0.05$) by one-way ANOVA followed by Tukey's HSD test. * $p < 0.05$.

Abbreviations: CP, Control fresh pasta; MP, fresh pasta samples with M-OPE (microencapsulated extract); FP, fresh pasta samples with F-OPE (free lyophilised extract); the numbers 1,2,3 indicate the 3 different concentrations of OPE used.

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Conclusion and future perspectives of Chapter 1

The aim of this first chapter of the thesis was to scale-up innovative solutions emerging from basic research to an industrial production environment, thereby supporting food companies in addressing global challenges, such as: i) promoting healthier diets; ii) reducing pollution, upcycling a by-product; iii) reducing food waste, improving the microbiological quality of products during storage. To translate these objectives into a concrete industrial application, the work focused on fresh pasta as a model product, using olive pomace as the upcycled ingredient. Accordingly, first of all, laboratory trials were preformed to assess the optimal olive pomace extract concentration (OPE) to be applied in fresh pasta samples. Then, the upscaling of the optimal formulations was performed at Maffei company (Cutrofiano, Lecce, Italy). The aims of these activities were to: i) improve the nutritional, sensory and physicochemical characteristics of fresh pasta samples, employing OPE, in order to promote healthier food; ii) enhance the microbiological and physicochemical quality of the samples during storage, in order to avoid food waste generation.

On the whole, these activities represent a successful scale-up of innovation in fresh pasta formulation, from basic research, up to industrial production, highlighting the pivotal role of the collaboration among researchers and food companies. Experimental samples, indeed, had higher total phenolic compound and higher antioxidant activity, compared to the control. The analysis of volatile organic compounds revealed that the addition of OPE did not negatively affect the volatile profile of fresh pasta, supporting the findings of the sensory analysis, in which all samples received positive scores in terms of overall acceptability. Promising results have been obtained during storage, as all samples up to 120 days, still complied with Italian law, according to water content ad a_w. Interestingly, OPE could be used as natural preservatives to extend the shelf-life of fresh pasta, as a result of reduced growth of spoilage microorganisms.

Future research should focus on the effective biological properties of the fresh pasta produced, in order to achieve health claims. It could also be interesting to evaluate the effect of OPE on extending the shelf-life of fresh pasta under thermal abuse conditions, simulating the real scenarios to which the fresh pasta is subjected, during transport and subsequent purchase. Thereafter, along the technology readiness scale (TRL), food companies could move on to TRL 8-9, in which all technical process and system to support commercial activity are ready and the product is full available for consumers. Hence, a fresh pasta rich in phenolic compounds from olive pomace and with longer shelf-life could be released on the market very soon, representing a concrete example of successful technology transfer in the food sector.

Chapter 2- Innovation in the formulation of traditional foods: a strategy to address non-communicable diseases

Introduction

This chapter describes the second case study, focused on the development of innovative food prototypes, that can be easily transferred to industrial production, and aimed at the nutritional improvement of a traditional food product. This section explores the small-scale production of reformulated *pão de queijo* (Brazilian cheese bread), developed to reduce saturated fat content, improving its fatty acid profile. Carried out in collaboration with the Nutrition Institute of Rio de Janeiro State University, this study shows how innovations can be integrated into traditional food matrices, without compromising sensory quality, contributing to the prevention of non-communicable diseases.

Non-communicable diseases (NCDs) are long term chronic diseases characterized by slow development or progression, representing a global public health challenge, as are responsible for sizeable deaths and disability worldwide (Habib & Saha, 2010). NCDs, indeed, account for about 38 million deaths each year, representing 68% of the world's total mortality rate (Hadian et al., 2021). They also contribute to death at a young age, especially in low- and middle-income countries, where 29% of NCD-related deaths occur in individuals under the age of 60, compared to 13% in high-income countries. If population growth continues at this rate, by 2030, 52 million people will die from these diseases each year (Hadian et al., 2021). The four most prominent NCDs are cancer, cardiovascular diseases (CVDs), chronic obstructive pulmonary diseases and diabetes. Risk factors such as tobacco and alcohol consumption, unhealthy diet, and sedentary behaviour have contributed significantly to the development of NCDs (Habib & Saha, 2010). However, evidence suggests that awareness and management of these risk factors could prevent up to 80% of premature cases of heart disease and stroke, 80% of type II diabetes, and approximately 40% of cancers (Habib & Saha, 2010). Thus, the emergence of NCDs as a global health crisis demands effective strategies for its prevention and management (Aderinola et al., 2024). Several studies (Claas & Arnett, 2016; Kaminsky et al., 2022) showed the critical role of lifestyle factors reducing the impact of these diseases, as well as in improving overall health outcomes. Particularly, diets have a pivotal role, emerging as a key approach in addressing the challenges posed by NCDs, as dietary interventions are sustainable in the long term, making them suitable for the chronic nature of NCDs. Reducing the consumption of salt, sugar and saturated fat could significantly reduce the development of NCDs (Aderinola et al., 2024). Thus, all individuals should be encouraged to adhere to a healthy eating plan. Focusing on dietary fat, it should contribute to 15–30% of a daily caloric intake, with saturated fat limited to 10% of total calories, while cholesterol intake should not exceed 300 mg per day (Habib & Saha, 2010). As a matter of fact, Kim et al. (2021) highlighted that diets rich in saturated fats have been linked to increased mortality from cardiovascular diseases and cancer, whereas higher intake of monounsaturated and polyunsaturated fats have been associated with reduced mortality from these conditions. Therefore, efforts are necessary to reduce the content of highly unsaturated fatty acids in food products, in order to pursue the 3rd Sustainable Development Goal of the 2030 Agenda, of reducing NCD-related deaths by a third by 2030 (Malta et al., 2020). However, many products that increase NCDs are profitable for companies; globalization of marketing and commerce offers many opportunities for companies to promote the consumption of ready-to-eat products, often characterized by high fat content, especially saturated fats. Such trends are most evident in nations with pronounced social disparities and limited educational opportunities (Hadian et al., 2021; Moodie et al., 2013). For this reason, it is expected that for cancer, type 2 diabetes and CVDs incidence, the estimated percentage increase by 2030, will be greater in low- (82%) and lower-middle-income countries (70%), compared with the upper-middle- (58%) and high-income countries (40%) (Wagner & Brath, 2012).

Brazil is a developing economy, in which 55% of deaths is related to NCDs (Feliciano et al., 2023). The incidence of these diseases has decreased in recent decades, because of reduced exposure to alcohol and tobacco use (Feliciano et al., 2023; Malta et al., 2016). However, unhealthy diet is recognized as the leading cause of NCDs in Brazil (Malta et al., 2016). Hence, there is a clear need for the prevention of these chronic diseases, with special attention to the role of nutrition. Bread is the staple food of Brazilian diet and is enjoyed in countries all around the world (Kazerski et al., 2022). In particular, cheese bread (*pão de queijo)* is a traditional Brazilian bread, which is currently available on foreign markets, being a gluten-free product (Papalia et al., 2015). It is produced using cassava starch, salt, oil, cheese and eggs, thus resulting in a product with a high content of saturated fatty acids (Teixeira et al., 2020). However, as the busy-modern lifestyle increases the demand for ready-to-eat or ready-to-bake foods (Kazerski et al., 2022), this products is commonly consumed at multiple times during the day, including breakfast and snack times, being a product ready for consumption (Clareto et al., 2006). These findings highlight the need to improve nutritionally this traditional bread, that is so widely consumed and could be associated with increased risk of CVDs for consumers. Considering that, this product is already manufactured at an industrial scale, there is a clear need to find solutions that can be easily adapted to large-scale applications. Therefore, in collaboration with the Nutrition Institute of Rio de Janeiro State University, innovations in cheese bread formulation, effective for the improvement of the fatty acid profile, have been tested in a small scale. Particularly, avocado pulp and emulsion-filled gel have been tasted as cheese substitute, in order to increase concentration of dietary fibre and reduce content of saturated fats. Although large-scale production was not investigated due to time constraints associated with pilot-scale experimentation, these experimental *pão de queijo* formulations are readily suitable for transfer to large-scale production, highlighting their potential for industrial application. The production process of cheese breads, indeed, is extremely simple, as it involves: i) dough making; ii) forming; iii) filling (if necessary). At industrial scale, these phases are carried out automatically within a single machine. Accordingly, progression to TRL 5-6 should involve pilot-scale validation using such integrated industrial equipment. Moreover, it is worth highlighting that there is no standardized technology for cheese bread production, which allows for the easy adaptation of new ingredients during scale-up, to large-scale production. Consequently, this study provides a preliminary technical basis for future pilot-scale assessment, showing that the simplicity of the production process enables realistic scale-up and future industrial validation.

The results presented in the following publication, including chemical-physical, rheological, nutritional characteristics, and consumer tests, concern the small-scale production of innovative *pão de queijo* (cheese breads). Recognizing this limitation, the concluding chapter identifies the technical and process-development steps required to reach TRL 5-6 through pilot plant production, while providing a framework for future research activities.

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Publication no. 3:

Reformulating Traditional Foods: Nutritional Enhancement of Brazilian Gluten Free Cheese Bread by combining Olive Oil Emulsion Filled Gel and Avocado Pulp

The following paper has been submitted to *Food Research International* as:

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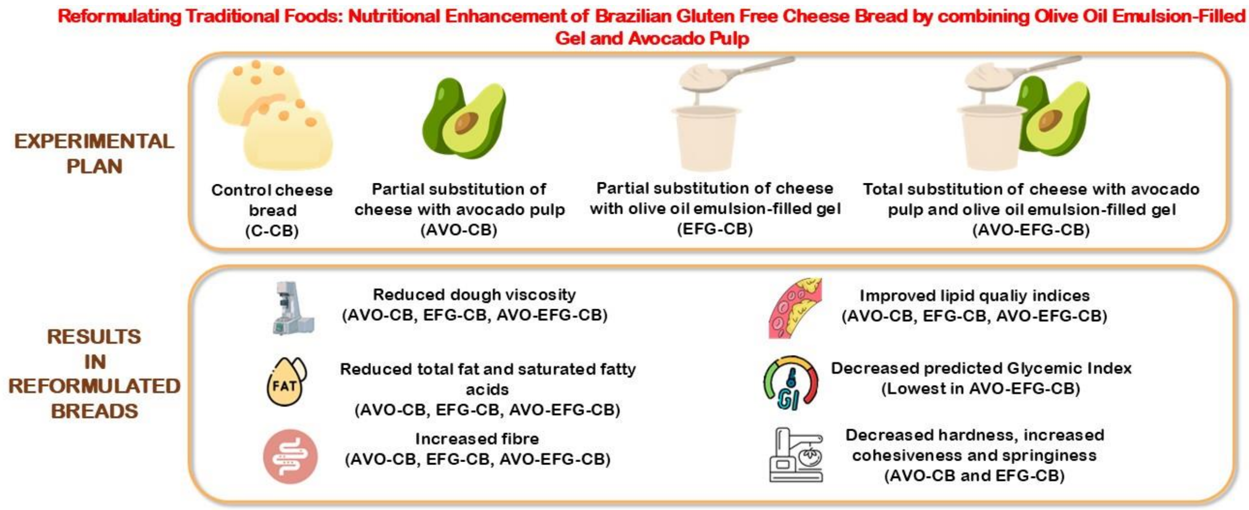
Graphical Abstract



Abstract

The worldwide rise in non-communicable chronic diseases led to a significant increasing demand for food products with higher fibre content and improved lipid profile, particularly needed in the gluten-free sector. Particularly, *pão de queijo* (cheese bread), a traditional and widely consumed Brazilian gluten-free food product, presents nutritional deficiencies, because of high concentration of saturated fats and low content of dietary fibre. Thus, the aim of the present study was to improve the nutritional profile of cheese bread by reformulating it by using avocado pulp and an olive oil emulsion-filled gel (EFG), structured with inulin, as cheese substitutes. The implications of these alternative ingredients on the nutritional composition, technological and rheological properties, and the consumer acceptance of the product were assessed. Results from rheological analysis showed that all doughs had a shear-thinning behaviour, with experimental samples showing lower viscosity values compared to the control. Cheese bread produced with the combined use of avocado pulp and EFG showed the highest fibre and unsaturated fatty acid content, which contributed to the lowest predicted glycaemic index (pGI) and improved lipid quality indices. These experimental formulations exhibited enhanced textural properties, demonstrating greater springiness and reduced chewiness, which combined with results from consumer test, indicated notable acceptability and potential for linking sensory quality with nutritional improvement. Overall, the findings align with worldwide health goals aimed at reducing saturated fats and increasing fibre intake through innovative food design.

Keywords: *pão de queijo*; avocado pulp; emulsion-filled gel; nutritional properties; fatty acid profile; dietary fibre; celiac disease.

1. Introduction

The worldwide rise in non-communicable chronic diseases has sparked increased interest in the reformulation of conventional foods to encourage healthy dietary practices (Frumuzachi et al., 2025; Yu et al., 2024). Excessive consumption of saturated fats and insufficient intake of dietary fibre are prominent dietary risk factors linked to these chronic illnesses (Fekete et al., 2025). These imbalances have been repeatedly reported especially in people following a gluten-free diet (Cardo et al., 2021; Vici et al., 2016). Consequently, there is a significant demand for the design of food products featuring enhanced lipid profiles and increased fibre content, while maintaining high sensory quality and assuring adequate customer acceptance (Mammolenti et al., 2025; Yang et al., 2025).

Cheese bread (*pão de queijo*) is a traditional product, extensively consumed in Brazil, which is gaining international recognition for its distinctive sensory qualities, including a soft texture and appealing flavour. It features a gluten-free composition, being basically composed of cassava starch, (Calderan et al., 2025; Kazerski et al., 2022) and does not contain yeast. Nevertheless, despite its cultural and culinary significance, Brazilian cheese bread demonstrates nutritional deficiencies, as it contains significant amounts of cheese, egg, and oil, resulting in high content of saturated fat and low dietary fibre (Carvalho & Silva, 2015). The nutritional imbalances of cheese bread make it a suitable candidate for reformulation, intended to aligning to modern dietary guidelines, especially those related to cardiovascular and metabolic health (Frumuzachi et al., 2025).

Recently, the application of structured emulsions, including oleogels, emulsion gels, and hydrogelled emulsions, has surfaced as a viable approach in the reformulation of baked goods and snacks (Difonzo et al., 2024; Giacomozzi et al., 2023; Kim et al., 2024; Totosaus et al., 2024), aimed at substituting saturated fats with healthier lipids, while enhancing the nutritional profile of products (Le et al., 2025). Emulsion gels integrate liquid oils with gelling agents, creating structured systems that replicate the technical properties of solid fats in food, while providing healthier lipid profiles, abundant in unsaturated fatty acids (Ferdaus et al., 2025). Inulin stands out itself among the many gelling agents investigated for the structuring of emulsion gels, due to its nutritional significance and technological efficacy. Derived from natural sources such as chicory root, inulin is a soluble dietary fibre that belongs to the fructan group and its prebiotic attributes, selectively enhancing advantageous gut microbiota and promoting metabolic health, are already well-established (Al-Adham et al., 2025). Furthermore, inulin acts as a suitable structuring agent, providing stable emulsion gels that facilitate the integration of liquid oils into solid-like matrices, appropriate for bakery applications (Correa et al., 2024), as previously demonstrated by Curti et al. (2018) and Rodríguez-García et al. (2014) in cookies and cakes, respectively.

Olive oil, due to its nutritional and functional properties, has been linked to various health benefits, including lower LDL cholesterol levels, enhanced insulin sensitivity, and protection from oxidative stress (Chiavarini et al., 2024; Fraga et al., 2025; Pessoa et al., 2022). The use of this ingredient in bakery products constitutes a significant advancement in the development of functional foods, which is closely associated with health benefits and a decreased risk of non-communicable diseases (Caponio et al., 2013). Nonetheless, directly substituting saturated fats with liquid oils might negatively influence food texture and stability. Structuring olive oil into an emulsion gel with inulin as gelling agent offers a novel approach that combines nutritional enhancement with the preservation of desirable sensory and technological characteristics (Giarnetti et al., 2015; Paradiso et al., 2015). Promising results have been obtained by Giarnetti et al. (2015) and Paciulli et al. (2020), employing emulsion-filled gel based on inulin and olive oil in shortbread cookies.

A potential strategy to improve the nutritional profile of bakery products is the use of avocado (*Persea americana*) pulp, which is rich in monounsaturated fats, dietary fibre, vitamins, and phytochemicals, such as carotenoids and tocopherols. Scientific evidence demonstrates, indeed, that avocado consumption is correlated with positive changes in lipid profiles, improving cardiovascular health (Candeloro et al., 2025). From a technological point of view, avocado pulp provides a creamy texture and moisture retention, thereby improving the quality of baked goods. The fruit offers natural pigments and flavours, that enhance the sensory acceptance of novel foods (Nascimento et al., 2025). Consistent with these evidences, several studies have already investigated the use of avocado pulp as a fat replacer in bakery products, such as muffins and pound cakes (Othman et al., 2018; Pokatong & Nathalie, 2021; Samaa, 2025).

The increasing worldwide interest in cheese bread has stimulated scientific efforts to enhance its quality and production process (Kazerski et al., 2022). For instance, Papalia et al. (2015) and Clareto et al. (2006) improved its nutritional profile applying guar gum and xanthan and a protein concentrate, respectively. Aplevicz et al. (2007) demonstrated that incorporating okara in cheese bread substantially increased the dietary fibre content, highlighting its potential as a functional ingredient. On the other hand, by incorporating gelatinized cassava starch, Kazerski et al. (2022) produced a clean label product with no modified starch, while retaining the typical sensory characteristics of cheese bread. However, the combined application of inulin-based emulsion-filled gels (EFG) with olive oil and avocado pulp could provide a synergistic strategy for enhancing the nutritional profile of cheese bread. Moreover, the gluten-free nature of this product enhances its appeal to individuals with celiac disease and those following gluten-free diets, thereby broadening the market potential for a nutritionally enhanced alternative.

In this context, this study aimed to enhance the nutritional profile of cheese bread by partially or totally replacing cheese with avocado pulp and an EFG structured with inulin and olive oil. In addition to the nutritional characteristics, the implications of these alternative ingredients on the technological and rheological properties, as well as on consumer acceptance, were investigated. This approach encourages the development of healthier food alternatives to traditional cheese bread, aligning with global public health recommendations, while preserving its cultural and gastronomic value.

2. Materials and methods

2.1 Materials and Chemicals

Inulin at high degree of polymerization (HDP, DP > 20, Fibruline® XL Chicory Root Fiber-Powder, ~99.50% inulin on dry matter, 94.50% dietary fibre, 4.00% moisture, 0.20% ash) was bought from Cosucra (Warcoing Industrie, Brussels, Belgium). Soy lecithin, olive oil and soybean oil were purchased from Mr.p Ingredients (York, England) Monini (Spoleto, Italy) and Valsoia (Bologna, Italy), respectively. Asiago Protected Designation of Origin (PDO) cheese, eggs, avocado, sour and sweet cassava starch and salt were purchased from local suppliers (Bari, Italy).

2.2 Emulsion-Filled Gel (EFG) and cheese bread preparation

Preparation of the EFG was performed as described by Paradiso et al. (2015) and Giarnetti et al. (2015), in order to obtain a homogeneous matrix, with the appearance of a white-yellowish cream, and a consistency similar to that of a commercial spreadable fat. The EFG was prepared with olive oil (37% w/w), inulin (19% w/w), soy lecithin powder (2% w/w) and water (42% w/w). Homogenization was performed at first with an Ultra-Turrax T-18 (IKA-Werke GmbH & Co. KG, Staufen, Germany) at 3000 rpm for 5 min and subsequently by means of high power ultrasound using a 200 W transducer (Sonopuls HD 3200, Bandelin Electronic, Berlin, Germany), with 6 mm diameter tapered tip (KE 76, Bandelin Electronic, Berlin, Germany) for 5 min. Control and experimental breads were prepared as follows: initially, scalding was performed, which involves gradually adding hot water (~80 °C) to the sweet and sour cassava starch, while continuously stirring, until a soft dough forms (approximately 15 min). Then, the other ingredients were added and mixed manually until the dough became homogeneous (approximately 30 min). Next, the bread was shaped into small balls (2.5 to 3 cm in diameter) and baked in oven (Smeg, Guastalla, Italy) at 160 °C for 20 min. The ingredients used in the control and experimental formulations of the cheese bread samples are presented in Table 1. The ingredient amounts for the control product were determined following the protocol of Silva et al. (2009). The levels of cheese replacement were defined by means of preliminary trials, following the procedure of Paciulli et al. (2020) and Sereti et al. (2023), with minor modification. Three different batches were prepared for each thesis.

Table 1. Cheese bread formulations.

Ingredients (g/100g)	C-CB	AVO-CB	EFG-CB	AVO-EFG-CB
Grated cheese (AsiagoPDO)	23.85	10.31	13.31	/
Sour cassava starch	20.49	21.26	22.88	24.41
Sweet cassava starch	20.49	21.26	22.88	24.41
Hot water	23.85	20.62	26.62	23.67
Soybean oil	2.38	2.06	2.66	2.37
Eggs	7.45	6.44	/	/
Salt	1.49	1.29	1.66	1.48
Avocado pulp	/	16.75	/	14.79
Olive oil emulsion-filled gel structured with inulin	/	/	9.98	8.88

Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin.

2.3 Rheological parameters

Small amplitude oscillatory shear measurements were carried out by a rheometer (HAAKE MARS iQ Air, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a parallel plate geometry (P35/Ti-02180932). First of all, the amplitude sweep was conducted as described by O'Connell and Hartel (2022). The strain was modified from 0.01 to 1000% at a constant frequency of 1 Hz, and a linear viscoelastic region (LVR) was determined. A shear ramp test was carried out to measure the viscosity of the doughs, as reported by Totaro et al. (2025). Briefly, the analysis was conducted at 25°C, using a 0.80 mm gap between the plates, by increasing the shear rate from 0 to 100 s^{-1}. The frequency-dependent behaviour was determined using a 0.80 mm gap between the plates by an oscillatory frequency sweep at 25 °C, carried out as described by Krystyjan et al. (2015), with minor modifications. The frequency varied from 0.10 to 10 Hz at 1% strain, within the LVR of the doughs. The elastic modulus (G′) and viscous modulus (G″) were evaluated as a function of the frequency. The loss factor (tan δ) was calculated as the ratio between G″ and G′. The temperature sweep analysis was conducted according to Totaro et al. (2025). The doughs were analysed at 1 Hz frequency, 0.10 % strain (within the linear viscoelastic regime), and with a 2 mm gap between plates. The temperature varied from 25 to 90 °C, at a heating rate of 5 °C min^{-1}. The geometry containing the sample was covered with a sample hood during the analysis to prevent water evaporation. The G' and G" moduli were recorded as a function of the temperature. All the determinations were carried out in triplicate.

2.4 Nutritional composition

The moisture content and water activity (a_w) of each sample was measured with a Thermobalance (Ladwag Mac 110/NP, Radwag, Poland) and an electronic hygrometer (Aqua-Lab 100–240 VAC, Pullman, WA, USA), respectively. The protein content (total nitrogen × 6.25) and ash content were estimated following the AACC 46–11.02 method (AACC, 2010) and the standard AOAC procedure, no. 923.03, respectively (AOAC, 2006). The lipid content was determined by a Randall apparatus (SER 148 extraction system, Velp Scientifica srl, Usmate, Italy), using diethyl ether as an extracting solvent, according to AOAC method 945.38 F (AOAC, 2006). The enzymatic-gravimetric procedure was used to determine total dietary fibre, as reported in AOAC method no. 991.43 (AOAC, 2006). The carbohydrate content was determined by subtracting the protein, ash, moisture, total dietary fibres, and lipid content from 100 (Oko et al., 2012). The results were expressed as g/100 g of product. Each sample was analysed in triplicate.

2.5 Fatty acids analysis

After fat extraction (performed as reported in 2.4 section), fatty acids were methylated according to the AOCS (American Oil Chemists Society) method Ch 1–91. Fatty acid profile of cheese breads was analysed using a gas-chromatograph 7890B (Agilent Technologies, Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a fused silica capillary column SP2340 (60 m × 250 μm × 0.2 μm film thickness) (Supelco Park, Bellefonte, PA, USA), as reported by Difonzo et al. (2022). The temperature of the split injector was 210 °C with a splitting ratio of 50:1. The temperature of the column was programmed in a temperature gradient of 80 °C for 5 min, with increments of 2 °C min^{-1}, until 200 °C for 5 min, followed by an increase of 10 °C min^{-1}, until 240 °C for 5 min. Helium was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. The temperature of the FID was set to 220 °C with an air flow of 400 mL min^{-1} and helium 40 mL min^{-1}. Identification and quantitative calculation of fatty acids were carried out by comparing the retention time with that of the corresponding methyl ester, using a standard mixture (C4–C24) (Supelco 37 component FAME Mix Bellefonte, PA, USA). The results were expressed as g of fatty acid/100 g of fat. The determinations were performed in triplicate.

2.6 Lipid Quality Indices

Lipid quality indices of cheese breads were assessed by calculating atherogenic index (AI), thrombogenic index (TI). The values of these indices indicate the quality for pro-atherogenicity, pro-thrombogenicity respectively. In addition, the Hypocholesterolemic/Hypercholesterolemic ratio (H/H) and the PUFA/SFA ratio were assessed to further characterise the lipid profile in terms of potential cardiovascular risks. These indices were determined using different combinations and ratios of fatty acids, such as total saturated fatty acids (ΣSFA), total monounsaturated fatty acids (ΣMUFA), total polyunsaturated fatty acids (ΣPUFA), according to Abbas et al. (2021) and Paszczyk & Tońska, (2022), using the following equations:

$$AI = (C12{:}0 + 4 * C14{:}0 + C16{:}0)/(\omega - 3\ PUFA + \omega - 6\ PUFA + MUFA) \quad (1)$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0)/(0.5 * MUFA + 0.5 * \omega - 6\ PUFA + 3 * \omega - 3PUFA + \omega - 3/\omega - 6) \quad (2)$$

$$H/H = (C18{:}1n - 9 + C18{:}2n - 6 + C18{:}3n - 3)/(C12{:}0 + C14{:}0 + C16{:}0) \quad (3)$$

2.7 In vitro starch hydrolysis

In vitro starch hydrolysis of cheese breads was determined according to Caponio et al. (2024), by simulating the *in vivo* digestion of starch. Briefly, 1 g of each sample was subjected to starch determination. Hydrolysed starch was measured as the amount of glucose released, using the D-glucose assay kit (GOPOD format) (Megazyme, Wicklow, Ireland). Starch was calculated as glucose (mg) × 0.90 (the conversion factor). Simulated digests were dialyzed (cut-off of the membrane: 12[thin space (1/6-em)]400 Da) for 180 min. Aliquots of dialysate, containing free glucose, and partially hydrolysed starch were sampled every 30 min and further treated with amyloglucosidase. Then, free glucose was determined using the above-mentioned enzyme-based kit and finally converted into hydrolysed (digested) starch in the cheese breads. Control white wheat bread was used as the control to estimate the hydrolysis index (HI = 100). The predicted glycaemic index (pGI) was calculated using the equation pGI = 0.549 × HI + 39.71, as described by Goñi et al. (1997). Each sample was analysed in triplicate.

2.8 Texture profile analysis

Texture profile analysis was performed using a Z1.0 TN texture analyser (Zwick Roell, Ulm, Germany), equipped with a cylindrical probe (36 mm diameter) and a 50 N load cell, according to the method described by Troilo et al. (2022), with minor modifications. Two compressive cycles were performed at a 3 mm/s probe compression rate and 40% sample deformation in both the compression, with 5 s pause before the second compression. Data were acquired by the TestXPertII version 3.41 software (Zwick Roell, Ulm, Germany). Values shown are means of three replicates.

2.9 Consumer test

Consumer sensory evaluation was conducted to assess the acceptability of control and experimental breads. A total of 73 participants were included in the study, comprising 48 women and 25 men. Participant ages ranged from 20 to 66 years, with an average of 36 years. The evaluation took place in a sensory analysis laboratory, conducted under controlled conditions. Each participant received the anonymized cheese bread sample, served in uniform portions at room temperature, and was asked to evaluate the following sensory attributes: appearance, aroma, texture, colour, taste, and overall liking, using a 9-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely). Before participation, all individuals provided written informed consent. The study was conducted in accordance with the ethical standards for sensory and consumer research, ensuring the voluntary and anonymous nature of participation, with no personal identifying data collected.

The second assessment involved Check All That Apply (CATA) questions, where consumer panels selected terms that best described their tasting experiences. The drivers of liking for the CATA questions were derived from previous studies and a professional focus group. The drivers of liking comprised 30 terms, including those refereed to appearance (Light colour, Dark colour, Attractive, Brown dots on the crust, Rounded loaf top, Uniform holes), texture (Soft, Chewy / elastic, Hard, Moist, Easy to chew, Hard to chew, Dry, Crunchy), flavour and taste (Intense cheese flavour, Mild cheese flavour, Tasty, Salty, Bitter, Sweet, Greasy / oily, Olive flavour, Spicy) and aroma (Intense cheese aroma, Mild cheese aroma, Pleasant aroma, Unpleasant aroma, Toasted cheese aroma, Olive aroma, Freshly baked bread aroma). These attributes were selected during a preliminary meeting of the expert panellists and following previous studies on gluten-free baked goods (Di Cairano et al., 2021; Iwamura et al., 2022; Kazerski et al., 2022).

2.10 Statistical analysis

The data were statistically processed by XLSTAT software (Addinsoft SARL, New York, NY, USA). The results of the analysis were subjected to one-way analysis of variance (ANOVA) followed by post-hoc Tukey's HSD (honestly significant difference) test, considering each formulation as independent variable. The differences were considered significant at 95% probability level ($p < 0.05$). Cochran's Q test ($p < 0.05$) was used to compare the frequencies of attribute selection from the CATA questions, and post hoc analysis was conducted using multiple pairwise comparisons with the McNemar test (Bonferroni) ($p < 0.05$). The mean consumer liking scores were an external preference mapping analysis, where they were regressed onto the first two dimensions of the PCA derived from the trained assessors' sensory data (Greenhoff & MacFie, 1994).

3. Results and discussion

3.1 Rheological properties of cheese bread doughs

3.1.1 Frequency sweep analysis

The frequency sweep analysis was carried out to evaluate the viscoelastic properties of the doughs, as a function of linear frequency. Figure 1 depicts the results of the frequency sweep test of cheese breads doughs, whereas the values of storage modulus (G ′), loss modulus (G″) and tan δ at 1 Hz, are reported in Table 2. As can be observed in Figure 1, both moduli exhibited an increase with rising frequency, a phenomenon previously documented in doughs of low-fat baked goods (Jung et al., 2020; Kaur et al., 2022). Particularly, G′ was more dependent on the frequency, than G″. This suggests the existence of a three-dimensional network stabilised by non-covalent bonds (Difonzo et al., 2024). Moreover, no evidence of crossover between G′ and G″ was observed, suggesting that the doughs remained stable over time (Totaro et al., 2025). Consistent with previous findings (Jung et al., 2020; Tsatsaragkou et al., 2021), all doughs exhibited a predominance of G′ over G″, indicating a solid-like behaviour (Kaur et al., 2022). This is also supported by the tan δ, which is defined as the ratio of G″ to G′, which reflects the liquid-like or solid-like behaviour of samples. The lower the tan δ, the more solid-like behaviour of the matrix. As shown in Table 2, tan δ values of all samples were less than 1, indicating a dominant elastic response of the doughs (Jung et al., 2020). Interestingly, all doughs were characterised by tan δ values within the recommended range of 0.15-0.44, which has been suggested as suitable for foods designed for people with swallowing difficulties (Sharma et al., 2022). In line with these results, Suebsaen et al. (2019) found that elderly with dysphagia preferred foods with higher tan δ for ease of swallowing. Greater tan δ values ($p < 0.05$), reported for experimental samples with partial cheese substitution (Table 2), indeed, are usually related with lower grainy and higher smooth measures (Sharma et al., 2022). Similarly, Kim et al. (2017) reported that the tan δ of the cake batters became higher as more amounts of shortening were replaced with oleogels in cakes. Among the formulations, the control sample (C-CB) displayed the highest values of both dynamic moduli, suggesting a more elastic-like behaviour, as also evidenced by its lower tan δ value. It was previously reported that the reduction of solid fat diminished dynamic moduli (Jung et al., 2020). Specifically, the replacement of cheese by fat replacers reduced G′ and G″ values of experimental samples, especially for AVO-CB sample. The latter also showed the highest tan δ, indicating that its inner structure was weaker (Hesso et al., 2015), compared to the other formulations. This phenomenon could be related to the higher moisture of this sample (Table 3). Peressini & Sensidoni (2009) reported that both dynamic moduli are dependent on moisture content, increasing as water decreases, corroborating our results. Therefore, the lower water content in EFG-CB (Table 3) could justify its higher G′ and G″ values, compared to AVO-CB. On the other hand, the significantly higher dynamic moduli in the formulation with full cheese replacement could be associated with its higher fibre content (Table 3), as already reported by Kaur et al. (2022). In view of an industrial production of the experimental cheese breads, further studies may focus on validation of these findings in an industrial environment.

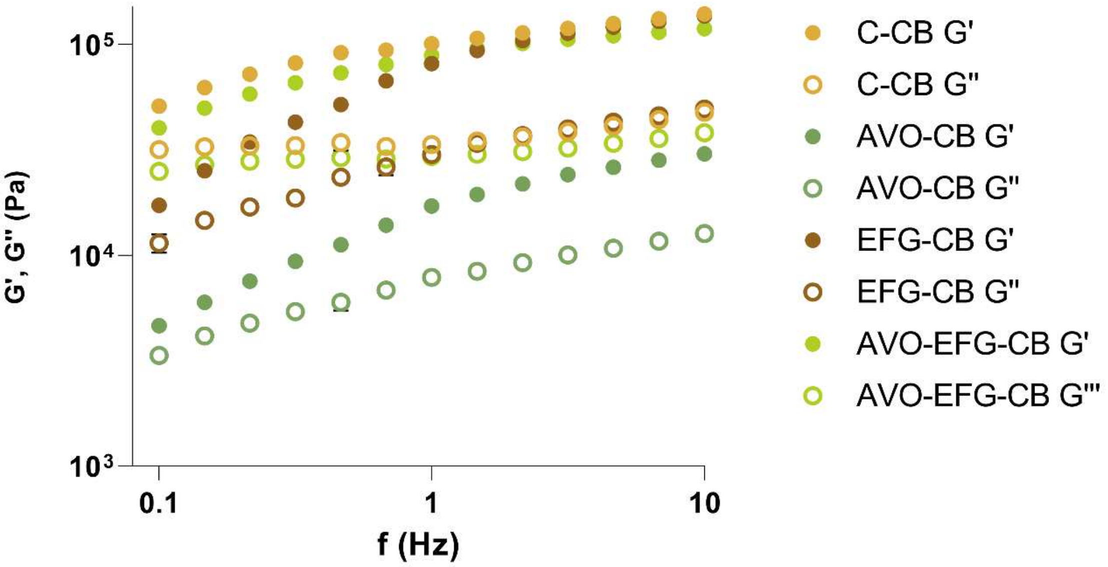


Figure 1. Mean storage modulus (G′, in Pa) and loss modulus (G″, in Pa) as a function of the regular frequency (f, in Hz). The error bars indicate the standard deviation.

Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin.

3.1.2 Flow curves of cheese bread doughs

Cheese bread dough viscosity was measured using a shear rate ramp test. The flow curves in Figure 2 illustrate the apparent viscosity as a function of shear rate. Moreover, Table 2 reports viscosity values (η) at shear rate ($\dot{\gamma}$) values of 10 s^{-1}, and 100 s^{-1}. The latter shear rate values were considered because mixing and pumping processes, as well as chewing and swallowing occur in a wide range of shear rate, ranging from 10 s^{-1} to 100 s^{-1} (Barnes, 2000). In all cases, doughs showed a shear thinning behaviour, since viscosity decreased, as the shear rate increased (Saeidy et al., 2023). This trend is typical of non-Newtonian pseudoplastic fluids. The re-arrangement of the molecules in the direction of flow, as well as the weakening of the intermolecular force promoted by the shear, could explain the reduction in viscosity (Difonzo et al., 2024). These findings are consistent with those reported by other authors (Lim et al., 2017; Saeidy et al., 2023). Notably, this shear-thinning behaviour is pivotal for sensory perception, as it contributes to create a favourable mouthfeel when foods are eaten (Wang et al., 2019a). The shear-thinning properties of the doughs could also be exploited in food processing applications, obtaining significant advantages, in terms of enhanced processability and energy efficiency (Li et al., 2019). Specifically, as the doughs become less viscous under shear stress, less mechanical force is required during mixing and pumping operations (Du et al., 2025), thereby decreasing overall energy consumption. Interestingly, experimental doughs exhibited lower viscosity than C-CB (Figure 2). In line with our results, Lim et al. (2017) reported that the muffin batters containing higher levels of oleogels exhibited lower viscosity than the control. Particularly, the doughs formulated with partial and full cheese replacement exhibited significantly lower viscosity values at a $\dot{\gamma}$ of 10 s^{-1}, compared to C-CB, with no statistically significant differences observed among experimental formulations (Table 2). Earlier investigations have reported that replacing saturated fats with unsaturated ones results in lower batter viscosity, likely due to alterations in the incorporation of air bubbles (Saghafi et al., 2019). On the other hand, considering viscosity at a $\dot{\gamma}$ of 100 s^{-1}, all doughs showed lower values compared to C-CB ($p < 0.05$); however, dough with partial substitution of cheese by EFG showed the lowest viscosity value (Table 2). This outcome may be attributed the higher content of EFG and soybean oil in this product, compared to the other formulations (Table 1), given documented evidence that oil diminishes the viscosity of baked goods (Qasem et al., 2017). Therefore, the latter dough could be advantageous for future industrial production, as it may help to prevent overprocessing and contribute to a reduction in energy consumption, while ensuring structural integrity and stability in the final baked product (Du et al., 2025). However, to fully understand how these doughs behave under real processing conditions, future studies should incorporate large strain amplitude tests, which are more representative of industrial-scale operations (Totaro et al., 2025).

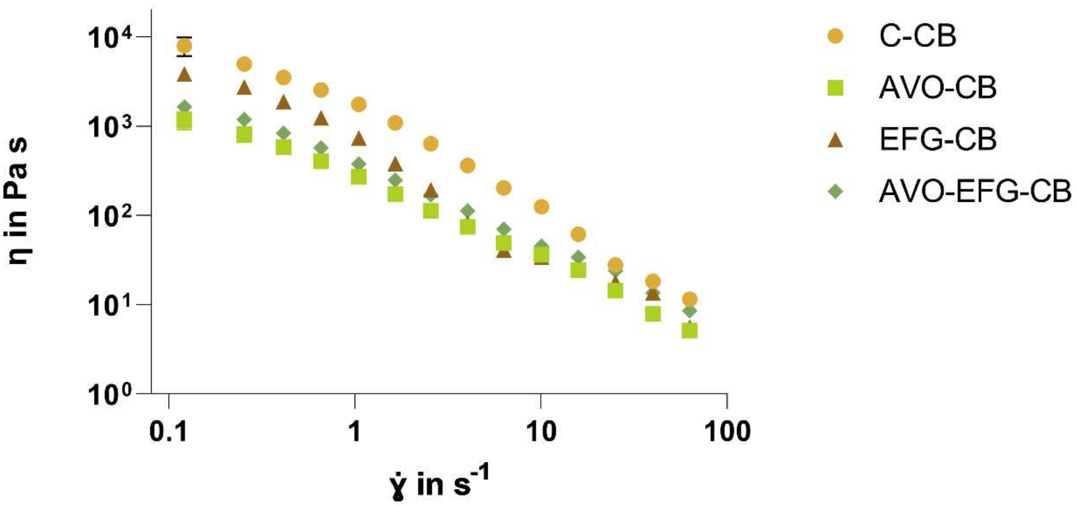


Figure 2. Mean of apparent viscosity (Pa s) as a function of the shear rate ($\dot{\gamma}$ s^{-1}). The error bars indicate the standard deviation. Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin.

3.1.3 Temperature sweep test

The temperature-dependent behaviour of the doughs is depicted in Figure 3. Temperature sweep analysis was conducted to simulate the baking process and then to investigate the main rheological phenomenon and structure modification in the doughs, providing information on viscoelasticity during baking (Difonzo et al., 2024). G′ was always higher than G″, throughout the temperature range studied, for both control and experimental doughs, indicating the predominance of an elastic-like behaviour. Recent work has demonstrated the elastic properties of avocado starch during heating and its potential use in bread (De Dios-Avila et al., 2022), which directly supports our results. During the early stages of heating, AVO-EFG-CB sample showed no change, whereas EFG-CB sample exhibited only a slight decrease in storage and loss moduli, indicating a firmer and more resilient network structure (Kaur et al., 2022). In agreement with the observations of Totaro et al. (2025) for inulin-based gels, both storage and loss moduli of these samples increased as the temperature rose from 40 to 50 °C; a response that can be ascribed to the inulin present in the EFG, promoting greater network entanglement. On the contrary, for C-CB and AVO-CB samples a decreasing trend of G′ and G″ values during heating was observed, which is consistent with fat melting, as already reported by other authors (Difonzo et al., 2024). Lucey et al. (2003) reported that when cheese is heated there is a decrease in the total number or strengths of bonds, which leads to melting phenomena, corroborating our results. These samples, indeed, were characterized by the highest fat content (Table 3) - likely resulting from the presence of cheese in the formulation - which could also explain the slightly delayed increase of the moduli, as fat inhibits starch gelatinization (Difonzo et al., 2024). On the whole, the increase of G′ and G″ values for all doughs could be related to starch gelatinization and protein unfolding and denaturation (Kaur et al., 2022), which are responsible for the increase of viscosity and elasticity of the matrices (Yovchev & Le-Bail, 2021). A closer examination reveals a significant effect of avocado pulp on G′ modulus recorded at the end of the analysis for AVO-CB and AVO-EFG-CB (Table 2), related particularly to protein type, which were responsible for strengthening the elastic behaviour of the doughs. Specifically, it has been reported that avocado proteins are rich in sulphur-containing amino acids, aspartic acid and glutamic amino acids (Wang et al., 2019b), which can increase G′ values, resulting in a more elastic system at the end of the analysis (Zhao et al., 2019). Conversely, the G′ value of C-CB was more than three times lower than that of the plant-based formulation. This difference may be attributed to the presence of fat, which can inhibit interactions between flour particles and water, resulting in a less elastic dough (Krystyjan et al., 2015).

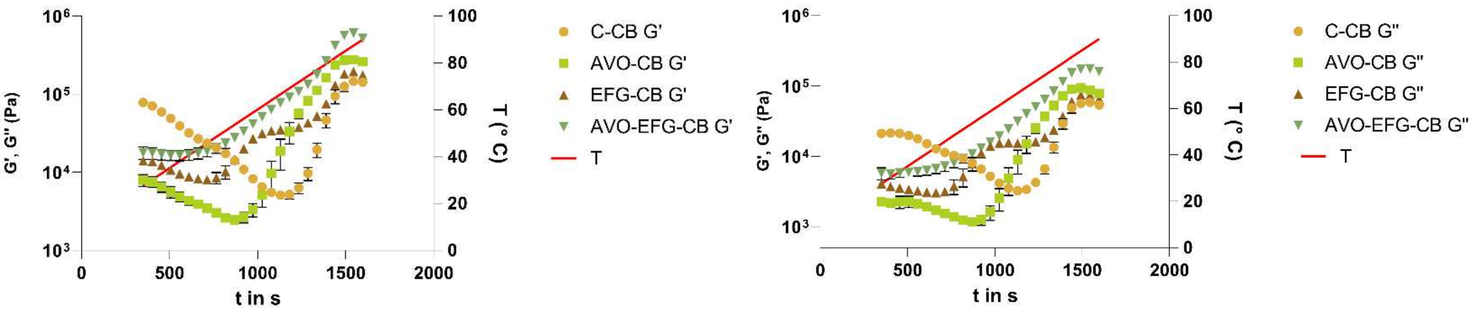


Figure 3. Mean of storage modulus (G′) and loss modulus (G″) development as a function of the temperature (°C). The error bars indicate the standard deviation. Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin.

Table 2. Rheological properties of control and experimental cheese breads.

	C-CB	**AVO-CB**	**EFG-CB**	**AVO-EFG-CB**
Frequency sweep at 1 Hz				
G′ (Pa)	$1.00540 \times 10^5 \pm 4.960 \times 10^3$ [a]	$1.7090 \times 10^4 \pm 3.50 \times 10^2$ [c]	$8.1060 \times 10^4 \pm 4.930 \times 10^3$ [b]	$8.8635 \times 10^4 \pm 4.375 \times 10^3$ [b]
G″ (Pa)	$3.3455 \times 10^4 \pm 2.565 \times 10^3$ [a]	$7.8555 \times 10^3 \pm 22.5$ [b]	$2.9980 \times 10^4 \pm 1.760 \times 10^3$ [a]	$2.9355 \times 10^4 \pm 1.855 \times 10^3$ [a]
tan δ	0.33 ± 0.01[c]	0.46 ± 0.01[a]	0.37 ± 0.01[b]	0.33 ± 0.01[c]
Flow curves				
η (Pa s at $\dot{\gamma}$ 10 s^{-1})	125.15 ± 10.05[a]	36.39 ± 2.15[b]	34.23 ± 2.57[b]	45.37 ± 0.71[b]
η (Pa s at $\dot{\gamma}$ 100 s^{-1})	15.01 ± 0.14[a]	3.07 ± 0.53[b]	1.51 ± 0.12[c]	3.44 ± 0.69[b]
Temperature sweep at 95 °C				
G′ (Pa)	$1.42700 \times 10^5 \pm 1.4900 \times 10^4$ [d]	$2.61000 \times 10^5 \pm 8.800 \times 10^3$ [b]	$1.75550 \times 10^5 \pm 1.2250 \times 10^4$ [c]	$5.19550 \times 10^5 \pm 1.2450 \times 10^4$ [a]
G″ (Pa)	$5.3740 \times 10^4 \pm 5.470 \times 10^3$ [c]	$7.8385 \times 10^4 \pm 1.515 \times 10^3$ [b]	$6.5310 \times 10^4 \pm 2.420 \times 10^3$ [bc]	$1.60850 \times 10^5 \pm 1.4650 \times 10^4$ [a]

Values shown are mean±standard deviation. [a,d] Different superscript letters in a row indicate significant differences, according to Tukey's HSD test ($p < 0.05$).

Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin.

*$p < 0.05$.

3.2 Nutritional profile of cheese breads

As shown in Table 3, the findings from the experimental cheese breads indicate nutritional and physicochemical alterations corresponding to the substitution of cheese and eggs with avocado pulp and EFG. The formulation incorporating avocado pulp (AVO-CB) demonstrated significantly higher moisture content and water activity (aw), in comparison to the control. These results corroborate findings from other authors, who demonstrated that using avocado pulp as fat replacers in cakes and cookies enhanced water content and aw (Marina et al., 2016; Nurul et al., 2016; Wekwete & Navder, 2008). Conversely, the incorporation of EFG alone as a fat substitute led to a reduced aw and moisture content ($p < 0.05$), consistent with findings on oil/water structured systems (emulgels or oleogels), that replaced traditional fats in baked goods (Gutiérrez-Luna et al., 2022; Malvano et al., 2022).
The significant decrease of fat content in experimental cheese breads is attributed to the reduction of traditional lipid sources (cheese and eggs). Particularly, samples with partial substitution of cheese showed on average a fat content of 8.50%, whereas AVO-EFG-CB sample exhibited a fat reduction of 50%. Studies aimed at the incorporation of avocado pulp as a substitute for conventional lipid ingredients in baked products demonstrated nutritional enhancements, particularly in terms of the total lipid content, with reductions ranging from approximately 20 to 80%, compared to their respective control formulations (Othman et al., 2018; Santos et al., 2024), corroborating our results. Similarly, incorporating EFG led to a marked reduction in fat content in brioches, decreasing it from 15% to 0.90% (Difonzo et al., 2024).
The increase in fibre content ($p < 0.05$) observed in the AVO-CB, EFG-CB, and AVO-EFG-CB formulations resulted from the incorporation of plant-based ingredients, notably avocado pulp, which contributes a significant amount of dietary fibre (Ford et al., 2023), and inulin from the EFG, a soluble fibre with well-documented prebiotic effects in the scientific literature (Alonso-Allende et al., 2024). According to European Regulation (EC) No. 1924/2006, foods containing at least 3 g of fibre per 100 g may be labelled as a “source of fibre”. In this context, the AVO-EFG-CB formulation, which reached a fibre content of 3.59%, satisfied this criterion and could therefore be considered a “source of dietary fibre”, reinforcing its potential as a nutritionally enhanced plant-based bakery product (Regulation (EC) No. 1924/2006).

The decrease in protein and ash content reflects the partial or complete removal of cheese and eggs, which are important sources of these nutrients in traditional cheese bread. Particularly, formulations with partial substitution of cheese showed a protein reduction of 50% compared to C-CB. However, as expected, the lowest protein content was detected in the plant-based formulation (AVO-EFG-CB). A similar trend has been reported for ash content. Comparable results have been observed in studies where dairy products and eggs were partially or entirely substituted with plant-derived components, which is an effective strategy from sustainability and functional standpoint, but results in reduced protein and ash levels, due to the lower nutrient density of some plant-based matrices (Majhenič et al., 2025; Nwosu et al., 2022). Overall, the reduction in fat, ash, and protein in the experimental treatments resulted in an increased carbohydrate content ($p < 0.05$). Although it is important to recognize these limitations and consider strategies for compensating in future formulations (i.e. the use of nutritional yeast, notably rich in protein and fibre), the nutritional benefits of the reformulated breads should not be overlooked. These products, indeed, exhibit an enhanced lipid profile (Table 4) and provide dietary fibre, attributes that comply to current nutritional recommendations. When used in a balanced diet, these reformulated cheese breads may contribute to healthy eating and overall dietary quality. Moreover, they are naturally gluten-free, rendering them appropriate for individuals with gluten-related disorders or those pursuing gluten-free options.

Table 3. Nutritional composition of control and experimental cheese breads.

Parameters	C-CB	AVO-CB	EFG-CB	AVO-EFG-CB
Aw	0.92 ± 0.01^{c}	0.94 ± 0.01^{a}	0.89 ± 0.01^{d}	0.93 ± 0.01^{b}
Moisture (%)	26.17 ± 0.61^{b}	29.83 ± 0.62^{a}	20.59 ± 0.19^{c}	27.14 ± 0.31^{b}
Protein (%)	8.61 ± 0.38^{a}	4.13 ± 0.02^{b}	3.68 ± 0.01^{b}	0.42 ± 0.01^{c}
Fat (%)	11.08 ± 0.24^{a}	8.97 ± 0.13^{b}	8.03 ± 0.15^{c}	5.23 ± 0.11^{d}
Carbohydrates (%)	50.81 ± 0.48^{d}	52.85 ± 0.60^{c}	63.53 ± 0.50^{a}	61.62 ± 0.52^{b}
Fibre (%)	0.27 ± 0.01^{d}	1.99 ± 0.09^{b}	1.20 ± 0.23^{c}	3.59 ± 0.41^{a}
Ash (%)	3.07 ± 0.02^{a}	2.22 ± 0.01^{c}	2.97 ± 0.01^{b}	2.02 ± 0.02^{d}

Values shown are mean±standard deviation. ^{a,d} Different superscript letters in a row indicate significant differences, according to Tukey's HSD test ($p < 0.05$). Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin.

*$p < 0.05$.

3.3 Fatty acid composition and lipid quality indices

The changes of cheese bread added by avocado pulp and a structured olive oil EFG markedly improved the fatty acid profile (Table 4). The progressive substitution of cheese and eggs with plant-derived lipid sources led to a significant decrease in total saturated fatty acids (SFA). Significantly, saturated fatty acids characteristics of dairy fat (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0) were markedly reduced or completely absent in the experimental formulations. C16:0, the main fatty acid of C-CB, diminished in experimental samples ($p < 0.05$); notably, its concentration in AVO-EFG-CB was approximately 50% lower than that of the control. Simultaneously, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were significantly elevated in formulations containing avocado and EFG. Indeed, in contrast to C-CB, mainly characterised by SFA, EFG-CB and AVO-EFG-CB were predominantly composed of MUFA. As regards PUFA, the plant-based formulation showed the highest concentration, followed by AVO-CB, EFG-CB and C-CB. Particularly, AVO-CB sample showed the highest content of C16:1, being avocado pulp rich in monosaturated fatty acids. C18:1 was the main fatty acid detected in EFG-CB sample (44.79 g/100 g fat), likely due to the presence of olive oil in the EFG; however, AVO-EFG-CB showed the highest concentration of this fatty acid, being two-fold higher, compared to C-CB. Interestingly, essential fatty acids (C18:2 and C18:3) showed the highest concentration in AVO-EFG-CB, followed by AVO-CB, EFG-CB and C-CB. This is noteworthy as essential fatty acids are vital in inflammation regulation, cell membrane integrity, brain function and cardiovascular health (Mititelu et al., 2024). These results corroborate those of other authors, who observed that the incorporation of fat replacers with a healthy fatty acid profile, including avocado pulp and olive oil emulsed gel, has consistently shown to enhance the lipid profile in baked goods, by reducing the proportion of SFA and increasing the levels of PUFA and MUFA (Paciulli et al., 2020; Othman et al., 2018).

The relationship between saturated and unsaturated fatty acid intake and cardiovascular health has been widely documented by the scientific literature. The review carried out by Venier & Capocasa, (2025) explored the association between the intake of various fatty acids and the risk of cardiovascular diseases. These authors stated that elevated consumption of SFA is correlated with raised serum levels of total cholesterol and low-density lipoprotein cholesterol (LDL-C), which are traditionally associated with an increased risk of atherosclerosis and cardiovascular occurrences. Conversely, the intake of unsaturated fatty acids, particularly MUFA like oleic acid, and PUFA such as omega-3 and omega-6, confers cardioprotective benefits by diminishing LDL cholesterol and triglyceride levels, while enhancing high-density lipoprotein (HDL-C).

In this context, the consumption of reformulated cheese bread could be a strategy to obtain beneficial effects on cardiovascular health, while aligning to global dietary guidelines, including those from the World Health Organization (WHO) and the EAT–Lancet Commission (2019), which strongly recommend the widespread adoption of plant-based diets, to enhance health and prevent chronic diseases (Willett et al., 2019; World Heath Organization, 2003).

Moreover, this reformulation strategy usually provides enhanced lipid quality indices, among them reduced atherogenic and thrombogenic indices (AI and TI, respectively), which reflect a healthier lipid profile (Burbano et al., 2025; Flores et al., 2019; Martínez et al., 2024; Othman et al., 2018).

The AI and TI indices, along with the PUFA/SFA and hypocholesterolemic/hypercholesterolemic ratios (H/H), are key nutritional indicators for lipid quality and cardiovascular health. Longitudinal epidemiological data from Menotti et al. (2024) indicated that consumption of foods with elevated AI and TI values, generally linked to low PUFA/SFA and H/H ratios, were highly associated with negative cardiovascular outcomes (Abbasi et al., 2025), leading to elevated cardiovascular mortality across multiple worldwide cohorts. Actually, AI represents the inclination of fat to settle in the walls of the arteries, while TI shows the tendency to form clots in the blood vessels. Moreover, H/H ratio is related to the functional activity of fatty acids in the metabolism of lipoproteins for plasma cholesterol transport and to the risk of cardiovascular disease (Abbas et al., 2021; Paszczyk & Tońska, 2022). The current study demonstrated that enhancements in fatty acid profile contributed to significant improvements in lipid quality indices (Table 4). Specifically, in reformulated cheese breads, AI and TI decreased, indicating high quantities of anti-atherogenic and anti-thrombogenic fatty acids, which are recommended for a healthy diet; whereas PUFA/SFA and H/H ratios rose, reducing cardiovascular risks, and lowering blood cholesterol levels, which prevents some contemporary diseases such as cancer and cardiovascular diseases (Abbas et al., 2021; Paszczyk & Tońska, 2022). In line with findings on fatty acid profile, the highest improvement of these indices was observed in the sample with full cheese replacement, as a consequence of the synergic effect of avocado pulp and olive oil. Botella-Martínez et al. (2023) showed that structured vegetable oils, such as olive oil, can improve lipid quality indices by replacing animal saturated fats. Similarly, avocado-based ingredients have been reported to reduce AI and TI values, enhancing overall lipid quality and cardioprotective potential (Ali, 2024; Zuñiga-Martínez et al., 2024).

Overall, these findings suggest that integrating avocado pulp and olive oil-based emulsions could enhance the nutritional value of traditional high-fat products. This is in line with the growing interest in plant-based functional ingredients and corresponds with worldwide public health goals aimed at reducing saturated fat and increasing fibre intake through innovative food design.

Table 4. Fatty acid profile (g/100 g fat) and lipid quality index of control and experimental cheese breads.

	C-CB	AVO-CB	EFG-CB	AVO-EFG-CB
C4:0	1.17 ± 0.04^{a}	0.69 ± 0.03^{b}	0.56 ± 0.01^{c}	nd
C6:0	1.10 ± 0.05^{a}	0.63 ± 0.03^{b}	0.54 ± 0.02^{c}	nd
C8:0	0.85 ± 0.06^{a}	0.47 ± 0.01^{b}	0.40 ± 0.01^{b}	nd
C10:0	2.27 ± 0.04^{a}	1.25 ± 0.03^{b}	1.02 ± 0.01^{c}	nd
C12:0	2.86 ± 0.10^{a}	1.57 ± 0.01^{b}	1.28 ± 0.01^{c}	nd
C14:0	8.86 ± 0.13^{a}	4.76 ± 0.03^{b}	3.97 ± 0.02^{c}	0.06 ± 0.01^{d}
C14:1	0.81 ± 0.01^{a}	0.53 ± 0.01^{b}	0.36 ± 0.01^{c}	nd
C15:0	0.97 ± 0.01^{a}	0.51 ± 0.01^{b}	0.40 ± 0.01^{c}	nd
C16:0	28.43 ± 0.78^{a}	23.45 ± 0.11^{b}	18.88 ± 0.01^{c}	13.18 ± 0.72^{d}
C16:1	1.25 ± 0.01^{c}	3.82 ± 0.15^{a}	0.94 ± 0.04^{d}	2.22 ± 0.11^{b}
C17:0	0.45 ± 0.03^{a}	0.35 ± 0.05^{b}	0.26 ± 0.01^{c}	0.07 ± 0.01^{d}
C17:1	0.33 ± 0.01^{a}	0.20 ± 0.01^{b}	0.20 ± 0.01^{b}	0.12 ± 0.01^{c}
C18:0	8.90 ± 0.24^{a}	6.00 ± 0.01^{b}	6.00 ± 0.02^{b}	3.24 ± 0.17^{c}
C18:1	25.94 ± 0.27^{d}	30.44 ± 0.1^{c}	44.79 ± 0.13^{b}	53.33 ± 2.69^{a}
C18:2	13.63 ± 0.20^{d}	22.23 ± 0.05^{b}	17.52 ± 0.11^{c}	24.37 ± 1.41^{a}
C20:0	0.20 ± 0.01^{b}	0.21 ± 0.01^{b}	0.42 ± 0.11^{a}	0.46 ± 0.01^{a}
C18:3	1.81 ± 0.05^{c}	2.68 ± 0.02^{a}	2.30 ± 0.01^{b}	2.90 ± 0.17^{a}
C20:1	0.37 ± 0.02^{a}	0.21 ± 0.02^{b}	0.16 ± 0.01^{c}	nd
∑SFA	56.06 ± 0.20^{a}	39.89 ± 0.09^{b}	33.73 ± 0.05^{c}	17.05 ± 1.00^{d}
∑MUFA	28.7 ± 0.28^{d}	35.2 ± 0.02^{c}	46.44 ± 0.06^{b}	55.68 ± 2.58^{a}
∑PUFA	15.44 ± 0.25^{d}	24.91 ± 0.07^{b}	19.82 ± 0.11^{c}	27.27 ± 1.58^{a}
IA	1.51 ± 0.02^{a}	0.73 ± 0.01^{b}	0.54 ± 0.01^{c}	0.16 ± 0.01^{d}
IT	1.73 ± 0.03^{a}	0.93 ± 0.01^{b}	0.74 ± 0.01^{c}	0.34 ± 0.02^{d}
PUFA/SFA	0.28 ± 0.01^{d}	0.62 ± 0.01^{b}	0.59 ± 0.01^{c}	1.60 ± 0.01^{a}
H/H	1.03 ± 0.02^{d}	1.86 ± 0.01^{c}	2.68 ± 0.01^{b}	6.10 ± 0.42^{a}

Values shown are mean±standard deviation. ^{a,d} Different superscript letters in a row indicate significant differences, according to Tukey's HSD test ($p < 0.05$). Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin; ∑SFA, total saturated fatty acids; ∑MUFA, monounsaturated fatty acids; ∑PUFA, polyunsaturated fatty acids; AI, Atherogenic index; TI, Thrombogenic index; H/H, Hypocholesterolemic and Hypercholesterolemic ratio. *$p < 0.05$.

3.4 In Vitro Starch Hydrolysis and Predicted Glycemic Index

The hydrolysis index (HI) and the predicted glycaemic index (pGI) are reliable indicators of the rate and extent of starch digestion in starchy foods, closely related to the release of rapidly available glucose during enzymatic hydrolysis (Suo et al., 2024). The data showed that reformulating cheese bread with avocado pulp and EGF significantly altered the starch digestibility profile, as demonstrated by the HI and the pGI values (Table 5). AVO-CB exhibited the highest HI and pGI values, followed by EFG-CB and AVO-EFG-CB (Table 5). This indicates that the type of lipid substitute used and how it interacts with the matrix influences glucose release during enzymatic digestion.

In AVO-CB sample, the higher moisture content (29.83 ± 0.62%) facilitates an amorphous, gelatinised structure, which increases susceptibility to starch hydrolysis, thereby raising the HI and pGI values (Adefegha et al. 2021). Conversely, the higher fibre content of AVO-EFG-CB created physical restriction to enzymatic diffusion, thereby slowing digestion and reducing the pGI (Ferrer-Mairal et al., 2012; Flores-Silva et al., 2015; Skřivan et al., 2024). Data from the literature reported that increase dietary fibre in snacks/baking products tend to lower pGI, highlighting the role of fibres in modulating starch digestion and glycaemic response (Caponio et al., 2022; Caponio et al., 2024; Caponio et al., 2025). In the EFG-CB formulation, the gel-like structure and lipid profile promoted amylose-lipid complexation and the formation of a dense matrix, which reduced the accessibility of α-amylase (Chen & Wang, 2024).

On the whole, despite showing an improved lipid profile (higher levels of unsaturated fatty acids and lower levels of total fat) and increased fibre content, the experimental formulations had slightly higher pGI values, compared to the dairy control (C-CB). This is due to the absence of the dairy matrix, whose protein-starch interactions and greater structural density limit gelatinisation and starch digestion (Graça et al. 2020; Huppertz et al. 2024). The literature confirms that the presence of milk or dairy products can slow gastric emptying and digestion compared to plant-based alternatives (Argyri et al., 2016; Chi et al., 2022).

Overall, these findings emphasise the challenge of balancing nutritional quality and glycaemic response in reformulated baked goods. Plant-based formulations, especially those containing avocado pulp and EFG alternatives, exhibited a superior lipid profile and higher fibre content. However, they partially lost the ability of the dairy matrix to modulate starch *in vitro* digestion. This result should not be interpreted as a limitation, but rather as a reflection of the structural characteristics of the dairy matrix, as modulators of digestive kinetics. This supports the idea that the digestibility and glycaemic response of starch-based products is determined by the matrix effect, as well as the chemical composition. Despite the slightly higher pGI, experimental samples offer broader metabolic and functional benefits, balancing lipid quality and glycaemic impact, making them nutritionally superior alternatives to the conventional product.

Table 5. Predicted glycemic index and hydrolysis index of control and experimental cheese breads.

Parameters	C-CB	AVO-CB	EFG-CB	AVO-EFG-CB
HI (%)	25.63 ± 0.95^{c}	41.10 ± 3.13^{a}	35.08 ± 3.10^{ab}	32.52 ± 2.14^{b}
pGI (%)	53.78 ± 0.52^{b}	62.28 ± 1.72^{a}	58.97 ± 1.70^{ab}	57.56 ± 1.17^{b}

Values shown are mean±standard deviation. ^{a,c} Different superscript letters in a row indicate significant differences, according to Tukey's HSD test ($p < 0.05$). Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin. *$p < 0.05$.

3.5 Texture profile analysis of cheese breads

The textural properties (hardness, springiness, cohesiveness and chewiness) of control and experimental cheese breads are shown in Table 6. These properties are important quality attributes, that consumer use to evaluate food acceptability and purchasing decision (Koç & Atar Kayabaşi, 2023; Rahman et al., 2021). Specifically, hardness was measured as the maximum force required to compress the crumb during the first compression cycle (Arifin et al., 2019; Sethi et al., 2022). Hardness values ranged from 8.46 to 25.13 N. The partial substitution of cheese in experimental samples caused significant variations in hardness. Among the samples, AVO-CB, indeed, exhibited the lowest hardness value, indicating the softest texture. This finding could be attributed to the higher moisture content observed in this sample, as reported in Table 3. Similar results have been obtained by El-Sayed, (2025), Pokatong & Nathalie, (2021) and Nurul et al. (2016), who applied avocado pulp as fat replacer in pound cakes and muffins, respectively. On the contrary, EFG-CB, characterized by the lowest moisture content, displayed the highest hardness value. Other researchers have shown that the use of different inulin preparations as fat replacers increased the hardness of shortbread cookies, biscuits, sponge cakes, cakes, or muffins, (Hager et al., 2011; Paciulli et al., 2020; Rodríguez-García et al., 2013; Rodríguez-García et al., 2012; Zahn et al., 2010), corroborating our results. Springiness refers to the ability of the bread crumb to recover its original shape after compression and is related to the elasticity of the bread crumb (Koç & Atar Kayabaşi, 2023). AVO-CB and EFG-CB exhibited higher values ($p < 0.05$) compared to the control and to the sample with full cheese replacement. Similarly, Zahn et al. (2010) observed that the replacement of 50% fat significantly increased springiness of the crumb, indicating that the strength of the bonds in the three-dimensional crumb network was increased. It is worth noting that consumers prefer bread with higher springiness value, as this attribute is commonly associated with product freshness and elasticity (Koç & Atar Kayabaşi, 2023; Matos & Rosell, 2012). Conversely, baked goods characterised by low springiness values are typically associated with crumb brittleness (Tóth et al., 2022). Cohesiveness is defined as the extent to which a material can be deformed before it ruptures (Matos & Rosell, 2012). Breads with higher cohesiveness are desirable as this property prevents product disintegration during mastication; whereas low cohesiveness indicates increased susceptibility of bread to crumble (Tóth et al., 2022), which represents a quality issue and may negatively affect consumer acceptance (Koç & Atar Kayabaşi, 2023; Tóth et al., 2022). Interestingly, significantly higher cohesiveness values were observed for AVO-CB and EFG-CB, as a result of a more compact crumb structure (Espert et al., 2023; Rodríguez-García et al., 2012). On the whole, springiness and cohesiveness results are in line with those obtained by other authors (El-Sayed, 2025; Nururl et al., 2016; Psimouli & Oreopoulou, 2013; Rodríguez-García et al., 2012; Totosaus et al., 2024). Chewiness is another key parameter of bread texture, that depends on springiness, hardness and cohesiveness (Koç & Atar Kayabaşi, 2023). Chewiness values ranged from 2.80 to 5.52 N. Among samples, C-CB showed the highest chewiness value, followed by EFG-CB, AVO-CB and AVO-EFG-CB. Lower chewiness values (p < 0.05), observed in the experimental samples, are preferred, as they indicate that the bread breaks easily in the mouth (Koç & Atar Kayabaşi, 2023; Matos & Rosell, 2012). These results align with those obtained by Martínez-Cervera et al. (2011). On the whole, the experimental formulations demonstrated improved textural properties, combining lower hardness and chewiness, with adequate springiness and cohesiveness, thereby enhancing overall quality.

Table 6. Texture profile analysis of control and experimental cheese breads.

	C-CB	AVO-CB	EFG-CB	AVO-EFG-CB
Hardness (N)	13.20 ± 0.90^{b}	8.46 ± 0.38^{c}	25.13 ± 1.96^{a}	14.43 ± 0.35^{b}
Springiness (−)	0.76 ± 0.05^{b}	0.95 ± 0.04^{a}	0.97 ± 0.04^{a}	0.80 ± 0.06^{b}
Chewiness (N)	5.52 ± 0.18^{a}	3.09 ± 0.05^{c}	3.87 ± 0.34^{b}	2.80 ± 0.07^{c}
Cohesiveness (−)	0.22 ± 0.01^{b}	0.37 ± 0.03^{a}	0.32 ± 0.01^{a}	0.24 ± 0.01^{b}

Values shown are mean±standard deviation. ^{a,c} Different superscript letters in a row indicate significant differences, according to Tukey's HSD test (p < 0.05). Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin. *p < 0.05.

3.6 Consumer test

The sensory analysis resulted from the CATA findings (Table 7) indicated that the addition of plant-based ingredients significantly modified the sensory attributes of the cheese bread formulations. The control sample (C-CB) maintained the characteristics often linked to the traditional cheese bread, including softness, chewiness, and a strong cheese flavour and aroma. Conversely, formulations of avocado pulp and emulsion-filled gel exhibited distinct sensory characteristics. The AVO-CB sample was often characterized by a darker colour, exhibiting evident brown spots on the crust. These results align with colour instrumental determinations (data not shown), which reported lower L^* values for formulations with avocado pulp, as polyphenol oxidase catalyses the oxidation of phenols to quinones, that subsequently polymerize into brown pigments (Donporn Wongwaiwech et al., 2024; Nurul et al., 2016). Moreover, AVO-CB formulation was seen as moister, highlighting the high-water retention ability of avocado pulp. Conversely, the EFG-CB exhibited increased hardness and crispiness, supporting instrumental analysis (Table 6). The replacement of cheese and eggs with plant-based ingredients resulted in a diminished intensity of salty and cheese-like flavours, while vegetal or olive-like aroma became more pronounced, particularly in AVO-EFG-CB.

The utilization of the Check-All-That-Apply (CATA) method in sensory studies has provided significant insights into the impact of substituting animal or saturated fats with plant-derived lipid components on consumer perception of bakery products. Lee & Ju (2024) and Tarancón et al. (2015) employed CATA to assess cookies made with various plant-derived oils and discovered that replacing traditional shortening markedly modified sensory descriptors associated to texture, appearance and aroma, while maintaining consumer preference. On the whole, CATA results obtained in this research suggested that including avocado and structured lipid systems altered the sensory characteristics of the product, resulting in formulations with particular plant-based attributes. These modifications result in an innovative sensory profile that meets clean-label and functional reformulation trends, even though they underline the importance for further optimization for balancing textural quality and flavour intensity, while maintaining consumer familiarity and acceptance.

Table 8. Relative frequency of sensory attribute mentions in the CATA questionnaire.

Sensory attribute	C-CB	AVO-CB	EFG-CB	AVO-EFG-CB	*p* value
			Appearance		
Light colour	59^{bc}	22^{a}	66^{b}	46^{c}	< 0.001*
Dark colour	0^{b}	13^{a}	0^{b}	1^{b}	< 0.001*
Attractive	26	23	15	15	0.042*†
Brown dots in crust	6^{ab}	16^{a}	4^{b}	2^{b}	< 0.001*
Rounded loaf top	35	29	33	26	0.320
Uniform alveoli	16	10	14	8	0.202
			Texture		
Soft	46^{a}	41^{ab}	11^{c}	26^{bc}	< 0.001*
Rubbery/springy	35	42	31	43	0.120
Hard	2^{a}	2^{a}	22^{b}	10^{ab}	< 0.001*
Moist	17^{ab}	21^{a}	7^{b}	18^{ab}	0.026
Dry	6	6	15	10	0.040*†
Crunchy	10^{a}	4^{a}	27^{b}	14^{ab}	< 0.001*
Easy to chew	42^{a}	32^{ab}	23^{b}	18^{b}	< 0.001*
Hard to chew	8	4	8	7	0.600
			Flavour/Taste		
Very cheese flavour	36^{b}	5^{a}	19^{b}	0^{a}	< 0.001*
Slightly cheese flavour	18^{ab}	30^{a}	23^{a}	7^{b}	< 0.001*
Tasty	36^{b}	19^{a}	21^{a}	12^{a}	< 0.001*
Salty	34^{b}	17^{a}	38^{b}	21^{ab}	< 0.001*
Bitter	0	4	3	5	0.154
Sweet	3	4	3	3	0.962
Greasy/oily	7	12	12	20	0.027*†
Olive flavour	6^{b}	27^{a}	8^{b}	15^{ab}	< 0.001*
Spicy	0	0	0	1	0.392
			Aroma		
Very cheese aroma	40^{c}	10^{ab}	21^{a}	2^{b}	< 0.001*
Slightly cheese aroma	16	19	12	10	0.246
Pleasant aroma	34	26	33	27	0.401
Toasted cheese aroma	17^{a}	6^{ab}	10^{ab}	2^{b}	0.001
Off aroma	2	7	3	8	0.146
Fresh baked aroma	5	8	9	8	0.701
Olive aroma	2^{b}	26^{a}	5^{bc}	18^{ac}	< 0.001*

* Significant difference according to Cochran's Q test ($p < 0.05$). Different letters in the same row indicate a significant difference in the McNemar post-hoc test with Bonferroni adjustment ($p < 0.05$). † No statistically significant difference was found in the post-hoc test. Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO-EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin.

Figure 4 shows the preference map that revealed distinct segmentation of customer acceptance among the assessed cheese bread recipes. This multivariate analytical approach correlates consumer preference with sensory attributes scaled according to their intensity. Clusters 1 and 2, situated adjacent to the control sample (C-CB), demonstrated a pronounced inclination towards the traditional formulation, presumably indicative of consumer familiarity with its sensory attributes. In contrast, Cluster 3, situated nearer to the AVO-CB and AVO-EFG-CB samples, exhibited a clear inclination towards the reformulated breads incorporating avocado pulp and emulsion-filled gel. This finding indicates the presence of a consumer subgroup receptive to new and nutritionally enhanced products, perhaps motivated by the perceived health advantages linked to an improved lipid profile and increased fibre content. The EFG-CB formulation, located around the centre of the map, demonstrated notable acceptability, suggesting its potential for linking sensory quality with nutritional improvement.

Recent studies utilizing preference mapping approaches have shown that substituting animal or saturated fats with plant-based lipid systems in baked goods can be accomplished without affecting consumer acceptance, highlighting that the sensory harmony of texture, flavour, and appearance is carefully preserved (Espert et al., 2023; Malvano et al., 2022; Merlino et al., 2022; Tarancòn et al., 2025). These studies collectively indicated that consumer preference is not exclusively influenced by familiarity with animal fats; rather, it can positively move towards healthier plant-based fat alternatives, when reformulation procedures successfully achieve desirable sensory characteristics. Although the traditional formulation remains the most widely accepted, the preference mapping results suggest that selected plant-based and functional formulations may appeal to a subset of consumers, indicating opportunities for further consumer-oriented research. These results provide valuable guidance for product development and scale-up strategies, indicating that reformulated cheese breads such as EFG-CB may achieve industrial feasibility by combining nutritional enhancement with sensory profiles that remain acceptable across consumers.

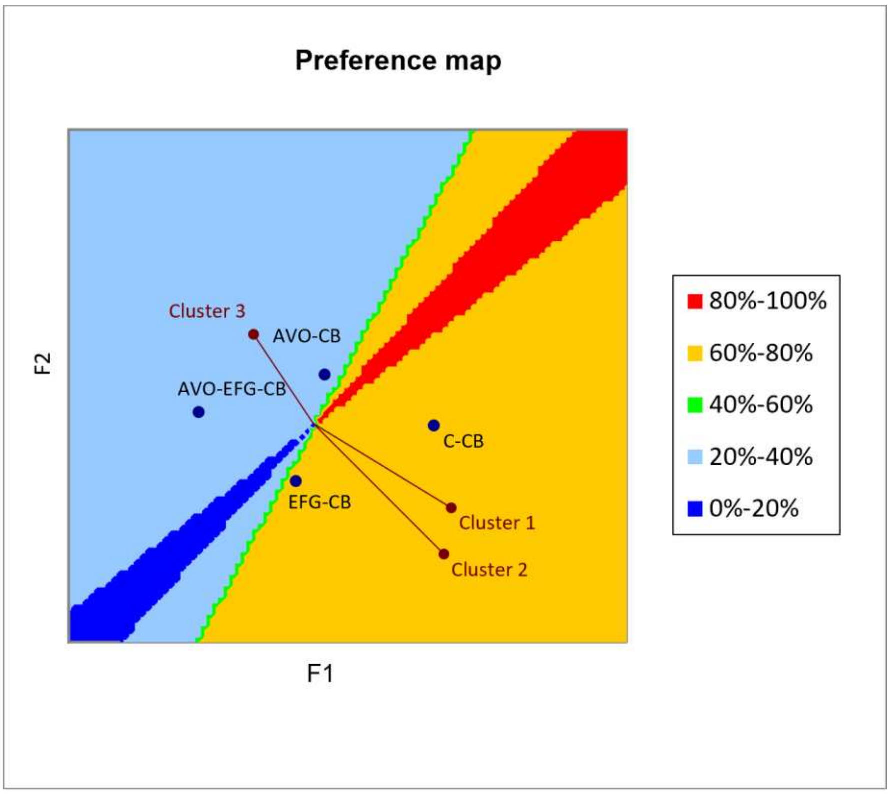


Figure 4. Preference map of consumer acceptance testing of cheese breads. Abbreviations: C-CB, control cheese bread; AVO-CB, cheese bread with partial substitution of cheese by avocado pulp; EFG-CB, cheese bread with partial substitution of cheese by olive oil emulsion-filled gel structured with inulin; AVO- EFG-CB, cheese bread with total substitution of cheese and eggs by avocado pulp and olive oil emulsion-filled gel structured with inulin.

4. Conclusions

This study assed the effect of avocado pulp and emulsion-filled gel (EFG), used as partial or total substitutes for cheese, on the physiochemical, rheological and nutritional properties of cheese bread (*pão de queijo*), a traditional Brazilian bread, typically rich in saturated fats and low in dietary fibre. A consumer test was also performed, to evaluate the sensory perception of the products. All doughs exhibited a predominant elastic behaviour and shear-thinning properties, indicating suitable rheological characteristics for processing and potential advantages in terms of energy efficiency. The incorporation of plant-based ingredients markedly enhanced the nutritional quality of the breads, particularly by increasing dietary fibre content and improving lipid composition. Formulations with partial substitution of cheese showed reduced total and saturated fat contents, alongside a significant increase in mono - and polyunsaturated fatty acids, resulting in more favourable lipid quality indices associated with cardiovascular health. The plant-based formulation, in particular, exhibited the highest fibre content, the lowest predicted glycaemic index among the tested samples, being also characterised by the highest content of polyunsaturated fatty acids. Results from texture profile analysis demonstrated that samples with plant-based ingredients had higher springiness and lower chewiness values, preferred by consumers. CATA results obtained in this research suggested that including avocado and structured lipid systems altered the sensory characteristics of the product, resulting in formulations with particular plant-based attributes. Although the traditional formulation remains the most accepted, preference mapping suggests that certain plant-based and functional formulations may appeal to specific consumers. On the whole, the incorporation of avocado pulp and emulsion-filled gels enables the development of clean-label cheese bread formulations with enhanced nutritional quality and desirable textural and rheological properties. Although the plant-based formulations showed promising technological and sensory results, future investigations should focus on their storage stability. Understanding how these natural ingredients influence moisture retention, textural evolution, and microbiological safety over time will be essential to fully assess the commercial viability of clean-label, functional *pão de queijo*. Such studies could further support the transition of these formulations from experimental prototypes to market-ready products. In addition, future research could explore strategies to increase the content of bioactive compounds in these formulations and evaluate their stability and concentration after digestion, providing deeper insight into their potential health benefits.

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Conclusion and future perspectives of Chapter 2

Cheese bread (*pão de queijo)* is a traditional Brazilian bread, which is currently available on foreign markets, being a gluten-free product. It is produced using cassava starch, salt, oil, cheese and eggs, thus resulting in a product with a high concentration of saturated fatty acids and low content of dietary fibre, which are dietary risk factors related to non-communicable diseases (NCDs). Nonetheless, this product is commonly consumed at multiple times during the day, including breakfast and snack times, being a product ready for consumption. These findings highlight the need to improve this traditional product, finding solutions that can be easily adapted on a large-scale production. Therefore, avocado pulp and emulsion-filled gel (EFG) have been used as cheese substitute in the *pão de queijo* production, evaluating the effects of these alternative ingredients on rheological, physicochemical and nutritional properties of the products, produced on a small scale. Results clearly showed that experiment formulations were characterised by a higher fibre content and lower concentration of saturated fats. Consequently, plant-based ingredients improved fatty acid profile and lipid quality indices, highlighting their enhanced effects on cardiovascular health. Moreover, although traditional *pão de queijo* is widely accepted by consumers, exists a promising segment for plant-based and functional cheese breads. The presented results concern the small-scale production of innovative cheese bread. Large-scale production (TRL 5-6) was not investigated due to time constraints associated with pilot-scale experimentation. Pilot- and industrial-scale trials would require access to dedicated large-scale processing facilities, and long-term experimental campaigns aimed at assessing process stability, reproducibility, and product consistency. However, these activities are identified as necessary steps for future development. Further studies, indeed, should aim at advancing the experimental cheese breads from laboratory-scale validation towards higher TRL. In particular, the transition to TRL 5-6 would require the production of these innovative cheese breads using automatic industrial equipment (Figure 1), which handles dough preparation, shaping and filling, when is required. This step is essential to assess process scalability, reproducibility, and compatibility with existing industrial workflows.

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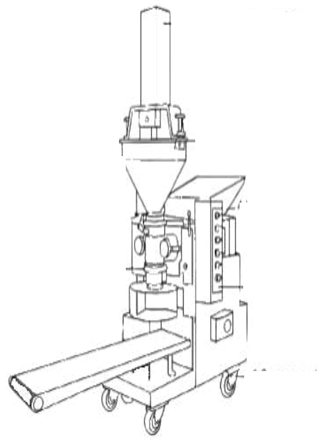

Figure 1. An example of an automatic machine used for the production of *pão de queijo.*

Given the use of emulsion-filled gels as fat replacers, large-scale production would additionally require the use of industrial turboemulsifiers, which operate through counter-rotating blades and homogenizing turbines to ensure the formation of stable and homogeneous emulsions. Such equipment is particularly well-suited for the food sector and specifically for manufacture of gels, creams, and sauces. The performance of these systems should be evaluated in terms of emulsion stability and integration within continuous processing lines. On the whole, these validation under relevant industrial conditions would represent necessary steps to demonstrate the feasibility of manufacturing plant-based cheese breads at pilot or pre-industrial scale, thereby facilitating their potential transfer to industrial production.

In Brazil, pre-prepared cheese bread can be mainly found on the market in two ways: i) the ready mix, which requires the addition of liquid ingredients for the dough preparation; ii) the frozen ready to bake cheese bread, which only needs to be baked. Therefore, assessing the effects of freezing on these new ingredients is essential to determine their technological performance in frozen product formulations, given the widespread commercial relevance of these products.

Furthermore, once baked, *pão de queijo* is typical consumed immediately while still hot, as its hardness tend to increase during storage. Thus, further investigations should optimize *pão de queijo* formulations, in order to increase their quality and prevent excessive hardening during storage.

From a nutritional perspective, the product could be further improved by incorporating nutritional yeast to enhance its protein content. In addition, future studies may focus on increasing the concentration of bioactive compounds, thereby strengthening the functional value of the final formulation. Accordingly, subsequent research should focus on evaluating the digestibility and bioaccessibility of the added bioactive compounds, with the aim of assessing their stability throughout gastrointestinal digestion and their actual bioactive potential.

Overall, these formulation and process optimizations should initially be assessed at small scale and subsequently validated at pilot scale (TRL 5-6) in order to support their transition toward large-scale production and assess their feasibility and compatibility with existing production lines.

In conclusion, the development of an improved, plant-enriched *pão de queijo* marks a significant step toward a healthier and more sustainable version of this traditional product. Its successful adaptation to industrial production could soon bring an innovative cheese bread to the market, meeting modern consumers' growing demand for nutritious and convenient foods, while contributing to public-health strategies designed to reduce the incidence of NCDs.

Chapter 3- Innovation in craft beer production: a strategic approach to address food safety concerns

Introduction

Chapter III presents the third case study, which exemplifies how basic and applied research can support future scale-up strategies, aimed at improving the safety and quality of fermented beverages. This research provides valuable insights and practical recommendations for brewers to reduce toxic compound formation, representing a critical preliminary step toward technological transfer and industrial application.

Outbreaks of foodborne diseases caused by pathogenic microorganisms, microbial toxins, and other contaminants from various food sources have raised public awareness of food safety (Younes et al., 2024); the latter is the process of preventing microbiological, chemical, and physically hazardous substances from the food supply chain at any point, during the production of food. Globally, the risk of foodborne diseases is high, especially among young, old, pregnant, immuno-compromised groups and people with underlying medical conditions (AL-Mamun et al., 2018). Food safety, indeed, represents a global public health challenge, contributing to 600 million cases of foodborne diseases and 420,000 deaths every year (Niyigaba et al., 2025). However, foodborne illnesses are often underestimated due to the challenges associated with their diagnosis, since they have various symptoms, including fatigue, fever, vertigo, diarrhoea and cramps (Makun, 2016). A substantial proportion of foodborne illness cases are linked to foods prepared outside the household, although home-cooked meals can also serve as vectors of contamination (Makun, 2016). The World Health Organisation (WHO) reported that foodborne illnesses are usually infectious or toxic in nature and caused by bacteria, viruses, parasites or chemical substances entering the body through contaminated food (WHO, 2015).

The most significant health concerns are related to foodborne intoxication (WHO, 2015), which can be stated as the disease caused by ingestion of food containing naturally occurring toxins and/or environmental pollutants, representing chemical hazards (AL-Mamun et al., 2018).

Interestingly, fermented foods and beverages have increasingly been associated with fatalities resulting from foodborne illnesses (underestimated), highlighting the need for safety measures in their production and consumption (Niyigaba et al., 2025). Fermented foods and beverages, traditionally consumed globally, have seen rapid growth in the global market, driven by increasing consumer demand for functional and minimally processed products (Niyigaba et al., 2025). A recent study indicated that the market would reach a valuation of USD 890.8 billion by 2031, with a compound annual growth rate (CAGR) of 6.20% from 2024 to 2031 (Cognitive Market Research, 2025). In spite of the many benefits associated with fermentation (inhibiting the growth of pathogenic microorganisms, improving the organoleptic properties and product digestibility, and being a valuable source of functional compounds and probiotics) (Skowron et al., 2022), it can also pose potential health risks, because of pathogenic microorganisms or toxins, contributing to cases of illness or even outbreaks. Indeed, in fermented foods and beverages, inadequate fermentation practices, starter cultures, insufficient hygiene measures, inadequate storage conditions, contaminated raw materials and improper processing and handling have been identified as the primary causes of foodborne illness (Niyigaba et al., 2025). These factors are very difficult to control, especially when the fermentation is carried out in small-scale industries or under households' conditions, where process standardization is often limited (Motarjemi, 2002). As a consequence, the accumulation of biogenic amines, the production of mycotoxins, and the proliferation of harmful microorganisms can occur, potentially causing adverse health effects such as allergic reactions, intoxication, foodborne illness, gastrointestinal disorders, and immunological issues (Niyigaba et al., 2025). Examples of foodborne disease outbreaks associated with fermented foods have been reported by Motarjemi, (2002), where *Clostridium perfringes*, *Clostridium botulinum*, strains of *Escherichia coli* and of *Salomenella* spp, have been identified as the major causative agents in dairy, meat and fish products. Interestingly, as regards foodborne intoxications, histamine (one of the most toxic biogenic amines) has been recognized as the main chemical substance related to outbreaks associated with fermented foods (Motarjemi, 2002).

Biogenic amines (BAs) are of great importance to public health, as contribute to numerous outbreaks of foodborne illness worldwide, which are often understated (Omer et al., 2021). From 2010 to 2017, the Europe Food Safety Authority (EFSA) reported 599 cases of foodborne illness related to histamine fish poisoning in Europe (Omer et al., 2021). Thus, there is an urgent need for more research on the content and effects of BAs, especially in fermented foods and beverages, to establish more precise safety thresholds and guidelines.

BAs are low-molecular weight organic bases produced by both microorganisms naturally present in the raw material and by starter cultures, especially in fermented foods (Del Rio et al., 2024). BAs are the product of amino acid decarboxylation, amination of aldehydes or ketones, or their transamination; therefore their formation is influenced not only by the type of microorganism that performs these processes, but also by the composition of the raw material (e.g., free amino acid content), the fermentation time, and the conditions under which food processing takes place (Saha Turna et al., 2024). However, BAs ingested with food could induce digestive, circulatory and respiratory disorders (Del Rio et al., 2024). These adverse effects may be enhanced by alcohol, since it increases BAs toxicity, reducing the effect of detoxification enzymes (monoamine oxidase and diamine oxidase) (Omer et al., 2021). Therefore, investigation about these toxic molecules in fermented alcoholic beverages is crucial for accurately assessing potential health risks associated with these products (Niyigaba et al., 2025).

Among alcoholic beverages, beer is one of the most consumed globally (Hong et al., 2025). Water, malt, hops, and yeast are the four main ingredients for beer manufacturing. Generally speaking, there are four basic stages in the brewing process: the preparation of the wort, fermentation, maturation, and filtration and/or stabilization (Ciont et al., 2022). However, craft beer is usually unpasteurized and unfiltered and brewed with unconventional ingredients (Baiano, 2021); thus, they are more prone to spoilage and to the accumulation of toxic compounds (Lorencová et al., 2020). Due to the growing demand for craft beer (Baiano, 2021), it is necessary to investigate potential contaminants that could affect consumers' wellbeing. Recently, Ciont et al. (2022) focused on the safety of beer products, highlighting microbiological, physical and chemical contamination sources. Among the latter, BAs are the main chemical molecules associated with foodborne illnesses in beer. In line with this statement, Hong et al. (2025) reported that BAs could be abundant in craft beer, thus their health risks should be taken seriously. Hypertensive crises have been observed in people with low BA detoxification capacity, after consumption of beer with a tyramine content higher than 10 mg/L. Moreover, it has been reported that beer products are capable of triggering allergic-like adverse reaction, because of histamine content (Kalač et al., 2002). In fact, limitations for BAs should be stricter in beer than in other foods without alcohol; however, the global industry has yet to establish unified limits on BAs content in these alcoholic beverages (Hong et al., 2025). Given the limited number of studies investigating the presence of BAs in craft beers (Choi et al., 2012; Li et al., 2023; Lorencová et al., 2020), further in-depth research is required to: i) establish accurate data on BA profiles across several brewing methods; ii) assess potential health risks associated with BAs consumption; iii) support the development of safety guidelines for beer production and consumption. Critical points of the brewing process should be detected, to subsequently validate and demonstrate on a pilot scale (TRL 5-6) innovative strategies to produce craft beers, which do not pose adverse health reactions in consumers.

As an efficient scale-up (transfer and adaption of technologies) relies on adequate basic scientific research and knowledge (Rolle & Satin, 2002), this research activity began with the investigation of BAs in beers produced in Italian and Romanian microbreweries, to collect data and identify critical steps. Proposing corrective actions to be applied during the brewing process, the objective was to produce craft beers that do not pose any health risks to all types of consumers. Unfortunately, the scale-up phase corresponding to TRL 5-6 was not implemented within the scope of this work. This limitation reflects structural constraints associated with pilot-scale development, including the requirement for dedicated manufacturing infrastructure and formal industrial partnerships. However, the article provides suggestions and practical applications for brewers to produce safe craft beers, laying the groundwork for subsequent technology transfer within the brewing industry, aligning with the 2nd Sustainable Development Goal of the 2030 Agenda, whose purpose is to guarantee safe, nutritious and sufficient food for all people. In the concluding chapter, the technical and process-development steps required to achieve TRL 5-6 are outlined, and directions for future research are presented, with the specific aim of reducing amine content in craft beers.

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Publication no. 4:

Hidden Hazards in Fermented Beverages: a Study on Biogenic Amines in Craft Beers from Italy and Romania

The following paper has been submitted to *Journal of Agriculture and Food Research* as:

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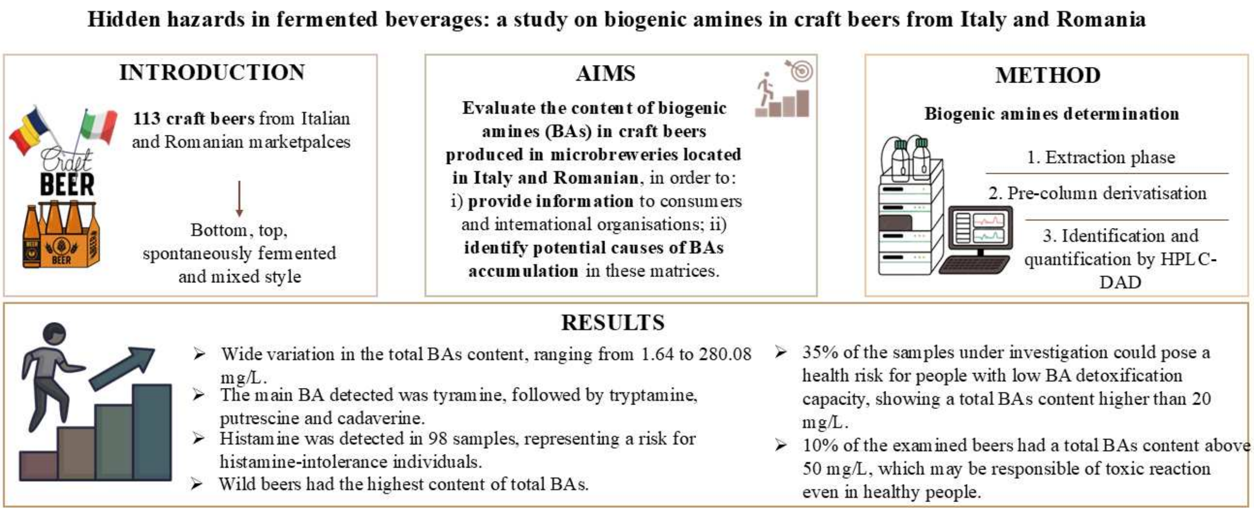
Graphical Abstract



Abstract

Biogenic amines (BAs) are nitrogenous compounds frequently found in fermented foods and beverages, potentially posing health risks for consumers. Craft beers, usually unfiltered and unpasteurized, offer conditions favourable to BAs formation. In this study, the concentrations of seven BAs were monitored in 113 craft beers purchased in the Italian and Romanian markets, categorized by fermentation type: bottom, top, spontaneously fermented beers and mixed style. Results showed that there was a wide variation in the total BAs content, ranging from 1.64 to 280.08 mg/L. The main BA detected was tyramine, followed by tryptamine, putrescine and cadaverine. Bottom fermented beers did not show harmful concentrations of tyramine, whereas top and spontaneously fermented beers, as well as mixed style beers, showed the most relevant tyramine content (sometimes even tryptamine) with a concentration above 10 mg/L, due to possible microbial contamination and the use of unconventional ingredients. Most of the samples (98) had a histamine concentration above the detectable level, which may cause adverse reactions in people with histamine intolerance. On the whole, 10% of the beers under investigation could cause negative physiological effects for healthy consumers, showing a total BAs content above 50 mg/L. However, 35% of the examined craft beers had a total BAs above 20 mg/L, resulting in a potential health hazard for consumers with low BA-detoxification capacity. An efficient food safety management system combined with the accurate selection of raw materials and starter cultures (selection of BA-degrading microorganisms), could guarantee that the products pose no safety risk for consumers.

Keywords: craft beers, biogenic amines, beer styles, food safety, microbreweries, unconventional ingredients.

1. Introduction

Beer is a fermented alcoholic beverage widely consumed around the world (Redruello et al., 2017). In recent years, the demand for craft beer has shown an upward trend, due to its uniqueness, originality and authentic taste (Gil et al., 2022; Poveda, 2019). Indeed, the Craft Beer Market is expected to reach 10.03 billion litres by 2029, growing at a CAGR (Compounded Average Growth Rate) of 9%, by the same year (Europe Craft Beer Market Size Report, 2024). The definition of craft beer is not shared across the world, but there are legal definitions established by national laws (i.e., USA, Italy, Canada) and several working definitions supplied by trade organizations (the US and German Brewers Association and the UK Society of Independent) (Baiano, 2021), which shear some features: i) the production conditions in microbreweries are usually significantly different from industrial plants (Lorencová et al., 2020), as craft breweries often produce beers with the addition of non-traditional ingredients (Gil et al., 2022); ii) microbreweries do not apply standardized processing conditions, crossing beers' styles (Baiano, 2021). Particularly, the Italian law defined craft beer as "the beer produced by small independent breweries and not subjected to pasteurization and microfiltration processes" (Law 154, 2016). Therefore, in craft beers, starter cultures and contaminating microorganisms may cause the appearance of organoleptic defects and undesirable compounds, such as biogenic amines (BAs) (Gil et al., 2022; Poveda, 2019). BAs are low-molecular weight nitrogen compounds, deriving mainly from decarboxylation of amino acids, but also transamination and or amination of aldehydes and ketones (Lorencová et al., 2020). Normally, small amounts of BAs could be detoxified by gut enzymes, such as monoamine oxidases (MAO), diamino oxidases (DAO) and histidine-methyltransferase (Lorencová et al., 2020; Loret et al., 2005). However, with a high BAs intake, the detoxification system may be insufficiently effective (Lorencová et al., 2020), causing toxic reactions with specific symptoms, such as cardiovascular diseases, headache, respiratory discomfort, neurological problems, urticarial, pseudo-allergic responses, vertigo and abdominal cramps (Gil et al., 2022; Pradenas et al., 2016; Vasconcelos et al., 2021). Other reasons for the BAs oxidation inefficiency are related to genetics, gastrointestinal diseases or due to the inhibitory effects of alcohol or some drugs with MAO and DAO inhibitors (MAOI and DAOI, respectively) (Dala-Paula et al., 2023; Omer et al., 2021b). Indeed, concentrations of BAs up to 100 mg/kg or 100 mg/L are considered to be safe for consumers (Lorencová et al., 2020); however, for the alcoholic beverages the recommended limits are much lower (Buňka et al., 2012), and for some certain BAs, may be units of milligrams per liter. Moreover, the evaluation of BAs in food products is pivotal not only for toxicological reasons, but also for their role as quality indicators (Buňka et al., 2012; Nalazek-Rudnicka et al., 2021; Spano et al., 2010; Vasconcelos et al., 2021). Ciont et al. (2022) and Tang et al. (2009) reported that histamine, tyramine, putrescine, cadaverine and phenylethylamine are the most BAs detected in beer. The most common sources of BAs in beers are the applied raw materials (especially malt), the starter cultures and contaminating microorganisms (mainly lactic acid bacteria) (Poveda, 2019). Moreover, Gil et al. (2022) and Matukas et al. (2022) focused on brewing techniques, reporting that mashing, fermentation temperatures and yeasts strains can influence the content of BAs in the final product. According to the European Food Safety Authority (EFSA), histamine and tyramine are the two most toxic and harmful BAs to human health (EFSA, Panel of Biological, 2011), as recently they have also shown cytotoxic effect *in vitro* (Linares et al., 2016); nevertheless, regulatory limits for the BAs content have not been set (Del Rio et al., 2024b; Galarce et al., 2016), except for histamine in fishery products (Commission Regulation (EC) 2073/2005). As high beer consumption in a short period of time may cause harmful effects on human health (Kalac & Krízek, 2003; Poveda, 2019; Tang et al., 2009), it is important to collect data of the content of these toxic compounds in craft beers, even though the toxic threshold is very difficult to establish, as it depends on individual response, diet composition, the presence of other BAs and other factors (Choi et al., 2012; Del Rio et al., 2024). Just a few studies have focused on the presence of BAs in craft beers, and due to the limited number of samples analysed, the overall exposure risk for consumers is still unclear. Indeed, contrary to Poveda (2019), who stated that, as long as a moderate consumption of beer is made, craft beers from the Spanish market do not entail any risk for the human health, Lorencová et al.(2020), Choi et al. (2012) and Li et al. (2023) detected a total BAs content above 50 mg/L, in most of the Korean, Chinese and Czech craft beers, representing a potential health risk for susceptible consumers.

No studies have been performed, to the best of our knowledge, in other geographical areas, such as Italy and Romania, in which the per capita consumption of beer is of 38 L/ year and 83 L/ year, respectively (The Brewers of Europe, 2025). Notably, in these countries the number of active microbreweries increased of three times in Romania and showed a growth of 44% in Italy, from 2017 to 2023 (The Brewers of Europe, 2025). Therefore, the aim of the present study was to evaluate the content of BAs in craft beers produced in microbreweries located in Italy and Romania in order to: i) provide information to consumers and international organisations, to support the establishment of legal limits for these molecules in alcoholic beverages; ii) identify potential causes of BAs accumulation in these matrices, in order to propose effective strategies to limit their formation.

2. Materials and methods

2.1 Materials, chemicals and standard solutions

Analytical standards of biogenic amines (98% or higher): tryptamine dihydrochloride-TRY, 2-phenylethylamine dihydrochloride-PHE, putrescine dihydrochloride-PUT, cadaverine hydrochloride-CAD, histamine dihydrochloride-HIS, tyramine dihydrochloride-TYM, spermidine-SPD were purchased from Sigma-Aldrich (Steinheim, Germany). The internal standard (IS) 1,7-diaminoheptane (HEP, 98%), dansyl chloride (used as a derivatization agent), were supplied by Sigma-Aldrich (Steinheim, Germany). The standard solutions of PUT, CAD, HIS, TYM, SPD, were prepared in TCA 5% solution. A solution of TCA 5%/ ACN (1:1, v/v) was used to prepare TRY and PHE standard solutions. 1,7-Diaminoheptane (IS) stock solution of 1 mg/L was also prepared in a 5% TCA solution. A volume of 25 µL and 1 mL of IS was added to both standard solutions and beer samples, respectively, with the aim of minimize the effect of random and systematic error during the analysis.

2.2 Craft beer samples

A total of 113 craft beers, 47 Romanian and 66 Italian, (packaged in glass bottles or polyethylene cans) were purchased from Italian and Romanian marketplaces (supermarkets, specialised stores and microbreweries), and were divided into groups according to their styles, as reported in Table 1. Craft beer samples were brought to the laboratory and placed in a refrigerator at 4 °C until analysis.

Table 1. Origin and styles of craft beers under investigation.

Country	Total microbreweries (no.)	Beer style			
		Bottom fermented (no.)	Top fermented (no.)	Spontaneously fermented (no.)	Mixed style (no.)
IT	41	6	39	3	18
RO	20	4	27	1	15

Abbreviations: IT, Italy; RO, Romania.

2.3 Craft beer analysis

2.3.1 Physicochemical analysis

Before analysis, the beer samples were degassed according to the EBC method 9.46 (European Brewery Convention, 2010) in an ultrasonic bath, model CP 104 Digit (CEIA S.p.A., Arezzo, Italy) and then filtered on laboratory filter papers (CordenonS, 77 Gsm, Milan, Italy). Beer alcohol content was measured by the automatic FermentoFlash analyser (Funke-Gerber, Germany), whereas pH was determined according to EBC method 9.35. (European Brewery Convention, 2010). The colour of the investigated beers was determined using a Cary 60 spectrophotometer (Cernusco, Milan, Italy), by measuring the absorbance of the beers (Abs) at a wavelength of 430 nm, according to EBC method 9.9.1. The absorbance results were converted to EBC units, using the following equation:

$$EBC = Abs430 \times 25.$$

All measurements were carried out in triplicate.

2.3.2 Analysis of the biogenic amines

The content of 7 biogenic amines (HIS, TYM, PHE, TRY, PUT, CAD, SPD) was determined on degassed craft beer samples, by liquid chromatography after derivatization with dansyl chloride. Derivatization procedure was carried out using the protocol described by Shen et al. (2017), with some modifications and optimized for a micro assay, as reported by Trani et al. (2010). Briefly, to 19 mL of degassed beer samples, were added 1 mL of IS (HEP, 1 mg/L in TCA 5%). The mixture was homogenised for 1 hour and then centrifugated at 4000 × *g*, for 10 min at 4 °C. The supernatant was collected and filtered with nylon syringe filters (0.45 μm). Then, 125 μL of the sample (or 100 μL of each BA standard solutions) were made alkaline by adding 20 μL of NaOH solution (2 mol/L) and 30 μL of saturated sodium bicarbonate ($NaHCO_3$) solution. Derivatisation was achieved with 125 μL of dansyl chloride solution in acetone (10 mg/mL), followed by incubation in a water bath for 45 min at 40 °C. Then, 10 μL of ammonia solution (33%) was added to terminate the reaction. A solution of sodium acetate (0.1 M) and acetonitrile (40:60, v/v) was added to the total volume of 500 μL, followed by centrifugation. The supernatant was filtered using 0.45 μm nylon syringe filter and injected into an UHPLC Ultimate 3000RS Dionex coupled with a DAD detector (Thermo Fisher Scientific, Waltham, MA, USA). The analyte separation was performed on a C18 column (Gemini 5U, 110 Å Phenomenex, Torrence, California, USA), heated to 38 °C, using sodium acetate (0.1 M)/acetonitrile (40:60, v/v) (solvent A) and acetonitrile HPLC grade (solvent B), as mobile phases. The flow rate was 0.50 mL/min with the following mobile phase gradient: 0-4 min 100% A, 4-18 min 17.5% A, 18-28 min 17.5% A, 28.1-43 min, 100 % A. The injection volume was 5 μL, and the detection wavelength was 254 nm. Each beer sample was analysed in triplicate, and concentrations were expressed as mg/L.

2.4 Statistical analysis

The data were statistically processed by XLSTAT software (Addinsoft, Paris, France). Results on BAs content and physicochemical analysis were subjected to descriptive analysis. Moreover, results on BAs were subjected to one-way ANOVA, followed by post-hoc Tukey's HSD procedure, considering their origin (Italy or Romania) and their brewing styles, as independent variables. The differences were considered significant at 95% probability level ($p < 0.05$).

3. Results and discussion

3.1 Biogenic amine content in craft beers: an overview

Figure 1 shows averages, minimum and maximum values of single and total BAs detected in 113 craft beer samples. There was a wide variation in the total BAs content, as it ranged between 1.64 - 280.08 mg/L, showing an average of 27.39 mg/L. These values exceeded those evidenced by Li et al. (2019) on Chinese craft beers. TYR (0-264.34 mg/L, average 10.24 mg/L), TRY (0-48.60 mg/L, average 6.49 mg/L), PUT (0.26-43.20 mg/L, average 4.05 mg/L), and CAD (0-55.70 mg/L, average 2.96 mg/L) were the main BAs detected. These results align with those of Lorencová et al. (2020), confirming the widespread presence of these molecules in craft beers. Moreover, Poveda (2019) analysed 26 beers from Spain, indicating a total BAs level ranging from 5.80 to 19.60 mg/L, with putrescine and tyramine presenting the highest content. Nevertheless, Spanish craft beers had a much lower content of tyramine (0.80-6.50 mg/L), and putrescine (3.60-8.90 mg/L), compared to ours. Conversely, Choi et al. (2012) reported higher average levels of putrescine (13.20 mg/L) and cadaverine (5 mg/L) in Korean craft beers. In our study, lower values of histamine were detected (0-28.22 mg/L, average 1.54 mg/L), followed by spermidine (0-6.73 mg/L, average 1.38 mg/L) and phenylethylamine (0-14.77 mg/L, average 0.73 mg/L). Similarly, most of the Czech craft beers had histamine and phenylethylamine content below 10 mg/L, corroborating our results (Lorencová et al., 2020). The determined mean value for spermidine was two-fold higher than those observed by Poveda (2019).

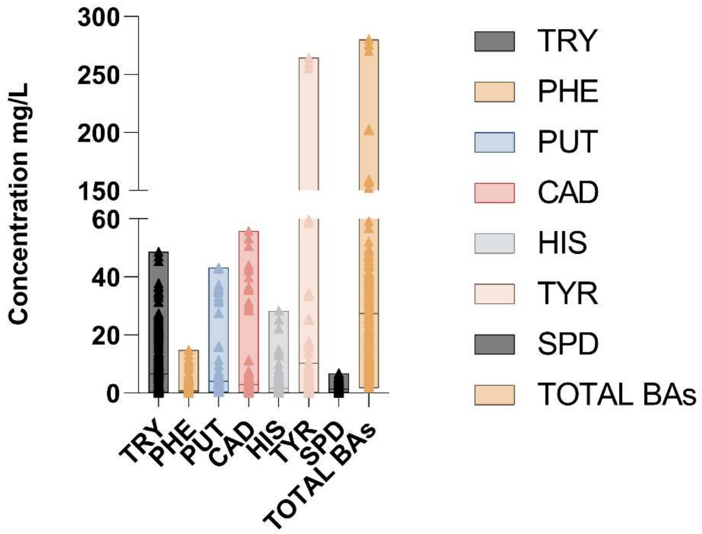


Figure 1. Biogenic amines averages, minimum and maximum detected in the investigated craft beers (mg/L).

Abbreviations: TOTAL BAs, total biogenic amines; TRY, tryptamine; PHE, phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPD, spermidine. The bands inside the bars represent the mean values.

3.2 Craft beer samples exceeding the suggested BAs limits for alcoholic beverages

According to several studies, histamine and tyramine are the most dangerous amines, as the former affects synaptic reactions, while both play a role in blood pressure control (Nalazek-Rudnicka et al., 2021; Visciano & Schirone, 2022). Tyramine and histamine, indeed, are often associated with food intoxication, responsible for 'cheese reaction' and 'scombroid food poisoning', respectively (Vasconcelos et al., 2021). However, there is no legal limit for these molecules, except for histamine in fishery products (threshold of 200 mg/kg for fresh or 400 mg/kg for cured products) (Commission Regulation (EC) 2073/2005). Despite the legal vacuum, there is broad consensus regarding the need to reduce the concentrations of BAs in food and beverages. Thus, limits have been suggested by international agencies and scientific literature. EFSA suggests for healthy individuals a histamine assumption < 50 mg/meal and for tyramine a consumption < 600 mg/meal (EFSA Panel of Biological, 2011). A much lower limit for tyramine, amounting to 50 and 6 mg/meal has been set by EFSA, for patients taking third-generation and classical MAOI drugs, respectively (Dala-Paula et al., 2023). However, it is worth mentioning that BAs health consequences could be enhanced by other molecules, as ethanol, which can inhibit the activity of MAO and DAO (Poveda, 2019); consequently, a high content of BAs in alcoholic beverages can pose a health risk, by delaying their detoxification in the human gut and allowing them to enter the circulatory system (Visciano & Schirone, 2022). Therefore, limits for BAs concentrations in alcoholic beverages have been proposed by the literature and are listed in Table 2, in which are also reported the number of craft beer samples that exceed them. Lorencová et al. (2020) suggested that a total BAs amount in the range of 20-50 mg/L in alcoholic beverages might be considered hazardous for susceptible consumers (children, elderly, pregnant women, people with healthy issues or taking MAOI or DAOI drugs). Additionally, the same authors stated that a total BAs content higher than 50 mg/L could result in a serious potential health risk, even for healthy consumers, in combination with alcohol. Interestingly, several authors (Kalac & Krízek, 2003; Tang et al., 2009) reported that a tyramine content over 10 mg/L in beers is considered as dangerous for people with low BA-detoxifying capacity, as hypertensive crises have been observed after beer consumption. Conversely, for alcoholic beverages, a toxic dose for tyramine comprised between 25 and 40 mg/L has been reported (Spano et al., 2010). As regards histamine, a content between 8-20 mg/L can cause negative physiological effects (Spano et al., 2010). However, for people with histamine intolerance, the no adverse effect level (NOEL) is below the detectable level (Dala-Paula et al., 2023). Putrescine and cadaverine, aliphatic amines, are considered important in food intoxication, as they can potentiate toxic effect of other BAs, such as histamine, tyramine and phenylethylamine (Buňka et al., 2012; Del Rio et al., 2024). Even if the literature sources rarely specify toxicological limits for cadaverine and putrescine, levels over 50 mg/L can be evaluated as hazardous in terms of food safety in beer products (Lorencová et al., 2020). According to Buňka et al. (2012), the toxic dose for phenylethylamine is above 3 mg/L, as this aromatic amine is associated with depression, hypertension, schizophrenia and Parkinson’s disease (Visciano & Schirone, 2022).

Table 2. Hazardousness of the collected craft beers according to the limits suggested in the literature.

Ref.	BAs suggested limits	Consumer type	Beer style			
			Bottom fermented (*no*=10)	**Top fermented (*no*=66)**	**Spontaneously fermented (*no*=4)**	**Mixed style (*no*=33)**
Total amines						
1	Total > 20 mg/L	Susceptible	1	28	3	8
1	Total > 50 mg/L	Healthy	/	7	3	2
Single amines						
2	TYR > 10 mg/L	Susceptible	/	7	3	3
2	TYR > 25-40 mg/L	Healthy	/	4	3	3
3	HIS > nd	HIS intolerant	9	53	4	32
4	HIS > 8-20 mg/L	Healthy	/	1	1	1
5	PHE > 3 mg/L	Healthy	4	2	/	1
5	PUT > 50 mg/L	Healthy	/	/	/	/
5	CAD > 50 mg/L	Healthy	/	1	/	/

Abbreviations: TYR, tyramine; HIS, histamine; PHE, phenylethylamine; PUT, putrescine; CAD, cadaverine. Ref.: 1 (Lorencová et al., 2020); 2 (Poveda, 2019); 3 (Dala-Paula et al., 2023); 4 (Spano et al., 2010); 5 (Buňka et al., 2012).

3.3 Biogenic amines content in different craft beer styles

Craft beer styles are the result of new brewing techniques with a combination of different traditional and unconventional ingredients (Villacreces et al., 2022). Usually, their classification depends on the types of yeast applied: top-fermented (ale), bottom-fermented (lager) or spontaneously-fermented beers (wild) (Lelièvre-Desmas et al., 2015). Moreover, The Brewers Association has been classifying beer styles, in hybrid/mixed, lager and ale beer styles (Brewers Association Beer Style Guidelines, 2024). Therefore, Italian and Romanian craft beers were divided into top, bottom, spontaneously fermented beers, and mixed styles (Table 1). To better understand the results and the influence of the brewing processes, the BAs content is discussed separately for each style, then a final comparison among the styles is provided to give a general overview of the hazardousness associated with different brewing processes.

3.3.1 Bottom fermented or lager beers

Lager beers are produced using bottom-fermenting yeast, traditionally referred as *Saccharomyces pastorianus* (syn. *carlsbergensis*), which ferments for 7-12 days at 6-15 °C (Harrison & Albanese, 2017).Within this beer group, different styles are collected, such as Pilsner, Vienna Lager beer and Bock, as reported by The Brewers Association Beer Style Guidelines (Brewers Association, 2025). These beers are usually malty with a balanced hoppiness and the colour of the final product ranges from 4 to 40 EBC (Harrison & Albanese, 2017), as shown for the craft beer samples under investigation (Table 3). As can be observed in Table 3, bottom fermented beers were characterised by a low alcohol content, 4.6-7% (v/v), and a pH of 4.69, corroborating data of Silva et al. (2022). Italian and Romanian lager beers presented a total BAs content from 2.87 to 23.96 mg/L, with an average value of 12.43 mg/L (Figure 2A), in line with values reported for Chilean and Chinese lager beers (Li et al., 2023; Pradenas et al., 2016). Interestingly, only one Italian beer (Pils Lager_2 - Table S1) could potentially entail a health risk for susceptible consumers, showing a total BAs content slightly above 20 mg/L (Table 2). Beers from Italy had a significantly higher content of total BAs compared to Romanian ones (Table 4), as well as other singles BAs, except for cadaverine. The latter was the main BA reported for lager Romanian beers (0.60-11.33 mg/L), being three-fold higher compared to Italians and to the results drown by Poveda (2019). Since this aliphatic amine is usually considered an indicator of contamination and of hygienic standards in beer production processes (Buňka et al., 2012), its higher content could reasonably be attributed to microbial pollution by microorganisms belonging to *Enterobacteriaceae* family and *Pseudomonas* genus (Ekici et al., 2019), likely resulting from inadequate hygiene conditions in the microbreweries. Actually, microorganisms belonging to the *Enterobacteriaceae* and *Pseudomonadaceae* families, capable of decarboxylating lysine into cadaverine, have been isolated from brewery surfaces, posing a hygiene and safety risk for alcoholic beverages (Maifreni et al., 2015). On the contrary, phenylethylamine was the most representative BA in Italian craft beers (0-14.77 mg/L) (Table 4). Other authors have reported mean values below 3 mg/L of this BA (Li et al., 2023; Poveda, 2019). As this molecule is mainly related to malts and hops (Almeida et al., 2012; Kalac & Krízek, 2003; Zotou & Loukou, 2009), it could be hypothesised that its higher content could be linked to the use of contaminated raw materials during the brewing processes. Four Italian lager beers (Lager_4, Pils Lager_1, Pils Lager_2, Pils Lager_3 - Table S1) exceeded the suggested toxic limit of phenylethylamine (Table 2). Concentrations of tyramine and tryptamine (Figure 2A) were consistent with the results reported by Li et al. (2023). Spermidine is expected in alcoholic beverages due to its role in nucleic acid metabolism (Lorencová et al., 2020). However, mean values of this BA detected in lager beers from Polonia (Slomkowska & Ambroziak, 2002) were almost three-fold higher compared to Italian and Romanian ones in the present study. On the whole, special attention should be paid by people with histamine intolerance, as regards consumption of these lager craft beers, as only in one sample (Lager_5 - Table S1), histamine was not detected (Table 2).

3.3.2 Top fermented or ale beers

Top fermented beers are traditionally fermented with *Saccharomyces cerevisiae* yeast, which ferments at warmer temperature, 18-22 °C, for 3-5 weeks (Goyal et al., 2023), producing significant amounts of esters and other flavour-imparting molecules (Polak et al., 2013). The result is often a beer with slightly "fruity" compounds, compared to the bottom fermented ones (Pavsler & Buiatti, 2009). As reported by Villacreces et al. (2022), several styles are classified in this category, such as Pale and Red Ale, Tripel, Porter, Stout, Italian Grape Ale (IGA), Gose and Barleywine. Physicochemical outcomes, reported in Table 3, corroborate those of Medina et al. (2023), Poveda (2019) and Silva et al. (2022). There was a wide variation in the BAs level, as the total BAs content ranged between 1.64-280.08 mg/L (Figure 2B), being these values higher than those reported in literature for craft beers from other nations (Choi et al., 2012; Li et al., 2023; Lorencová et al., 2020; Poveda, 2019). Actually, concerning the total concentration of these toxic compounds, almost half of the craft beers belonging to this style may pose risks for people with low BA - detoxification capacity and 10% of them could also represent a health hazard for healthy people (Table 2). Analysing the BA profile, tyramine, tryptamine, putrescine and cadaverine showed the highest concentration, both in craft beers from Italy and Romania (Figure 2B). However, samples crafted in Romania had a significantly higher content of total BAs, together with tyramine, histamine and phenylethylamine, compared to samples from Italy (Table 4). Particularly, as regards tyramine content, the consumption of these beers in conjunction with other fermented foods notably rich in biogenic amines (cheeses, fishes, cured meats) should be carefully considered, as almost 10% of them could be responsible of hypertensive crisis after their ingestion in susceptible people and four of them could also entail negative physiological effects in healthy consumers (Table 2). Among ale craft beers, the highest content of tyramine was observed in two Romanian barleywine beers (Barleywine_1 and Barleywine_3 - Table S1), which are typically barrel aged ones. This outcome could explain the high tyramine level, as recently, microorganism belonging to *Pediococcus* genus (associated with tyramine production) (Kalac & Krízek, 2003) have been isolated from barrel aged beers (Bossaert et al., 2022). Moreover, four stout craft samples (Stout_8, Stout_16, Stout_19 and Stout_21 - Table S1) and one craft pale ale (Pale Ale_11 –Table S1) had a tyramine content higher than 10 mg/L (Table 2). These samples shared the use of unconventional ingredients during the brewing processes, such as coffee, cocoa and chocolate, which can be sources of BAs and especially of tyramine, as previously reported in other studies (Özdestan, 2014; Restuccia et al., 2015). Pale Ale_11 (Table S1), an Italian hemp-brewed sample, exceeded safety limits for tyramine and cadaverine (Table 2), corroborating findings of Maciel et al. (2024) on BAs levels in hemp seeds. Moreover, Pale Ale_10 showed the highest putrescine levels, likely due to the use of lentils, known to be rich in this BA (Mockus et al., 2024; Sánchez-Pérez et al., 2018). On the whole, Pale Ale and Stout&Porter beers from China and Poland showed a much lower content of tyramine and putrescine, compared to our results (Li et al., 2023; Slomkowska & Ambroziak, 2002). Phenylethylamine was detected in concentration below the suggested toxic dose (Table 2), except for 2 samples (Barleywine_3 and Pale Ale_5 - Table S1). The former was a dark barleywine beer (data not shown); this could justify the highest content of this aromatic compound (Poveda, 2019). Indeed, Stout&Porter beers were the top fermented ones characterised also by the highest tryptamine content (Table S1). These findings align with Li et al. (2023) and Poveda (2019) who attributed phenylethylamine and tryptamine formation to heat-induced decarboxylation of amino acids during the high-temperature malt treatment in dark beer production. However, in comparison with previous studies, it must be pointed out that craft beers from Spain had a much lower content of tryptamine and phenylethylamine (Poveda, 2019), whereas higher values were detected in Chinese beers (Li et al., 2023). Histamine content was comparable to that observed in red ale and dark ale from Spain (Poveda, 2019) and lower compared to Korean craft beers (Choi et al., 2012). As can be observed in Table S1, only one sample (Stout_17 - Table S1) had a histamine level higher than the suggested safety threshold (Table 2) for all consumers. Moreover, it is important to highlight that 80% of the ale beers under investigation could cause toxic reactions in people with histamine intolerance.

Among top-fermented beers, the Italian Grape Ale (IGA) style, formally recognized by the Beer Judge Certification Program (BJCP) in 2015 (Becchi et al., 2025), is included in this study. IGA style is the expression of the link between beer and wine, as for this kind of production, brewers can add grape or wine must, at different stages during the brewing process (boiling, primary or second fermentation and bottling), in a range from 5% to 40% of the total wort composition (De Simone et al., 2021). This new process, used by many regional microbreweries, highlights the connection to the territory by combining the biodiversity of Italian grape varieties with the brewer's creativity (Garavaglia, 2020; Siesto et al., 2023). There is the necessity to investigate on the safety of this new beer style, as only one study focused on the content of putrescine, cadaverine, histamine and tyramine in home-made IGA (Aponte et al., 2022). Total BAs content ranged from 4.09 to 52.03 mg/L, with an average of 20.53 mg/L (Table S1). Contrary to tyramine and cadaverine, which showed comparable values to those reported by Aponte et al. (2022), putrescine presented a higher level. Actually, among IGA, sample IGA_4 (Table S1) showed the highest putrescine content. This may probably be due to the addition of mango and pineapple during the brewing process (Table S1), as putrescine is the main BA detected in those tropical fruits (Santiago-Silva et al., 2011). Interestingly, IGA_5 (Table S1), brewed in Stout style, showed the highest tryptamine content, supporting findings discussed above on this BA in dark beers. Thus, brewers should consider the possibility of mixing different styles for the IGA production and should also carefully choose the raw materials, considering that also wort can be a source of BAs (Moreira et al., 2024).

3.3.3 *Spontaneously fermented beers*

Spontaneously fermented beers are characterised by the natural fermentation of microorganisms from raw materials, equipment, or environment, which influence the microbiota associated with the final product (Mudoor Sooresh et al., 2023). Wild samples under investigation were characterised by a low alcohol content and a pale colour, as shown in Table 3, corroborating results of Hinojosa-Avila et al. (2025). Total BAs showed concentrations ranging from 15.77 to 202.73 mg/L, with a mean value of 104.91 mg/L (Figure 2C). As regards total content of these toxic compounds (Table 4), all the analysed Italian wild beers could result in a serious potential health risk, even for healthy consumers (Table 2). This supports earlier reports of high total BA levels in Polish gueuze and lambic beers (Nalazek-Rudnicka et al., 2021). Furthermore, tyramine seemed to pose the highest health hazard, as 75% of the samples exceeded the safety threshold set in beers at 25- 40 mg/L, representing a high risk for all consumers (Table 2). Actually, tyramine was the highest amine accumulated in craft samples from Italy (Table 4), followed by putrescine, cadaverine and histamine, in line with other researchers (Nalazek-Rudnicka et al., 2021; Saad & Tofalo, 2020). In view of these results, it seems that these craft beers were characterised by an intense metabolic activity of decarboxylase positive microorganisms, as a result of uncontrolled fermentation processes (Mudoor Sooresh et al., 2023; Rodríguez-Saavedra et al., 2020; Toro-Funes et al., 2015). Our outcomes corroborate those of Loret et al. (2005), who isolated decarboxylating microorganisms during spontaneous fermentation of beers; particularly, enteric bacteria prevailed the first step of fermentation, while lactic acid bacteria (LAB) occurred during its last step. The pH of beer is important in amine accumulation, as these molecules are produced by microorganisms to counteract their acidic stress (Natrella et al., 2024). In line with this statement, these beers showed lower pH value (average of 3.65), compared to other beer styles (Table 3). This finding is consistent with data of Loret et al. (2005), who reported a pH of 3.62 for spontaneous fermented beers from Belgian market. The Romanian wild sample was mainly characterised by tryptamine and spermidine (Table 4), surpassing values drawn by Nalazek-Rudnicka et al. (2021). The higher tryptamine content in this wild beer (Table S1) may be due to rye malt, which is rich in free tryptophan (20.6 mg/kg), and can be decarboxylated into tryptamine (Comai et al., 2007). On the whole, spontaneous fermented craft beers had tyramine, putrescine and cadaverine concentrations higher than those found in the literature (Nalazek-Rudnicka et al., 2021; Saad & Tofalo, 2020). However mean values of histamine above 10 mg/L, were observed in spontaneous fermented beers from Belgium, largely exceeding our results (Loret et al., 2005). Only one Italian sample (Wild_1, Table S1) exceeded the toxic histamine limit for healthy consumers (Table 2). However, all wild beers may pose risks for those with histamine intolerance. Given the small sample size and growing popularity of this style (Mudoor Sooresh et al., 2023), further studies are needed to confirm these findings.

3.3.4 Mixed beer styles

According to the beer styles listed from the Brewers Association Beer Style Guidelines (Brewers Association, 2025) and to the scientific literature (Modzelewska et al., 2023; Tan et al., 2021; Villacreces et al., 2022), within the "mixed" style we have included wheat-based, sour&fruit and smoked beers (Table S1). Values of alcohol content, colour (EBC) and pH, reported in Table 3, match those found by other researchers (Aponte et al., 2022; Poveda, 2019; Silva et al., 2022). Total BAs content ranged between 2.47-90.56 mg/L, showing an average of 17.62 mg/L (Figure 2D). Craft beers produced by Italian microbreweries showed a significantly higher total BAs content, compared to those produced by Romanian ones (Table 4). Indeed, as regards total BAs, almost 25% of samples (most Italians) could be potentially dangerous for susceptible people, otherwise 2 samples could also represent a health hazard for healthy people (Table 2). Analysing the BAs profile, tryptamine, tyramine and putrescine were the most representative ones (Figure 2D), being these last two significantly higher in samples from Italy, compared to those from Romania. Particularly, one Italian sour beer (Sour&Fruit_11, Table S1) had tyramine levels seven times above the toxic dose causing hypertension in susceptible individuals (Table 2).This result was expected, as microorganisms belonging to the *Pediococcus* genus, related to tyramine production (Kalac & Krízek, 2003; Loret et al., 2005), are the dominant LAB isolated in sour beers (Aponte et al., 2022). Moreover, researchers stated that strains of *L. brevis* are the major tyramine producers among LAB isolates from craft breweries. Thus, as some strains of *L. brevis* are used as commercial starter cultures for sour beer production, brewers should carefully select LAB for their brewing processes (Rodríguez-Saavedra et al., 2020). Li et al. (2023) reported similar findings for sour craft beers from China, as tyramine was detected at a maximum concentration of 80 mg/L. In contrast to previous research (Li et al., 2023) this study showed that investigated wheat beers were mainly characterised by tyramine and putrescine (Table S1). A similar pattern of results was obtained by Poveda (2019). Saad & Tofalo (2020) stated that in wheat beers a lactic acid fermentation can occur, leading to BAs formation. In line with these results, together with Sour&Fruit_11 sample, also two Italian wheat beers (Wheat_7 and Wheat_8 - Table S1) could be responsible of intoxication even in healthy people, because of tyramine content (Table 2). Recently, Aponte et al. (2022) reported significantly lower mean histamine levels (0.10 mg/L) in sour and fruit beers than those found in our samples. Nevertheless, concerning histamine level, among analysed mixed craft beers, only one sample (Sour&Fruit_9 - Table S1) may cause negative physiological effects in healthy consumers. In good agreement with results obtained for craft beers from Korea (Choi et al., 2012), mixed beers under investigation could cause intoxication in people with histamine intolerance (Dala-Paula et al., 2023) (Table 2). Sour craft beers from China exhibited phenylethylamine levels slightly above 5 mg/L (Li et al., 2023), consistent with findings for the Sour&Fruit_12 sample (Table S1); the latter, indeed, exceeded the safety threshold as regards phenylethylamine (Table 2). Tryptamine belonged to the most frequently occurring BAs in smoked beers (Table S1). This could be related to the Maillard reaction during malt baking (Li et al., 2023). Considering the other results obtained, it is interesting to note that the use of unconventional ingredients, mainly fruit products, caused a higher accumulation of putrescine and spermidine. The highest content of the latter BA was observed in Sour&Fruit_6 sample (Table S1), brewed with passion fruit. According to Santiago-Silva et al. (2011) passion fruits had a significantly higher content of spermidine (on average 30.5 mg/kg), compared to other tropical fruits, corroborating our results. Furthermore, the sample Sour&Fruit_11 was the one with the highest content of putrescine. It could be hypothesised that the oat and mango addition could have increased its content, in line with the outcomes of other researchers (Jia & Yu, 2022; Sánchez-Pérez et al., 2018; Santiago-Silva et al., 2011). These results highlight the importance of the accurate selection of the raw materials. Indeed, also fruit products could be sources of these toxic compounds which can be accumulated in the wort, leading to a high BA content in the final products. Moreover, the microbiota associated with the organic raw materials and unconventional ingredients, added to get a unique beer aroma and taste, could increase the risk of spoilage and of BAs formation (Rodríguez-Saavedra et al., 2020). In line with this statement, LAB (*L. brevis, L. paracasei, L. plantarum, P. acidilactici*) have been isolated on the external surfaces of filler machines of Spanish craft breweries, as these species were part of vegetable and fruit products, barley grain, wheat and malt microbiota. Rodríguez-Saavedra et al. (2020) reported that among LAB isolates, *L. brevis* exhibited the major BAs production, playing a significant role in relation to food safety of fermented beverages. Thus, brewers should consider that the risk of contamination by LAB is not only due to their spoilage activity, but also to their potential as BAs producers.

Table 3. Physicochemical characterization of Italian and Romanian craft beers.

	Average	Minimum value	Maximum value
		Bottom fermented	
pH	4.69	4.38	5.10
Alcohol (%)	5.20	4.60	7.00
Colour (EBC)	13.34	2.75	40.00
		Top fermented	
pH	4.41	3.55	5.10
Alcohol (%)	8.06	0.50	14.00
Colour (EBC)	172.72	3.05	818.88
		Spontaneously fermented	
pH	3.65	3.45	3.95
Alcohol (%)	5.50	4.80	5.90
Colour (EBC)	16.16	7.55	38.13
		Hybrid/mixed	
pH	4.06	3.05	5.11
Alcohol (%)	5.40	3.20	9.00
Colour (EBC)	28.10	3.23	289.00

Abbreviation: EBC, European Brewing Convention.

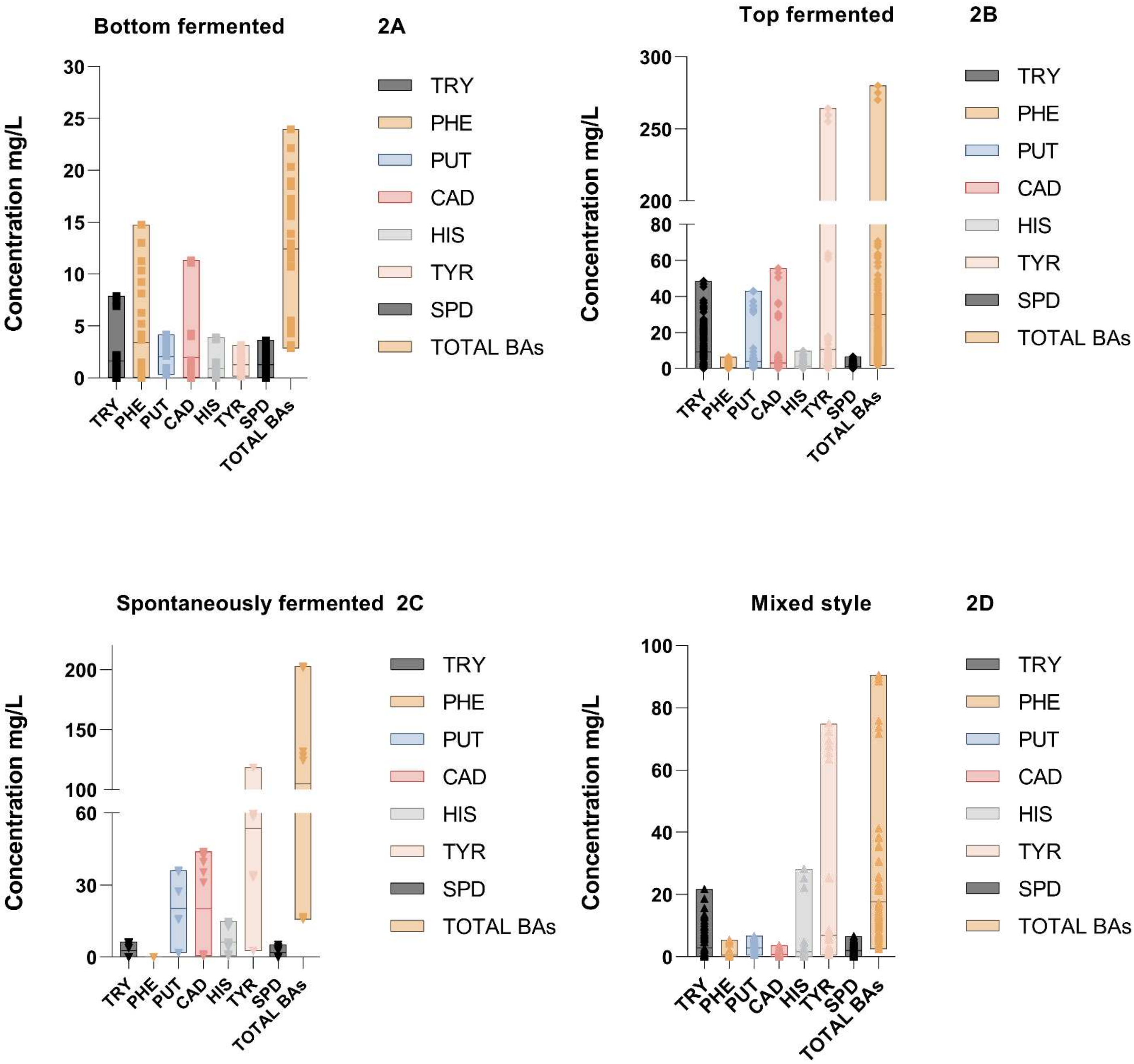


Figure 2. Biogenic amines content (mg/L) in different craft beer styles; 2A, biogenic amines content in bottom fermented beers; 2B, biogenic amines content in top fermented beers; 2C, biogenic amines content in spontaneously fermented beers; 2D, biogenic amines content in mixed style beers. Abbreviations: TRY, tryptamine; PHE, phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPD, spermidine; TOTAL BAs, total biogenic amines. The bands inside the bars represent the mean values.

Table 4. Biogenic amines content in different craft beer styles from Italy and Romania.

	Biogenic amine (mg/L)							
	TRY	PHE	PUT	CAD	HIS	TYR	SPD	TOTAL BAs
Bottom fermented								
IT	2.35 ± 2.45^{a}	5.23 ± 4.91^{a}	3.08 ± 0.7^{a}	0.91 ± 1.5^{b}	1.37 ± 1.17^{a}	1.89 ± 0.52^{a}	1.46 ± 1.11^{a}	16.3 ± 3.97^{a}
RO	0.58 ± 0.43^{b}	0.63 ± 0.58^{b}	0.49 ± 0.24^{b}	3.56 ± 4.63^{a}	0.07 ± 0.05^{b}	0.33 ± 0.18^{b}	0.97 ± 0.27^{a}	6.63 ± 4.45^{b}
Top fermented								
IT	7.97 ± 9.44^{a}	0.24 ± 0.48^{b}	5.38 ± 9.34^{a}	4.06 ± 10.77^{a}	1.03 ± 1.22^{b}	4.07 ± 9.9^{b}	1.13 ± 1.34^{a}	23.88 ± 26.65^{b}
RO	11.12 ± 13.2^{a}	0.72 ± 1.38^{a}	2 ± 1.05^{b}	1.87 ± 1.93^{a}	1.74 ± 2.36^{a}	20.16 ± 54.31^{a}	0.89 ± 1.09^{a}	38.48 ± 57.75^{a}
Spontaneously fermented								
IT	1.64 ± 2.51^{b}	0 ± 0^{a}	26.44 ± 8.71^{a}	26.54 ± 19.48^{a}	8.09 ± 4.41^{a}	70.68 ± 37.57^{a}	1.07 ± 1.62^{b}	134.45 ± 56.27^{a}
RO	6.14 ± 0.22^{a}	0 ± 0^{a}	1.84 ± 0.09^{b}	0.5 ± 0.03^{b}	0.9 ± 0.39^{b}	2.7 ± 0.05^{b}	4.23 ± 1.04^{a}	16.3 ± 0.53^{b}
Mixed style								
IT	3.28 ± 5.43^{a}	0.65 ± 1.14^{a}	3.21 ± 1.39^{a}	0.62 ± 0.7^{b}	0.9 ± 1.1^{a}	11.28 ± 21.33^{a}	2.16 ± 1.85^{a}	22.09 ± 23.24^{a}
RO	2.38 ± 3.6^{a}	0.54 ± 0.51^{a}	2.33 ± 0.98^{b}	0.98 ± 0.78^{a}	2.48 ± 6.21^{a}	1.58 ± 0.72^{b}	1.97 ± 1.53^{a}	12.26 ± 8.42^{b}

Values shown are mean±standard deviation. [a,b] Different superscript letters in a column indicate significant differences, according to Tukey's HSD test ($p < 0.05$).

Abbreviations: TOTAL BAs, total biogenic amines; TRY, tryptamine; PHE, phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPD, spermidine; IT, Italy; RO, Romania.

*$p < 0.05$.

3.4 Comparison of craft beer styles

Finally, beer styles were compared to identify which generally has the highest BA content and could cause potential health risk (Table 5). As expected, spontaneously fermented beers had a higher content of total BAs ($p < 0.05$), together with the individual BAs of bacterial origin (histamine, tyramine, putrescine, cadaverine), compared to the other beer styles. These results suggest a heavy contamination of the microbreweries by decarboxylase positive microorganisms (Loret et al., 2005), as mentioned in the section 3.3.3. Similarly, spontaneously fermented beers from Belgium had the highest BAs content (Loret et al., 2005). In line with results reported by Li et al. (2023), top fermented beers were characterised by a significantly higher content of tryptamine, as this style included Stout and Porter beers (section 2.3.3.2). Phenylethylamine showed the highest level ($p < 0.05$) in craft bottom fermented beers. Interestingly, our findings are not consistent with data reported by Poveda (2019), who stated that dark beers are the ones characterised by the highest phenylethylamine content. Craft beers of mixed style exhibited the highest spermidine concentration, not showing significant differences with wild products. This could be related to the use of contaminated raw materials during the brewing processes, as mentioned in the section 3.3.4.

Table 5. Comparison of biogenic amines content (mg/L) in different craft beers styles.

	TRY	PHE	PUT	CAD	HIS	TYR	SPD	TOTAL BAs
Bottom fermented	1.64 ± 2.09^{b}	3.39 ± 4.42^{a}	2.04 ± 1.41^{b}	1.97 ± 3.34^{b}	0.85 ± 1.1^{b}	1.27 ± 0.88^{b}	1.27 ± 0.9^{b}	12.43 ± 6.32^{b}
Top fermented	9.26 ± 11.21^{a}	0.44 ± 0.98^{b}	4 ± 7.39^{b}	3.16 ± 8.42^{b}	1.32 ± 1.81^{b}	10.65 ± 36.31^{b}	1.03 ± 1.25^{b}	29.85 ± 42.72^{b}
Spontaneously fermented	2.76 ± 2.96^{b}	0 ± 0^{b}	20.29 ± 13.37^{a}	20.03 ± 20.37^{a}	6.29 ± 4.97^{a}	53.69 ± 44.41^{a}	1.86 ± 2.04^{ab}	104.91 ± 71.82^{a}
Mixed style	2.87 ± 4.69^{b}	0.6 ± 0.91^{b}	2.81 ± 1.29^{b}	0.78 ± 0.76^{b}	1.62 ± 4.31^{b}	6.87 ± 16.43^{b}	2.07 ± 1.7^{a}	17.62 ± 18.66^{b}

Values shown are mean±standard deviation. ^{a,b} Different superscript letters in a column indicate significant differences, according to Tukey's HSD test ($p < 0.05$).

Abbreviations: TOTAL BAs, total biogenic amines; TRY, tryptamine; PHE, phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPD, spermidine. *$p < 0.05$.

4. Conclusions

The occurrence of 7 individual BAs (and their total concentration) was evaluated in 113 craft beer samples produced in microbreweries located in Italy and Romania. Results showed that there was a wide variation in the total BAs content, with tyramine, tryptamine, putrescine and cadaverine being the major detected ones. Although histamine was found in low concentrations, people with histamine intolerance should pay attention to craft beer consumption, as 86% of the samples had histamine content above the detectable level. Moreover, 35% of the samples under investigation could pose a health risk for people with low BA detoxification capacity, showing a total BAs content higher than 20 mg/L. Furthermore, 10% of the examined beers had a total BAs content above 50 mg/L, which may be responsible of toxic reaction even in healthy people. Raw materials played a pivotal role for the accumulation of these toxic compounds, especially for Stout and Sour&Fruit beers, in which unconventional ingredients (fruit products, chocolate, coffee, cocoa, etc) were added during the brewing processes. Therefore, microbreweries should pay attention to the selection of the raw materials and particularly to their quality and to the microbiota associated with unconventional ingredients, as well. Moreover, as Wild, Barleywine and Sour beers, showed the highest content of BAs related to microbial contamination, more attention should be paid to the selection of starter cultures and to the brewery hygiene conditions. An efficient food safety management system with more frequent external controls should be implemented in microbreweries, to guarantee that the products do not entail any safety risk for consumers and meet the desired quality characteristics. On the other hand, researchers should focus on the isolation of BA-degrading microorganisms from beers, in order to reduce BAs content, without affecting sensory properties. Moreover, further research is needed to assess the evolution of biogenic amines during storage of craft beers.

Supplementary Table 1. Biogenic amines in craft beers from Italian and Romanian markets (mg/L).

	ID	STYLE	Origin	Ingredients/processes	TRY	PHE	PUT	CAD	HIS	TYR	SPD	TOTAL BAs
Bottom fermented	LAGER_1	LAGER	RO		0.72 ± 0.02	0.27 ± 0.04	0.38 ± 0.01	11.21 ± 0.12	0.09 ± 0.01	0.31 ± 0.01	0.9 ± 0.09	13.88 ± 0
	LAGER_2	LAGER	RO		0.48 ± 0.05	1.44 ± 0.05	0.3 ± 0.02	1.48 ± 0.2	0.08 ± 0	0.23 ± 0.02	1.19 ± 0.04	5.19 ± 0.34
	LAGER_3	LAGER	RO		1.13 ± 0.11	0.82 ± 0	0.38 ± 0.01	0.6 ± 0	0.13 ± 0.01	0.17 ± 0.04	1.2 ± 0.06	4.43 ± 0.15
	LAGER_4	LAGER	IT		1.93 ± 0.29	9.24 ± 1.1	3.56 ± 0.41	0 ± 0	0.55 ± 0.04	1.99 ± 0.08	0 ± 0	17.27 ± 1.68
	LAGER_5	LAGER	RO		0 ± 0	0 ± 0	0.88 ± 0.09	0.94 ± 0.1	0 ± 0	0.61 ± 0.02	0.59 ± 0.06	3.02 ± 0.15
	PILS LAGER_1	LAGER	IT		2.15 ± 0.03	5.2 ± 1.07	2.17 ± 0.05	0.26 ± 0.06	0.5 ± 0.01	1.29 ± 0.09	0.32 ± 0.09	11.89 ± 1.07
	PILS LAGER_2	LAGER	IT		0 ± 0	13.02 ± 1.76	4.13 ± 0.05	0.56 ± 0.04	1.12 ± 0.17	1.73 ± 0.16	1.58 ± 0.01	22.14 ± 1.81
	PILS LAGER_3	LAGER	IT		0.86 ± 0.06	3.93 ± 0.26	2.78 ± 0.53	0 ± 0	0.94 ± 0.05	1.57 ± 0.01	1.52 ± 0.2	11.6 ± 0.88
	VIENNA LAGER_1	LAGER	IT		1.74 ± 0.23	0 ± 0	2.9 ± 0.5	4.14 ± 0.14	3.81 ± 0.1	2.86 ± 0.31	3.02 ± 0.59	18.48 ± 1.86
	BOCK_1	LAGER	IT		7.4 ± 0.5	0 ± 0	2.96 ± 0.12	0.52 ± 0.15	1.33 ± 0.18	1.91 ± 0.03	2.33 ± 0.18	16.45 ± 0.44
Top fermented	PALE ALE_1	ALE	IT		1.18 ± 0.2	0.95 ± 0.05	1.95 ± 0.08	1.28 ± 0.02	0 ± 0	2.26 ± 0	1.03 ± 0.05	8.65 ± 0.15
	PALE ALE_2	ALE	IT		0 ± 0	0.14 ± 0.04	2.65 ± 0.09	0.67 ± 0.06	0.43 ± 0.04	3.57 ± 0.21	0 ± 0	7.46 ± 0.02
	PALE ALE_3	ALE	IT		0.06 ± 0.01	0.19 ± 0.01	1.45 ± 0.02	0.98 ± 0.02	0.12 ± 0.09	1.42 ± 0.19	0 ± 0	4.22 ± 0.13
	PALE ALE_4	ALE	IT		0.51 ± 0.05	0.72 ± 0.06	2.25 ± 0.43	0.97 ± 0.01	0.55 ± 0.11	2.48 ± 0.19	2.29 ± 0.01	9.77 ± 0.65
	PALE ALE_5	ALE	RO		0.87 ± 0.06	3.67 ± 0.22	1.55 ± 0.01	1.45 ± 0.04	0.98 ± 0.03	1.83 ± 0.01	1.05 ± 0.04	11.4 ± 0.22
	PALE ALE_6	ALE	IT		2.16 ± 0.06	0.65 ± 0.09	1.53 ± 0.25	0.87 ± 0.1	0.39 ± 0.02	2.38 ± 0.31	0.45 ± 0.07	8.41 ± 0.78
	PALE ALE_7	ALE	RO		6.6 ± 0.07	0.16 ± 0.07	0.71 ± 0.07	0.77 ± 0.07	0.04 ± 0.04	1.72 ± 0.07	0.8 ± 0.07	10.8 ± 0.38
	PALE ALE_8	ALE	RO		0 ± 0	0.24 ± 0.07	1.06 ± 0.09	0.84 ± 0.27	0.15 ± 0.05	1.84 ± 0.06	0.78 ± 0.38	4.91 ± 0.8

PALE ALE_9	ALE	RO		0 ± 0	0 ± 0	1.16 ± 0.02	0.56 ± 0.13	0 ± 0	1.35 ± 0.47	0.31 ± 0.08	3.38 ± 0.44
PALE ALE_10	ALE	IT	lentils	0 ± 0	0 ± 0	33.09 ± 1.91	36.13 ± 0.47	0 ± 0	0 ± 0	0 ± 0	69.22 ± 1.44
PALE ALE_11	ALE	IT	hemp	0 ± 0	0 ± 0	42.94 ± 0.26	53.1 ± 2.6	0 ± 0	62.29 ± 1.45	0 ± 0	158.33 ± 0.89
PALE ALE_12	ALE	IT	honey, chestnut	0 ± 0	0 ± 0	3.82 ± 0.09	29.37 ± 1	0 ± 0	8.29 ± 0.85	0 ± 0	41.48 ± 1.93
PALE ALE_13	ALE	IT	oat	0 ± 0	0 ± 0	0.6 ± 0.05	0.54 ± 0.04	0.43 ± 0.17	0.39 ± 0.05	0 ± 0	1.96 ± 0.32
DARK ALE_1	ALE	RO	cinnamon	0 ± 0	0 ± 0	2.42 ± 0.09	0 ± 0	3.88 ± 0.15	0 ± 0	0 ± 0	6.3 ± 0.25
RED ALE_1	ALE	IT		0 ± 0	0 ± 0	2.56 ± 0.28	0.94 ± 0.12	2.4 ± 0.06	4.8 ± 0.75	0 ± 0	10.7 ± 0.3
RED ALE_2	ALE	IT		7.2 ± 0.87	0 ± 0	2.54 ± 0.16	1.36 ± 0.14	2.05 ± 0.46	1.59 ± 0.15	0.34 ± 0.01	15.07 ± 1.48
RED ALE_3	ALE	IT		11.01 ± 0.83	0 ± 0	2.57 ± 0.24	4.25 ± 0.3	0 ± 0	2.22 ± 0.27	0.37 ± 0.02	20.42 ± 0.49
RED ALE_4	ALE	RO	refermented in bottle	0 ± 0	2.11 ± 0.11	2.28 ± 0.04	2.13 ± 0.16	0.29 ± 0.03	2.25 ± 0.01	0.49 ± 0.05	9.55 ± 0.28
RED ALE_5	ALE	RO		1.23 ± 0.15	0.35 ± 0.03	0.68 ± 0.04	1.75 ± 0.07	0.46 ± 0.06	0.34 ± 0	0.96 ± 0.04	5.76 ± 0.17
TRIPEL_1	TRIPEL	IT		2.82 ± 0.28	0.28 ± 0.06	2.16 ± 0.1	0.67 ± 0.03	0.53 ± 0.08	1.26 ± 0.12	0 ± 0	7.72 ± 0.55
TRIPEL_2	TRIPEL	IT		2.52 ± 0.08	0.84 ± 0.09	1.73 ± 0.02	0.97 ± 0.04	0.21 ± 0.01	0 ± 0	0.85 ± 0.05	7.12 ± 0.09
TRIPEL_3	TRIPEL	IT		7.21 ± 0.37	1.17 ± 0.12	2.17 ± 0.12	1.19 ± 0.14	0.27 ± 0.06	0.96 ± 0.03	0 ± 0	12.98 ± 0.85
TRIPEL_4	TRIPEL	RO	refermented in bottle	0 ± 0	0 ± 0	3.47 ± 0.13	1.41 ± 0.38	1.36 ± 0.04	1.11 ± 0.12	1.71 ± 0.01	9.06 ± 0.34
TRIPEL_5	TRIPEL	RO	refermented in bottle	0 ± 0	0 ± 0	0.9 ± 0.1	6.46 ± 0.42	0 ± 0	1.2 ± 0.05	1.45 ± 0.25	10.01 ± 0.61
PORTER_1	PORTER	RO		1.08 ± 0.05	2.63 ± 0.08	2.27 ± 0.02	1.59 ± 0.04	0.86 ± 0.09	1.54 ± 0.04	0.19 ± 0.01	10.16 ± 0.09
PORTER_2	PORTER	IT		1.88 ± 0.23	0.92 ± 0.17	2.61 ± 0.21	1.17 ± 0.14	0.41 ± 0.05	2.28 ± 0.06	1.05 ± 0.02	10.33 ± 0.17
PORTER_3	PORTER	RO		12.44 ± 0.52	0.39 ± 0.08	1.37 ± 0.06	3.43 ± 0.21	0.78 ± 0.35	0.76 ± 0.07	0.81 ± 0.07	19.99 ± 1.11
PORTER_4	PORTER	IT		11.72 ± 1.66	0 ± 0	2.66 ± 0.24	1.29 ± 0.26	0.49 ± 0.01	0.72 ± 0.04	0.32 ± 0.01	17.21 ± 1.74

PORTER_5	PORTER	RO	refermented in bottle	7.41 ± 0.43	0.47 ± 0.16	0.39 ± 0.13	1.09 ± 0.15	0.31 ± 0.07	0.86 ± 0.07	0.31 ± 0.13	10.84 ± 1.14
PORTER_6	PORTER	RO		21.82 ± 1.39	0 ± 0	2.01 ± 0.02	0.3 ± 0.07	3.07 ± 0.01	2.26 ± 0.31	0 ± 0	29.44 ± 1.19
PORTER_7	PORTER	IT	candy sugar, oat	15.26 ± 1.18	0 ± 0	9.34 ± 1.94	0 ± 0	1.35 ± 0.56	1.4 ± 0.28	0.6 ± 0.17	27.95 ± 3.79
PORTER_8	PORTER	IT		21.25 ± 1.4	0 ± 0	3.87 ± 0.13	0 ± 0	4.57 ± 0.07	2.06 ± 0.02	0 ± 0	31.75 ± 1.22
STOUT_1	STOUT	IT		2.17 ± 0.09	1.15 ± 0.08	3.52 ± 0.05	0.87 ± 0.31	0.48 ± 0.12	5.08 ± 0.57	4.59 ± 0.17	17.86 ± 0.06
STOUT_2	STOUT	IT		10.79 ± 1.12	2.22 ± 0.08	2.88 ± 0.48	5.22 ± 0.12	1.11 ± 0.29	3.13 ± 0.13	1.55 ± 0.05	26.9 ± 2.1
STOUT_3	STOUT	RO	chocolate, coconut	24.54 ± 2.9	0 ± 0	1.71 ± 0.08	6.3 ± 1.2	0 ± 0	2.49 ± 0.22	0.86 ± 0.11	35.9 ± 2.11
STOUT_4	STOUT	RO	salty caramel, barrled aged	19.14 ± 2.54	0.63 ± 0.03	1.45 ± 0.24	4.48 ± 0.09	0.17 ± 0.02	1.5 ± 0.13	0.79 ± 0.09	28.15 ± 2.74
STOUT_5	STOUT	RO		21.39 ± 0.73	0 ± 0	2.36 ± 0.01	2.58 ± 0.27	0 ± 0	1.09 ± 0.21	0.81 ± 0.05	28.22 ± 0.21
STOUT_6	STOUT	IT		5.55 ± 0.07	0.23 ± 0.07	2.97 ± 0.07	1.76 ± 0.07	0.08 ± 0	1.72 ± 0.07	1.42 ± 0.07	13.74 ± 0.42
STOUT_7	STOUT	RO	refermented in bottle	9.28 ± 1.36	0 ± 0	0.74 ± 0.04	1.32 ± 0.14	0 ± 0	0.42 ± 0.02	0.13 ± 0.03	11.88 ± 1.46
STOUT_8	STOUT	RO	coffee	33.78 ± 2.48	2.6 ± 0.2	2.25 ± 0.1	3.48 ± 0.41	0.16 ± 0.02	104.86 ± 1.93	4.9 ± 0.04	152.03 ± 5.18
STOUT_9	STOUT	IT	oat, cinnamon	34.56 ± 3.23	0 ± 0	2.05 ± 0.05	0 ± 0	4.45 ± 0.45	3.16 ± 0.19	0 ± 0	44.21 ± 2.54
STOUT_10	STOUT	IT	coffee, cocoa	26.23 ± 0.85	0 ± 0	5.7 ± 0.4	0 ± 0	1.64 ± 0.69	4.05 ± 0.15	1.73 ± 0.49	39.35 ± 0.49
STOUT_11	STOUT	IT	cocoa, vanilla, coconut	14.22 ± 1.51	0 ± 0	3.34 ± 0.19	0 ± 0	3.28 ± 0.08	0.99 ± 0.15	0.59 ± 0.01	22.42 ± 1.26
STOUT_12	STOUT	IT		0 ± 0	0 ± 0	3.09 ± 0.68	0 ± 0	2.35 ± 0.17	0 ± 0	0 ± 0	5.45 ± 0.51
STOUT_13	STOUT	IT	cocoa, coffee, candy sugar	22.15 ± 4.22	0 ± 0	3.03 ± 0.52	0 ± 0	0.58 ± 0.07	1.93 ± 0.14	2.47 ± 0.28	30.17 ± 4.25
STOUT_14	STOUT	IT	barrel aged	22.08 ± 1.23	0 ± 0	2.74 ± 0.62	3.55 ± 1.02	0 ± 0	1.45 ± 0.24	1.95 ± 0.33	31.75 ± 0.98
STOUT_15	STOUT	IT	cocoa, coffee	25.04 ± 1.45	0 ± 0	2.11 ± 0.28	0 ± 0	2.93 ± 0.84	3.25 ± 1.18	5.67 ± 1.06	38.99 ± 1.9
STOUT_16	STOUT	IT	oat, cocoa	12.41 ± 3.24	0 ± 0	2.7 ± 0.03	6.91 ± 0.72	0 ± 0	16.89 ± 0.88	0 ± 0	38.91 ± 1.67

	STOUT_17	STOUT	RO	coffee, vanilla	18.61 ± 1.27	0 ± 0	3.47 ± 0.03	0 ± 0	8.12 ± 1.74	6.17 ± 0.53	0 ± 0	36.37 ± 1.03
	STOUT_18	STOUT	RO	cocoa, vanilla	20.52 ± 0.08	0 ± 0	3.43 ± 0.13	0 ± 0	6.47 ± 0.03	8.15 ± 0.15	2.25 ± 0.34	40.81 ± 0.36
	STOUT_19	STOUT	RO	coconut, chocolate	19.59 ± 0.05	0 ± 0	3.97 ± 0.96	3.67 ± 0.37	4.06 ± 0.01	16.22 ± 0.75	0 ± 0	47.52 ± 2.02
	STOUT_20	STOUT	RO	coffee	46.98 ± 1.62	0 ± 0	2.81 ± 0.52	0 ± 0	3.65 ± 0.03	9.15 ± 0.05	0 ± 0	62.59 ± 1.08
	STOUT_21	STOUT	RO	chocolate, coffee	34.85 ± 1.9	0 ± 0	3.91 ± 0.57	0 ± 0	6.67 ± 0.9	13.65 ± 2.8	0 ± 0	59.07 ± 2.36
	IGA_1	IGA	IT	grape must, oat	0 ± 0	0 ± 0	0.99 ± 0.1	0 ± 0	0.68 ± 0.03	0.69 ± 0.35	1.86 ± 0.42	4.22 ± 0.14
	IGA_2	IGA	IT	grape must	0 ± 0	0 ± 0	1.91 ± 0.03	0.18 ± 0.04	0.7 ± 0.14	2.86 ± 0.11	2.21 ± 0.06	7.87 ± 0.1
	IGA_3	IGA	IT	grape must	2.93 ± 0.68	0 ± 0	3.2 ± 0.12	0.27 ± 0.03	0.27 ± 0.11	1.14 ± 0.1	1.04 ± 0.13	8.85 ± 0.61
	IGA_4	IGA	IT	grape must, mango, pineapple	6.96 ± 0.95	0 ± 0	34.67 ± 2.57	2.44 ± 0.37	0 ± 0	2.25 ± 0.11	2.44 ± 1.17	48.76 ± 3.27
	IGA_5	IGA	IT	grape must	25.45 ± 1.26	0 ± 0	2.94 ± 0.04	0 ± 0	1.66 ± 0.31	1.03 ± 0.26	1.89 ± 0.25	32.97 ± 1.03
	BARLEY WINE_1	Barleywi ne	RO	vanilla, barrel aged	0 ± 0	0.6 ± 0.04	1.23 ± 0.08	4.95 ± 0.15	0.68 ± 0.23	102.27 ± 1.02	0.78 ± 0.15	110.51 ± 0.92
	BARLEY WINE_2	Barleywi ne	IT	barrel aged	9.32 ± 0.9	0 ± 0	3.23 ± 0.68	0 ± 0	1.83 ± 0.22	3.34 ± 0.15	2.16 ± 0.07	19.88 ± 1.72
	BARLEY WINE_3	Barleywi ne	RO	barrel aged	0 ± 0	5.62 ± 0.78	2.73 ± 0.33	0 ± 0	3.87 ± 0.73	259.75 ± 4.6	3.19 ± 0.86	275.17 ± 4.92
	BARLEY WINE_4	Barleywi ne	IT	barrel aged	1.18 ± 0.32	0 ± 0	1.2 ± 0.14	0.93 ± 0.13	2.18 ± 0.12	1.91 ± 0.1	3.01 ± 0.11	10.41 ± 0.91
	GOSE_1	GOSE	RO	basil	0 ± 0	0 ± 0	1.31 ± 0.02	1.56 ± 0.24	0.52 ± 0.07	0.74 ± 0.1	0.94 ± 0.11	5.07 ± 0.32
	GOSE_2	GOSE	RO	tomatoes, salt	0 ± 0	0 ± 0	2.27 ± 0.04	0.28 ± 0.04	0.31 ± 0.04	0.8 ± 0.1	0.4 ± 0	4.06 ± 0.23
	GOSE_3	GOSE	IT	passion fruit, mango, pineapple	5.03 ± 0.07	0 ± 0	2.15 ± 0.05	0.3 ± 0.2	0.4 ± 0	0.95 ± 0.05	2.22 ± 0.12	11.05 ± 0.15
	GOSE_4	GOSE	IT	coriander, sea water	0 ± 0	0 ± 0	2.91 ± 0.09	0 ± 0	1.25 ± 0.59	2.47 ± 0.06	0 ± 0	6.63 ± 0.44
Spontaneously fermented	WILD_1	WILD	IT		4.91 ± 1.07	0 ± 0	15.91 ± 0.15	1.11 ± 0.27	13.88 ± 1.03	33.83 ± 0.48	3.21 ± 0.42	72.85 ± 2.46
	WILD_2	WILD	RO	rye malt	6.14 ± 0.22	0 ± 0	1.84 ± 0.09	0.5 ± 0.03	0.9 ± 0.39	2.7 ± 0.05	4.23 ± 1.04	16.3 ± 0.53

	WILD_3	WILD	IT	refermented in bottle	0 ± 0	0 ± 0	35.93 ± 0.2	43.07 ± 0.9	4.76 ± 0.54	118.49 ± 0.09	0 ± 0	202.26 ± 0.47
	WILD_4	WILD	IT	refermented in bottle	0 ± 0	0 ± 0	27.46 ± 0.01	35.44 ± 4.27	5.61 ± 0.63	59.72 ± 1.2	0 ± 0	128.24 ± 3.69
Hybrid/mixed	WHEAT_1	WHEAT	IT	orange	0 ± 0	0.68 ± 0.23	2.98 ± 0.16	0.65 ± 0.11	0.58 ± 0.18	0.76 ± 0.08	0.68 ± 0.04	6.33 ± 0.79
	WHEAT_2	WHEAT	IT		9.67 ± 0.13	0.79 ± 0.04	4.08 ± 0.16	2.88 ± 0.79	2.47 ± 0.33	0.92 ± 0.04	1.5 ± 0.09	22.31 ± 1.19
	WHEAT_3	WHEAT	IT		0 ± 0	0.72 ± 0.01	3 ± 0.02	1.43 ± 0.17	0.32 ± 0.12	1.11 ± 0.03	0.76 ± 0.06	7.35 ± 0.04
	WHEAT_4	WHEAT	IT		0 ± 0	0.57 ± 0.06	2.47 ± 0.07	0.65 ± 0.08	0.41 ± 0.02	1.33 ± 0.02	2.1 ± 0.34	7.52 ± 0.35
	WHEAT_5	WHEAT	RO		0.65 ± 0.21	1.09 ± 0.22	5.01 ± 0.14	1.79 ± 0.11	0.42 ± 0.12	1.02 ± 0.03	2.88 ± 0.08	12.87 ± 0.23
	WHEAT_6	WHEAT	RO		0.23 ± 0.03	0.41 ± 0.09	1.79 ± 0	1.23 ± 0.1	0.26 ± 0.01	0.63 ± 0.01	0.54 ± 0.01	5.11 ± 0.03
	WHEAT_7	WHEAT	IT		0 ± 0	0.61 ± 0.34	2.62 ± 0.11	0.57 ± 0.01	0.56 ± 0.09	25.36 ± 0.25	0.89 ± 0.2	30.6 ± 0.17
	WHEAT_8	WHEAT	IT	orange	0 ± 0	0 ± 0	4.44 ± 0.05	0.24 ± 0.03	0.74 ± 0.19	65.47 ± 2.12	2.77 ± 0.28	73.66 ± 2.11
	WHEAT_9	WHEAT	IT	barrel aged	0 ± 0	0 ± 0	0.58 ± 0.06	0 ± 0	0.52 ± 0.14	0.92 ± 0.14	0.68 ± 0.3	2.7 ± 0.23
	WHEAT_10	WHEAT	IT	oat	2.47 ± 0.3	0.35 ± 0.01	3.29 ± 0.43	0.68 ± 0	0.43 ± 0.05	0.94 ± 0.03	1.01 ± 0.09	9.18 ± 0.07
	WHEAT_11	WHEAT	RO		0.64 ± 0.05	1.6 ± 0.03	1.45 ± 0.07	1.42 ± 0.01	1.29 ± 0.06	1.39 ± 0.1	1.29 ± 0.02	9.08 ± 0.13
	WHEAT_12	WHEAT	IT	coriander	0 ± 0	1.64 ± 0.4	1.98 ± 0.09	0 ± 0	1.25 ± 0.26	2.98 ± 0.32	0 ± 0	7.85 ± 1.07
	SMOKE_1	SMOKED	IT		1.42 ± 0.1	0.79 ± 0.15	2.2 ± 0.07	0.56 ± 0.13	0.15 ± 0.04	2.75 ± 0.01	1.76 ± 0.12	9.63 ± 0.1
	SMOKE_2	SMOKED	RO		0 ± 0	0 ± 0	1.8 ± 0.08	0.32 ± 0	0.32 ± 0	0.81 ± 0.05	0.83 ± 0.14	4.08 ± 0.16
	SMOKE_3	SMOKED	RO		10.78 ± 0.86	0.58 ± 0.08	3.16 ± 0.02	2.65 ± 0.19	0.78 ± 0.09	2.7 ± 0.5	0.99 ± 0.06	21.63 ± 0.09
	SMOKE_4	SMOKED	RO		1.13 ± 0.27	0.97 ± 0.19	2.4 ± 0.09	1.81 ± 0.14	0.69 ± 0.05	1.53 ± 0.04	1.06 ± 0.13	9.61 ± 0.47
	SMOKE_5	SMOKED	IT		18.66 ± 3.01	0 ± 0	5.28 ± 0.62	0 ± 0	4.72 ± 0.18	5.58 ± 0.12	3.94 ± 0.9	38.19 ± 3
	SOUR&FRUIT_1	SOUR&FRUIT	RO	rasperry, strawberry	0 ± 0	0.34 ± 0.16	1.89 ± 0.01	1.15 ± 0.05	0.21 ± 0.02	2.67 ± 0.08	3.59 ± 0.01	9.85 ± 0.01

SOUR&FRUIT_2	SOUR&FRUIT	IT	rasperry, strawberry	0.61 ± 0.01	0.47 ± 0.2	2.7 ± 0.11	1.37 ± 0.03	0.29 ± 0	3.36 ± 0.04	0.73 ± 0.03	9.54 ± 0.28
SOUR&FRUIT_3	SOUR&FRUIT	RO	strawberry	0.77 ± 0.08	0.77 ± 0.02	0.94 ± 0.05	1.66 ± 0.08	0.6 ± 0.08	1.83 ± 0.01	4.51 ± 0.39	11.09 ± 0.54
SOUR&FRUIT_4	SOUR&FRUIT	RO	lemon, cranberry	0.55 ± 0.04	0.42 ± 0.01	2.75 ± 0.13	0.44 ± 0.02	0.49 ± 0	0.82 ± 0.05	1.4 ± 0.31	6.86 ± 0.29
SOUR&FRUIT_5	SOUR&FRUIT	RO	mango	0 ± 0	1.23 ± 0.14	3.32 ± 0.18	0.95 ± 0.17	0.69 ± 0.01	1.72 ± 0.15	3.85 ± 0.41	11.77 ± 0.77
SOUR&FRUIT_6	SOUR&FRUIT	IT	passion fruit	0 ± 0	0.17 ± 0.02	4.7 ± 0.12	0.33 ± 0.04	0.18 ± 0.04	0.74 ± 0.09	6.52 ± 0.1	12.63 ± 0.22
SOUR&FRUIT_7	SOUR&FRUIT	RO	passion fruit, peach, vanilla, cocnonut, blueberry	7.49 ± 0.39	0.73 ± 0.09	1.24 ± 0.04	0 ± 0	0 ± 0	0.68 ± 0.1	3.01 ± 0.04	13.14 ± 0.39
SOUR&FRUIT_8	SOUR&FRUIT	IT	raspberry, mint	13.11 ± 0.4	0 ± 0	2.74 ± 0.15	0.15 ± 0.05	0.3 ± 0.01	6.36 ± 0.03	2.96 ± 0.42	25.61 ± 0.24
SOUR&FRUIT_9	SOUR&FRUIT	RO	blueberry, passion fruit	4.64 ± 0.04	0 ± 0	2.43 ± 0.06	0 ± 0	25.15 ± 3.07	2.03 ± 0.52	4.27 ± 0.24	38.51 ± 2.81
SOUR&FRUIT_10	SOUR&FRUIT	IT	apricot	6.18 ± 0.57	0 ± 0	2.17 ± 0.26	0.45 ± 0.03	0.64 ± 0.03	2.24 ± 0.2	5.44 ± 0.34	17.13 ± 0.16
SOUR&FRUIT_11	SOUR&FRUIT	IT	mango, oat	6.83 ± 0.95	0 ± 0	6.43 ± 0.3	0.18 ± 0.02	0.28 ± 0.02	72.19 ± 2.76	3.57 ± 0.44	89.49 ± 1.07
SOUR&FRUIT_12	SOUR&FRUIT	IT	cinnamon, passion fruit, lactobacilli	0 ± 0	4.9 ± 0.58	2.08 ± 0.02	0.51 ± 0.1	1.57 ± 0.19	1.83 ± 0.18	3.53 ± 0.39	14.43 ± 0.73
SOUR&FRUIT_13	SOUR&FRUIT	IT	oat, coriander, orange, barrel aged	0 ± 0	0 ± 0	4.1 ± 0.28	0.44 ± 0.01	0.78 ± 0.08	8.14 ± 0.58	0 ± 0	13.46 ± 0.21
SOUR&FRUIT_14	SOUR&FRUIT	RO	refermented in bottle, *Saccharomyces spp.*, *Brettanomyces spp.*, lactobacilli, *Pediococcus spp.*	0 ± 0	0 ± 0	2.45 ± 0.19	0.45 ± 0.15	2.34 ± 0.08	2.46 ± 0.22	0 ± 0	7.71 ± 0.19
SOUR&FRUIT_15	SOUR&FRUIT	RO	barrel aged, lactobacilli, *Brettanomyces spp.*	0 ± 0	0 ± 0	1.85 ± 0.09	0 ± 0	1.38 ± 0.12	1.31 ± 0.09	1.29 ± 0.1	5.82 ± 0.04
SOUR&FRUIT_16	SOUR&FRUIT	RO	refermented in bottle, lactobacilli, *Pedioccus spp.,*	8.79 ± 0.83	0 ± 0	2.45 ± 0.17	0.79 ± 0.19	2.59 ± 0.43	2.14 ± 0.27	0 ± 0	16.75 ± 0.66

Values shown are mean ± SD. Abbreviations: TOTAL BAs, total biogenic amines; TRY, tryptamine; PHE, phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPD, spermidine; IT, craft beers from Italy; RO, craft beers from Romania.

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Conclusion and future perspectives of Chapter 3

Despite growing attention in recent years, the body of research on food safety interventions remains limited compared with the substantial literature on nutritional approaches. Therefore, more in-depth research is needed, particularly on foodborne intoxication caused by chemical hazards, such as biogenic amines (BAs). These toxic compounds contribute to numerous foodborne outbreaks worldwide, often underestimated when the effects are mild for several reasons, among which the similarity of their symptoms to those of influenza. While mild cases are often underestimated, severe intoxications can lead to pronounced physiological effects. In this context, alcohol plays a crucial role, as it can intensify the respiratory, circulatory, and gastrointestinal discomfort caused by BAs. Therefore, alcoholic beverages deserve particular attention in food safety assessment. Craft beers are usually unfiltered and unpasteurized and are brewed in small-scale companies, microbreweries. Thus, their production processes may result in the accumulation of harmful levels of BAs, posing potential health risks for consumers. Suitable strategies to reduce their formation should be proposed, ensuring the safety of these products. Therefore, researchers and food companies have a pivotal role in researching, testing, validating and demonstrating effective solutions for BAs reduction, which can be scaled up to industrial level, preventing thousands of outbreaks and restoring consumer confidence. In this context, this project began with a survey on the content of BAs in Italian and Romanian craft beers. Although laboratory trials and pilot scale production (TRL 5-6) were not carried out, as it would require dedicated pilot-scale infrastructure, extensive process optimization, and industrial partnerships, this study provides: i) valuable information to consumers and health organizations on the average content of such molecules in different beer styles; ii) details on potential causes of BAs accumulation in these matrices, in order to propose effective strategies to limit their formation. Therefore, these findings provide a strong foundation for future scale-up, guiding the translation of laboratory research into practical applications, within the brewing industry.

Results showed that tyramine, tryptamine, putrescine and cadaverine were the main BAs detected in craft beers. Wild beers showed the highest content of total BAs, as well as BAs of microbial origin (histamine, tyramine, cadaverine), compared to ale, lager and mixed style beers. Top fermented beers were characterized by the highest content of tryptamine, because of malt baking. On the other hand, unconventional ingredients led to a higher accumulation of spermidine in mixed style beers. Despite histamine was found in low concentrations, 86% of the samples had histamine content above the detectable level, representing a health hazard for people with histamine intolerance. Craft beers consumption in combination with other fermented foods should be carefully considered by susceptible consumers, as 35% of the samples analysed had a total BAs content higher than the limit suggested by literature for this type of consumers (20 mg/L). The risk is lower for healthy subjects, because only 10% of beer products had a total BAs level higher than 50 mg/L, regarded as harmful for this type of consumer.

Moreover, results highlighted that some practices applied in the production of craft beers could pose a high food safety risk. The selection of raw materials, especially unconventional ingredients, malt baking, mashing, fermentation process and the selection of starter cultures and generally hygiene conditions have been identified as critical points for the accumulation of BAs in craft beers. Thus, according to these results and to previous research, critical points have been highlighted also on a craft beer production plant (Figure 1).

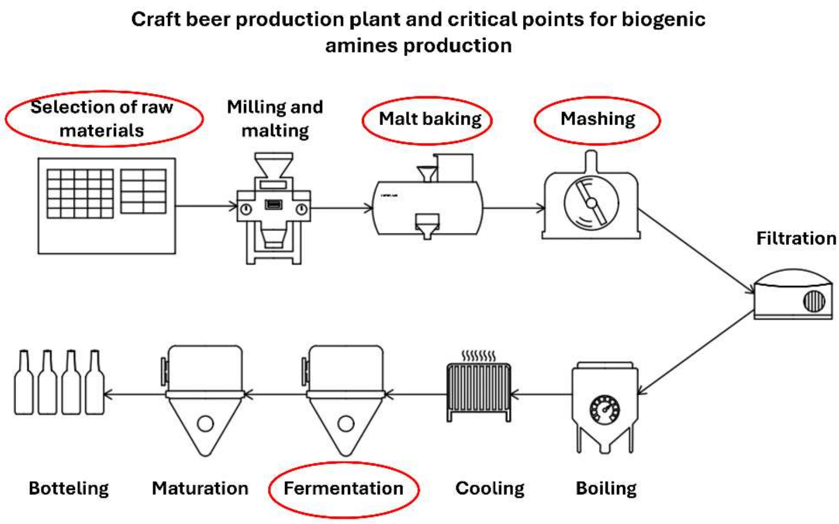

Figure 1. Craft beer production plant and critical point for biogenic amines production.
Red circles are used to represent the critical points for biogenic amines production in the processing plant.

To advance current knowledge on biogenic amines in craft beer from laboratory-based observations towards higher Technology Readiness Levels (TRL 5-6), it is essential to translate the identification of critical control points into validated mitigation strategies applicable along the brewing process. Thus, brewers should be advised on these critical phases and apply control measures. Future research should test, validate and demonstrate strategies for BAs control and reduction at laboratory level and subsequently on industrial scale. The accurate selection of raw materials, based on the evolution of i) associated microbiota that could produce BAs; ii) presence of free amino acids, which could be decarboxylated into biogenic amines, could be a first step towards managing and reducing these toxic substances. Moving forward in the beer brewing process, malt baking and mashing parameters should be optimized to reduce the formation of BAs, especially tryptamine and phenylethylamine, produced during malt treatments. Temperature and accurate starters culture selection are critical factors to be considered during fermentation stage. These optimizations represent necessary steps for future pilot-

and industrial-scale implementation, supporting the scale-up of controlled brewing strategies aimed at reducing toxic compound accumulation in craft beer production.
Furthermore, future research aimed at advancing the mitigation of biogenic amines towards higher TRL should focus on the isolation and laboratory-scale validation of biogenic amine–degrading microorganisms from beer products. At this stage, their effectiveness in reducing BAs content, as well as their impact on the sensory characteristics of craft beer, should be systematically assessed. Once their functionality and safety are demonstrated under controlled laboratory conditions, these microorganisms could be subsequently validated at pilot and industrial scale, thereby supporting the transition from laboratory-based findings to scalable and applicable solutions for the production of safer and high-quality craft beers.
Moreover, as for craft beers, the major issue is related to people with histamine intolerance, further research is required to investigate this issue in greater depth. In this regard, research on the feasibility of applying the enzyme that oxidizes histamine (diamino-oxidase enzyme - DAO) extracted from various sources, such as microorganisms, pig kidney and legume sprouts, would have a significant impact. Several studies, in fact, have already optimised sprouting processes of legumes, to enhance DAO activity, offering clear advantages over current porcine-derived products. This approach may lead to the production of a functional craft beer that can be safely enjoyed by all consumers, including those with histamine intolerance, as well as vegetarians, vegans, and individuals adhering to religious dietary restrictions. Thus, future research should initially focus on laboratory-scale investigations (TRL 3-4) to accurately assess DAO stability, activity, and interaction with the beer matrix, as well as its impact on sensory properties. It is essential to define operational parameters and feasibility under controlled conditions. On this basis, subsequent validation at pilot scale (TRL 5-6) would be required to evaluate process integration and enzyme performance during production, supporting the potential industrial application of DAO for the development of histamine-reduced craft beers.
Overall, by integrating all the aforementioned strategies into industrial processes, it will be possible to scale-up innovations from research to large scale production. These strategies, indeed, hold the potential to innovate the craft beer sector, offering products that combines safety, inclusivity and sensory appeal; a craft beer for everyone is no longer just a possibility, it is now an achievable goal.

Chapter 4

Conclusions of the thesis

This doctoral research thesis has explored and demonstrated how scientific innovations developed within academic environments could be effectively translated into industrial applications, through models of technology transfer and scale-up in the food sector, generating outcomes useful in helping food companies in facing global challenges, such as non-communicable diseases (NCDs), food waste, and food safety. The results obtained across the three case studies confirmed that innovation in products, processes, and technologies represents a decisive strategy for achieving sustainable growth and aligning the food industry with the Sustainable Development Goals (SDGs) of the 2030 Agenda.
The first chapter demonstrated that natural bioactive compounds, such as polyphenols extracted from olive pomace, can be successfully scaled up from laboratory scale to industrial-level applications, improving the nutritional and microbiological quality of fresh pasta, thus contributing to waste reduction and promoting healthier dietary patterns. These findings highlight how circular economy principles and valorisation of by-products can be integrated into industrial processes, transforming waste into valuable resources and reducing the environmental footprint of food production.
The second chapter focused on the nutritional improvement of a Brazilian traditional product, *pão de queijo*, to address the global challenge of NCDs, related to diets rich in saturated fats and with a low content of dietary fibre. The reformulation with avocado pulp and emulsion-filled gels, based on inulin and olive oil, demonstrated the feasibility of developing healthier alternatives, without inducing perceptible sensory modifications and while preserving industrial scalability, thereby indicating a favourable potential for consumer acceptance. Although this study was limited to laboratory scale formulation and validation, it emphasized the importance of designing innovations that can be easily adapted to automated production lines, thereby facilitating technology transfer and adoption by food companies. Future research should prioritize pilot-scale experimentation aimed at validating process robustness, optimizing key processing parameters, and assessing shelf-life under conditions representative of industrial production. Such activities are necessary to enable effective technology transfer and to advance the proposed reformulation strategies towards higher Technology Readiness Levels (TRL).
The third chapter addressed the issue of food safety, particularly the presence of biogenic amines (BAs) in craft beers, which pose potential health risks to consumers. Through a survey on the content of BAs in Italian and Romanian craft beers, critical points (raw material selection, fermentation conditions, and hygiene practices) of the brewing process related to the accumulation of these toxic compounds were identified. The study was limited to laboratory-scale investigation and did not include pilot or industrial validation. Nevertheless, these findings provide a scientific basis for future

research aimed at validating mitigation strategies at pilot and industrial scale (TRL 5-6), thereby supporting the development of safer production practices in the craft brewing industry.

Overall, the thesis demonstrated that the successful scale-up of food innovations requires a systemic approach, integrating scientific research, technological feasibility, and industrial applicability. Collaboration between research institutions and food companies (which represent the strength of this thesis) emerges as the cornerstone of this process, bridging the gap between basic research and industrial application. By combining sustainability, nutrition, and safety, the outcomes of this doctoral research thesis provided a comprehensive framework for promoting innovation in the food sector.

In conclusion, this work highlighted the strategic role of innovation as a driver of competitiveness and sustainability and illustrated that bridging the gap between laboratory-scale discoveries and industrial implementation is not only possible, but pivotal for addressing contemporary global challenges. This doctoral thesis, indeed, provided models for future initiatives, aimed at enhancing the health orientation, safety and sustainability of the global food system.

Conferences and workshops

Attendance at international conferences

1. 3rd International Trainee Symposium in Agri-Food, Nutrition and Health - Oral presentation - "Extending the shelf-life of fresh pasta with free and microencapsulated olive pomace extracts" Winnipeg, Manitoba, Canada, October 15-17, 2025.
2. 2nd International trainee symposium in agri-food, nutrition and health - Oral presentation - "Application of D-optimal mixture design for the formulation of inulin-based fat replacers" Winnipeg, Manitoba, Canada, January 25-26, 2024.

Attendance at national conferences and workshops

1. 29th Workshop on the Developments in the Italian PhD Research on Food Science and Technology and Biotechnology - Oral Presentation - "Free and microencapsulated olive pomace extracts to improve the phenolic content and shelf-life of fresh pasta". Teramo, Italy, September 10-12, 2025.
2. Convegno nazionale della Società di Scienze e Tecnologie Alimentari - Transizione verso un sistema alimentare sostenibile 2025 - Oral presentation - "Valutazione della shelf-life di pasta fresca addizionata con estratto di sansa in forma libera e microincapsulata" Bologna, Italy, June 10-12, 2025.
3. Convegno nazionale della Società di Scienze e Tecnologie Alimentari - Transizione verso un sistema alimentare sostenibile 2025 - Poster presentation - "Indagine sul contenuto delle ammine biogene nelle birre artigianali italiane e rumene" Bologna, Italy, June 10-12, 2025.
4. Dottorati STEM: un'opportunità di sviluppo- Poster Presentation- "Biogenic amines in craft beers: preliminary results of a survey on the Italian market" Bari, Italy, November 4, 2024.
5. 28th Workshop on the Developments in the Italian PhD Research on Food Science and Technology and Biotechnology - Poster Presentation - "From the research to company: Case study on the biogenic amines in craft beers and cheeses" Catania, Italy, September 18-20, 2024.
6. Convegno nazionale della Società di Scienze e Tecnologie Alimentari - Transizione verso un sistema alimentare sostenibile 2024 - Poster presentation - "Survey on the content of biogenic amines in Italian and Romanian craft beers" Bari, Italy, June 12-13, 2024.
7. 27th Workshop on the Developments in the Italian PhD Research on Food Science and Technology and Biotechnology - Poster Presentation - "From research to business: Technology transfer models in the field of Food Science" Portici, Italy, September 13-15, 2023.

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