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FERMENTATION AS A TOOL FOR DEVELOPMENT OF INNOVATIVE ORGANIC FOOD PRODUCTS WITH ADDED VALUE

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Dedication

To my mother, whose strength never asked for recognition and whose sacrifices formed the ground I stand on—every page of this work is built on your love.

To my brother, my backbone. Thank you for standing beside me through every version of myself.

To my best friends, who shared with me all the memories—this journey would have been unbearable without you.

To my supervisors, who believed in me. Thank you for your guidance, patience, and trust. You taught me how to grow into this role. I am deeply grateful.

And to the memory of my father:

The last words you spoke to me were a poem and a wish—to see me become a doctor. You are not here to witness this day, yet I have carried those words in my heart for fourteen years.

Today, I have fulfilled my promise and I hope, somehow, you are smiling and proud.

This doctorate exists because of you.

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Introduction and aim of the thesis

The organic industry is currently undergoing a phase of dynamic growth, not only in the European Union but also in other regions. Italy is known for its robust organic market [1], where consumers show great enthusiasm for both locally sourced organic products and those with organic certification, demonstrating a high level of awareness regarding organic standards [2]. This growth phase is indicative of the increasing demand for organic products, which has been attributed to various factors such as the rise in health and environmental awareness among consumers [3,4]. It is clear now that conventional agriculture systems contribute significantly to greenhouse gas emissions, biodiversity loss, freshwater depletion, and soil degradation, making current patterns of production incompatible with long-term ecological resilience [5]. To minimize this risk, many approaches have been employed recently such as shifting towards healthy diets, waste valorization, better agriculture practices, such as organic agriculture, and sustainable food processing techniques, such as organic food processing with minimal technologies. Organic production systems prioritize ecological balance, biodiversity protection, and responsible use of natural resources. Importantly, organic processing is governed by strict regulatory frameworks such as EU Regulation 2018/848, which emphasizes minimal processing, prohibits most synthetic additives, and encourages techniques that preserve the intrinsic qualities of raw materials. These regulatory constraints redefine the technological toolbox available to organic processors and highlight the importance of biological processes, such as fermentation, that naturally enhance safety, quality, and shelf-life without compromising organic integrity.

Fermentation represents one of the most powerful and versatile tools available to the organic food sector. Historically employed as a means of preservation, fermentation has evolved into a sophisticated biotechnological platform capable of improving nutritional value, generating desirable sensory attributes, and transforming simple substrates into high-value foods [6]. Unlike many modern processing technologies, fermentation is inherently aligned with organic principles because it is based on metabolism of living organisms, namely microorganisms inhabiting natural environment or as “active principle” of starter preparations. Because in fermented food items chemical additives are not used in most cases and/or intensive thermal treatments are not applied, fermentation is

considered as minimal processing technique. Microorganisms such as lactic acid bacteria (LAB), yeasts, and acetic acid bacteria (AAB) play central roles in shaping the biochemical and sensory profile of fermented products through the production of organic acids, aromatic compounds, vitamins, exopolysaccharides, and antimicrobial substances [7,8]. At the same time, fermentation has become increasingly important as a mechanism for valorizing underutilized organic resources. A significant portion of agricultural losses — particularly in the fruit and vegetable sectors — arises from the discarding of aesthetically defective but otherwise edible produce [9]. Their transformation into innovative products not only reduces waste but also creates opportunities for added economic value within local supply chains. This aligns strongly with circular economy goals and with organic principles that emphasize efficient use of natural resources. The organic sector in many regions, including the Apulia region (Southern Italy), is characterized by a high density of small and medium-sized enterprises (SMEs). These SMEs operate with limited processing infrastructure and rely on affordable, low-energy techniques. Fermentation is particularly suitable in this context because it requires relatively simple equipment, supports decentralized production, and allows processors to differentiate their products through craftsmanship and microbiological complexity [10]. However, despite its potential, there is a lack of standardized fermentation protocols specifically adapted to organic matrices and SME-scale operations. This gap represents a barrier to innovation and restricts the ability of organic processors to develop new products that meet consumer expectations for authenticity, health, and sustainability.

This thesis investigates fermentation as a key strategy for developing innovative organic food products and adding value to underutilized raw materials within the organic sector. By focusing on the role of natural and commercial starter cultures and the microbial mechanisms that drive fermentation, the research applies these biological processes to selected organic matrices to enhance product quality, improve resource efficiency, and provide feasible technological solutions for small and medium-sized enterprises. In doing so, the work contributes to advancing sustainable organic food processing and strengthening circularity across the organic value chain.

To guide the reader through the development of this work, the thesis is structured into four main chapters, each contributing to the central objective of exploring fermentation as a tool for creating innovative organic food products and enhancing the valorization of underutilized organic resources. Chapter 1 provides the scientific and regulatory background necessary to contextualize the research, including definitions of organic food, market trends, and the microbiological principles of fermentation. Chapter 2 offers an extensive review of natural and commercial starter cultures, tracing their historical evolution, microbial ecology, technological functions, and relevance within the constraints of organic production. This chapter establishes the conceptual framework that underpins the experimental studies. Chapter 3 presents the first experimental investigation, focused on developing an organic sourdough bread enriched with germinated seed pastes. Here, fermentation is applied as a technological tool to enhance nutritional value, sensorial properties, and sourdough robustness—directly contributing to the thesis aim of innovating organic cereal products. Chapter 4 describes the second experimental study, which evaluates the potential of converting third-category organic fruits into vinegar through sequential alcoholic and acetic fermentation. That chapter demonstrates how fermentation can support circularity and reduce waste within organic supply chains. Finally, the thesis concludes by integrating the findings across chapters to highlight the broader implications of fermentation for sustainable organic food processing and to propose future research directions.

Abstract

Chapter 1 provides the conceptual, regulatory, and scientific framework underpinning the thesis by contextualizing fermentation within the organic food system. It first outlines the definition of organic food, the evolution of organic agriculture, and current market trends at global, European, and national levels, with particular emphasis on the regulatory constraints imposed by EU Regulation 2018/848. These constraints, which limit the use of synthetic additives and promote minimal processing, redefine the technological options available to organic food processors and highlight the importance of biologically driven processes. The chapter then introduces fermentation as a key processing strategy aligned with organic principles, describing its historical role, technological relevance, and sustainability potential. A detailed overview of the fermentation process is provided, focusing on the metabolic activities and functional roles of lactic acid bacteria, acetic acid bacteria, and yeasts. Their contributions to food safety, shelf-life extension, sensory development, nutritional enhancement, and resource efficiency are discussed. By integrating regulatory, microbiological, and technological perspectives, this chapter establishes the theoretical basis for the subsequent review and experimental studies, framing fermentation as a central tool for innovation, value addition, and circularity in organic food processing.

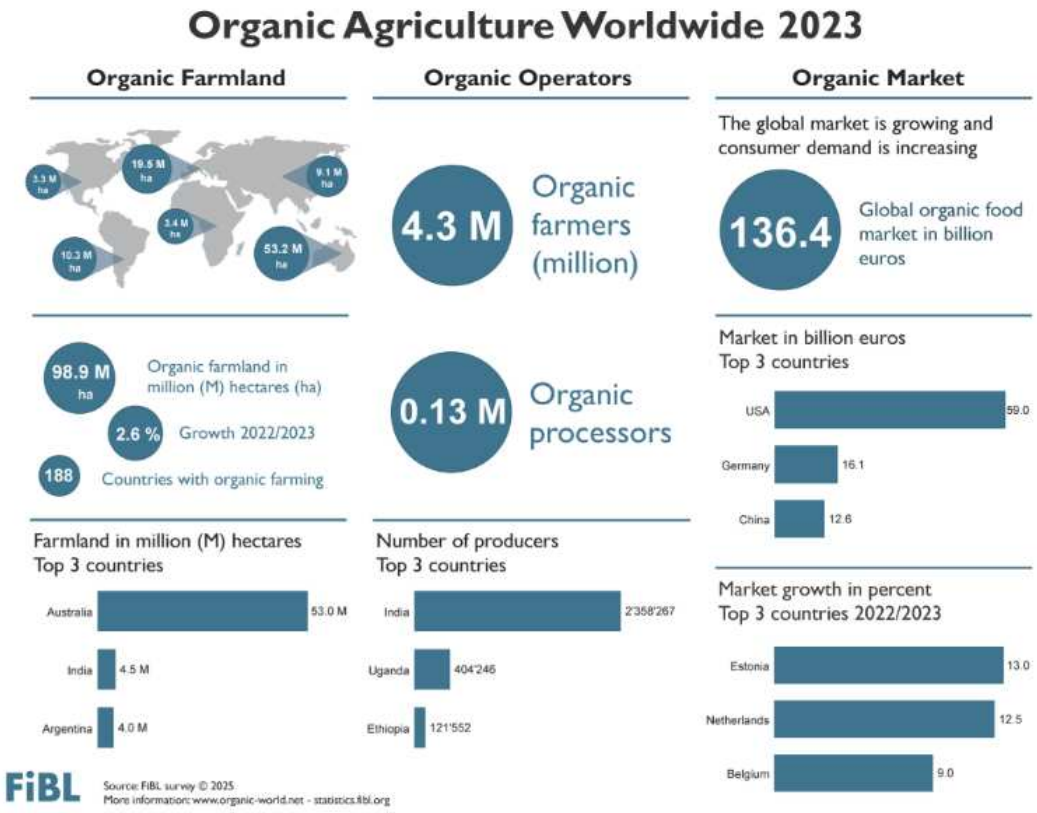
1.1 Organic food: definition, regulation and market trends

Organic food is produced within agricultural systems that aim to sustain soil fertility, protect biodiversity and minimize the use of synthetic inputs. In the European Union, the legal basis is Regulation (EU) 2018/848, which defines organic production rules across the whole chain—from primary production to processing, labelling and control—and stipulates that organic food should be obtained "by the use of processes that do not involve the use of genetically modified organisms and that are based on natural substances and processes" [11]. Globally, organic agriculture continues to expand in both area and market value. According to the latest FiBL/IFOAM statistics for 2023, organic farming is practiced on about 98.9 million hectares, corresponding to 2.1% of the world's

agricultural land, with approximately 4.3 million organic producers worldwide (**Figure 1**) [12,13].

Figure 1. Organic agriculture statistics presented on February 2025. (Source: IFOAM)

Market analyses converge on strong medium-term growth expectations. For example, one recent industry report estimated the global organic foods market at 177.08 billion USD in 2023, with projections of 199.35 billion USD in 2024 and over 529 billion USD by 2032, corresponding to a compound annual growth rate of around 13% [14]. In the United States alone, organic sales reached 71.6 billion USD in 2024, an increase of 5.2% compared to 2023, with particularly strong growth in



organic meat, poultry, seafood and yogurt [15].

Europe and the European Union are among the main global hubs for organic food production and consumption. The latest FiBL media release on Europe reports that, in 2023, 19.5 million hectares in Europe and 17.7 million hectares in the EU were organically farmed, meaning that organic agriculture accounted for 10.9% of

total farmland in the EU. Retail sales of organic products in Europe reached 54.7 billion euros in 2023, of which 46.5 billion euros were generated within the EU; Germany remained the largest single market with 16.1 billion euros in sales [16].

These developments are closely linked to EU policy targets. Under the Farm to Fork Strategy, the European Commission has set an objective for at least 25% of the EU's agricultural land to be under organic management by 2030, accompanied by measures to stimulate demand, strengthen value chains, and support farmers in conversion [17,18]. Organic consumption has shown remarkable resilience despite economic uncertainty: recent market monitoring indicates that, after a small contraction in 2022, the European organic market grew again by about 3% in 2023, with particularly dynamic trends in some northern and central European countries.

Italy is one of the leading countries in Europe in terms of organic area and number of operators. According to the most recent national indicator compiled by ISPRA on the basis of SINAB, Eurostat and ISTAT data, Italian organic farming covered 2,456,020 hectares in 2023, representing 19.8% of national utilized agricultural area (UAA) and involving 94,441 organic operators. Organic farming is particularly concentrated in a few regions—Sicily, Apulia, Tuscany, Calabria and Emilia-Romagna, which together account for more than half of the national organic area and operators—highlighting the strategic relevance of these territories for organic production and processing [19,20].

Taken together, these data show that organic food is no longer a niche market but a rapidly expanding segment of the global agri-food system, with strong policy support in the EU and significant regional hubs such as Italy and the Apulia region. For organic processors and small- and medium-sized enterprises, this expansion creates both opportunities and challenges: it increases demand for high-quality, differentiated organic products, but also requires processing technologies that comply with strict regulatory standards, respond to consumer expectations for "natural" and minimally processed foods, and make efficient use of limited and often costly organic raw materials. Fermentation-based approaches, as explored in this thesis, are particularly well suited to this context.

1.2 Fermentation process

Fermentation is a microbial process in which bacteria, yeasts, or filamentous fungi convert organic substrates—primarily carbohydrates—into simpler metabolites such as organic acids, alcohols, gases, and other compounds, usually under (micro)anaerobic conditions [21]. Historically developed as a low-input preservation method, fermentation today is recognized as a key biotechnological tool for improving the safety, stability, and functionality of foods in both artisanal and industrial systems [22,23]. Food fermentations are commonly classified according to their dominant metabolic end products and leading microbial groups: lactic acid fermentations driven mainly by LAB; alcoholic fermentations driven largely by yeasts; acetic acid fermentations mediated by AAB; and mixed or secondary fermentations in which several microbial groups interact in sequence or consortium [22,24]. Through these pathways, microorganisms acidify the matrix, consume oxygen, and produce antimicrobial metabolites (organic acids, ethanol, bacteriocins, hydrogen peroxide), thereby suppressing spoiling and pathogenic microorganisms and extending shelf life [24,25]. Beyond preservation, fermentation substantially modifies the nutritional and functional profile of foods. It can degrade anti-nutritional factors (e.g., phytates, tannins), improve protein digestibility, increase mineral bioavailability, and transform phenolic compounds into more bioactive or bioavailable forms [26,27]. Additionally, it enhances the organoleptic properties of food, improving taste and aroma. Fermented foods and fermented protein ingredients have been associated with enhanced antioxidant capacity and the generation of bioactive peptides with antihypertensive, immunomodulatory, and other health-relevant activities [28,29]. Recent reviews emphasize that these microbial transformations are particularly relevant for plant-based and minimally processed foods, where fermentation provides a “natural” route to improve sensory quality, functionality, and nutritional value while aligning with sustainability goals and clean-label expectations [30,27,21].

The characteristics of fermented products can vary significantly based on the specific microorganisms employed. Among the microorganisms most utilized in driven fermentation are LAB, AAB, and yeasts, each contributing distinct qualities to the final product.

1.2.1 Lactic Acid Bacteria

LAB are a broad group of Gram-positive, non-spore-forming cocci and rods that share the ability to ferment carbohydrates predominantly into lactic acid via substrate-level phosphorylation. They are aerotolerant anaerobes, typically catalase-negative, with low G+C DNA content, and are widely associated with plant material, fermented foods, and the gastrointestinal tract of humans and animals [31]. Historically, the core LAB genera included *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus* and *Weissella*. Relatively recent whole-genome phylogenies led to a major reclassification of the former genus *Lactobacillus* into 25 genera (e.g. *Lactiplantibacillus*, *Lacticaseibacillus*, *Latilactobacillus*, *Limosilactobacillus*) and to the union of the former families *Lactobacillaceae* and *Leuconostocaceae* into an expanded *Lactobacillaceae* [32]. This revised taxonomy groups species into clades that better reflect their ecology and metabolic capabilities, for example vertebrate-adapted species, insect-associated species, or plant/food-associated lineages.

In food systems, LAB colonize nutrient-rich, often mildly or strongly acidic environments such as dairy products, sourdoughs, fermented vegetables, meats, and plant-based beverages. Fermented foods are now recognized as a primary niche for LAB activity and a major source of viable cells and metabolites in the human diet [33]. The success of LAB in these habitats is linked to rapid sugar uptake and acidification, tolerance to low pH, and the ability to cope with osmotic and oxidative stresses.

LAB are traditionally classified as homofermentative or heterofermentative based on their pathways for hexose fermentation and major end products [34]. In details:

- **Obligately homofermentative LAB** (e.g. *Lactococcus*, *Streptococcus*, *Enterococcus* and many *Lactobacillus*-related genera) metabolize glucose via the Embden–Meyerhof–Parnas (EMP) pathway to pyruvate, which is then reduced to lactic acid. One mole of glucose yields two moles of lactic acid and two moles of ATP, with NAD^+ regeneration achieved through lactate dehydrogenase activity [34]. This efficient ATP yield underpins fast growth and strong acidification, which are crucial technological traits in many food fermentations.
- **Obligately heterofermentative LAB** (e.g. *Leuconostoc*, *Weissella*, *Oenococcus* and some *Lactobacillus*-derived genera) lack a complete EMP

pathway and utilize the phosphoketolase branch of the pentose phosphate pathway. Glucose-6-phosphate is oxidized to 6-phosphogluconate, decarboxylated to pentose-5-phosphate, then cleaved into glyceraldehyde-3-phosphate and acetyl phosphate. The typical end products are equimolar lactic acid, CO_2 and ethanol or acetate, with a net yield of one mole of ATP per mole of glucose [34,35]. The production of CO_2 contributes to dough leavening and bubble structure in cereal fermentations.

- **Facultatively heterofermentative LAB** (many *Lactiplantibacillus* and *Lacticaseibacillus* species) can use the EMP pathway for hexoses and the phosphoketolase pathway for pentoses, allowing metabolic flexibility depending on substrate availability and redox balance [31].

Beyond simple hexoses, LAB metabolize a wide array of plant-derived carbohydrates, including fructans, resistant starches and non-starch polysaccharides, via secreted and cell-associated glycosidases. This capacity to degrade complex substrates is central in cereal- and legume-based fermentations and underpins applications in valorization of fiber-rich or by-product matrices [31, 36]. Although lactic acid is the main fermentation product, LAB can redirect pyruvate and related intermediates to alternative pathways, modulating both energy yield and flavor formation [34]. Under carbohydrate-limited conditions or in the presence of external electron acceptors (e.g. oxygen, citrate, fructose), pyruvate can be converted to acetate, sometimes via acetyl-phosphate and acetyl-CoA. This shift generates an additional mole of ATP per mole of glucose and contributes to sharper, more acidic sensory profiles [36].

Citrate and other organic acids may be co-metabolized, leading to production of CO_2, diacetyl and acetoin, which contribute buttery and creamy notes desirable in certain dairy and cereal fermentations [36].

LAB also catabolize amino acids such as arginine, glutamate and branched-chain amino acids. The arginine deiminase pathway, for example, yields ATP and ammonia, enhancing acid tolerance and contributing to pH homeostasis in acidic environments [35].

From a technological perspective, key LAB traits include rapid acidification, tolerance to low pH, salt and osmotic stress, and the capacity to grow at refrigeration or ambient temperatures depending on the application. Many strains synthesize exopolysaccharides (EPS) that improve dough rheology, crumb softness,

viscosity and water-holding capacity in dairy and cereal-based foods [37]. EPS can be produced in situ during fermentation or added as purified biopolymers, providing “clean-label” texture modification in organic and minimally processed foods.

LAB proteolytic systems—comprising cell-envelope proteinases and a suite of intracellular peptidases—liberate peptides and free amino acids from food proteins. These catabolites act as flavor precursors and can be further transformed into volatile compounds (e.g. aldehydes, alcohols, sulfur compounds) that shape the aroma of breads, cheeses and fermented plant products [31].

LAB produce a manifold spectrum of metabolites far beyond lactic acid, encompassing organic acids (such as lactic, acetic, propionic and succinic acids), ethanol, carbon dioxide, hydrogen peroxide, vitamins, bioactive peptides, and bacteriocins. Together, these compounds significantly impact food preservation, sensory quality, nutritional value, and health-promoting properties [38].

- **Organic acids** including lactic and acetic acid lower pH, restrict spoilage and pathogenic microbes and thus contribute to microbial safety and extended shelf-life in fermented foods [39].
- **EPS** from LAB enhance texture (thickening, viscosity, mouth-feel) and also exhibit potential functional benefits such as immunomodulation and prebiotic effects. The yield and structural diversity of EPS are strain- and condition-specific, and their application in clean-label formulations is growing [40].
- **Bacteriocins** are ribosomally synthesized antimicrobial peptides that act against closely related or even broader microbial species. They are increasingly explored as natural biopreservatives in foods and as part of hurdle technologies for minimally processed products [41].
- **Bioactive peptides** released during LAB-driven proteolysis of food proteins show promise for functional food development. These peptides may exert antihypertensive, antioxidant, immunomodulatory and other bioactivities, thereby linking fermented foods with health-oriented markets [42].

Recent research also reveals that LAB-derived metabolites interact with the gut microbiota, contribute to intestinal barrier function, modulate immune responses,

and exert anti-inflammatory effects—thus bridging the technological and nutritional functionality of fermented foods [43].

Because LAB can produce multiple metabolites in parallel and respond to substrate, environmental, and strain variables, their functional output is highly tunable. From the perspective of organic food processing, exploiting LAB's metabolite portfolio enables the development of foods with added value (e.g., enhanced texture, improved nutritional profile, extended shelf life) while adhering to regulation-friendly processing (minimal additives, low input).

1.2.2 Acetic Acid Bacteria (AAB)

AAB are a group of Gram-negative, strictly aerobic α-Proteobacteria characterized by their ability to incompletely oxidize a wide range of alcohols and sugars into the corresponding organic acids, with acetic acid as the hallmark product. AAB are typically rod-shaped or ellipsoidal, motile or non-motile, catalase-positive and oxidase-negative. They are obligate aerobes: oxygen is essential, as their energy metabolism relies on membrane-bound dehydrogenases linked to a respiratory chain. This strict dependence on oxygen explains their typical localization at the air–liquid interface in fermentations, where they often form a characteristic surface film or pellicle [44,48].

From a taxonomic perspective, the AAB group currently encompasses more than twenty genera, including *Acetobacter*, *Komagataeibacter*, *Gluconobacter*, *Gluconacetobacter*, *Kozakia* and *Tanticharoenia*, among others [45]. These genera differ in their preferred substrates and oxidation patterns: for example, *Acetobacter* and *Komagataeibacter* efficiently oxidize ethanol to acetic acid and can further oxidize acetate to CO_2 and H_2O, whereas *Gluconobacter* specializes in the incomplete oxidation of sugars and sugar alcohols at the cell surface. Together, AAB play central roles in oxidative fermentations such as kombucha, water kefir, traditional wine, cereal and fruit vinegars, and in mixed ecosystems like cocoa and coffee fermentations [46,47].

Ecologically, AAB colonize niches rich in simple carbohydrates and ethanol, including flowers, fruits (especially damaged fruits), plant saps and exudates, insect guts, sugary beverages, and spontaneously fermented foods. In these habitats they frequently coexist with yeasts and LAB, engaging in metabolic interactions that structure the community and influence flavor formation. In cocoa fermentation, for instance, AAB follow yeasts and LAB in the classic

succession and oxidize yeast-derived ethanol to acetic acid, contributing to bean death and flavor precursor formation [47]. Similar successions are observed in traditional vinegars and other spontaneous acetic acid fermentations [46,49]. Recent reviews emphasize that AAB are not only “vinegar bacteria” but versatile biocatalysts with relevance for novel beverages, functional ingredients (e.g. levan, bacterial cellulose) and biotechnological oxidations [44,50].

The defining metabolic feature of AAB is their ability to perform incomplete, periplasmic oxidation (Pox) of substrates such as ethanol, sugars (glucose, fructose), sugar alcohols (sorbitol, mannitol) and other polyols. Substrates are oxidized at the outer face of the cytoplasmic membrane by pyrroloquinoline quinone (PQQ)-dependent or flavin-dependent dehydrogenases, with electrons transferred through ubiquinone to terminal oxidases in the respiratory chain [51,52].

Ethanol oxidation proceeds in two main steps: membrane-bound alcohol dehydrogenase (ADH, often PQQ-ADH) oxidizes ethanol to acetaldehyde and membrane-bound aldehyde dehydrogenase (ALDH) oxidizes acetaldehyde to acetic acid. Thank to their peripheral location, both enzymes allow rapid release of acetic acid into the medium. This surface-bound, respiratory oxidation is highly efficient for energy conservation, as electrons feed into the electron transport chain and drive proton translocation and ATP synthesis while the end product accumulates externally [45,51].

AAB can also perform incomplete oxidation of carbohydrates. *Gluconobacter* spp., for example, oxidize glucose to gluconic, 2-ketogluconic and 5-ketogluconic acids via membrane-bound glucose and gluconate dehydrogenases, with limited flux through the tricarboxylic acid (TCA) cycle. This capacity is exploited industrially to produce gluconic acid and related compounds [50].

When external substrates are depleted, AAB may shift to acetic acid peroxidation, re-importing acetate and converting it to acetyl-CoA via acetyl-CoA synthetase. Acetyl-CoA is then oxidized completely through the TCA cycle and oxidative phosphorylation, which reduces the acetic acid concentration and supports secondary growth. This dual capacity—production and subsequent utilization of acetate—is important in long fermentations and for strain robustness [45].

Recent omics-based analyses in industrial vinegar systems have elucidated how AAB adjust central carbon fluxes, respiratory chains and cofactor use under changing ethanol, oxygen and acetic acid levels, highlighting strain- and process-specific strategies for maintaining activity and yield [52].

During oxidative fermentations, AAB are exposed to challenging environments: high ethanol at the start of the process, rising acetic acid concentrations, low pH and often elevated temperatures. These conditions are growth-inhibitory or lethal to many bacteria, but selected AAB strains have evolved a suite of stress-response mechanisms.

Recent work synthesizes these mechanisms into several main strategies [45,53]:

- **Membrane and cell envelope adaptation**
    - Change in fatty acid composition (e.g. increased cyclopropane or saturated fatty acids) to reduce membrane permeability to undissociated acetic acid.
    - Modification of capsular and lipopolysaccharide components that limit acid influx.
- **Enhanced activity of membrane-bound dehydrogenases**
    - Up-regulation and stabilization of PQQ-ADH and ALDH, which both generate energy and contribute to acetic acid extrusion.
    - PQQ itself appears to support tolerance to strong acid and high temperature.
- **Efflux and detoxification systems**
    - Proton-motive-force-driven efflux pumps and ABC transporters export acetate anions and protons, reducing cytosolic acidification.
    - Increased expression of enzymes that oxidize or assimilate acetate in the cytoplasm, lowering its toxic impact.
- **General stress response networks**
    - Up-regulation of molecular chaperones and proteases that maintain protein homeostasis.
    - Activation of quorum-sensing and regulatory circuits that coordinate stress responses at the population level.

Comparative studies of thermotolerant and acid-tolerant strains confirm that robust AAB typically show higher ethanol and acetic acid tolerance, maintain respiratory activity at elevated temperature, and are thus better candidates as

industrial starters [48,54]. These properties are crucial when designing processes with higher substrate loading or shorter fermentation times.

In many fermentations, AAB grow preferentially at the air–liquid interface, forming a robust biofilm or pellicle. This lifestyle optimizes access to oxygen and helps cells withstand high acid and ethanol in the bulk liquid. Surface growth is particularly pronounced in *Komagataeibacter* spp., which also synthesize bacterial cellulose, a highly crystalline extracellular polysaccharide that contributes to pellicle structure [44].

Bacterial cellulose produced by AAB has attracted interest as a food ingredient and structuring agent, for example in gluten-free baked goods or as a fat replacer, due to its water-holding capacity, mechanical strength and clean label perception [55,56]. Some AAB also produce EPS such as levan, which can contribute to texture and may have prebiotic or immunomodulatory properties. Selected strains of *Tanticharoenia* and other genera have been explored for high-yield levan production and potential functional applications [57].

Beyond acetic acid itself, AAB participate in the formation and modulation of a wide array of flavor-active metabolites. During acetic fermentation they can generate or transform organic acids (acetic, gluconic, lactic, succinic), aldehydes, ketones, higher alcohols, esters and volatile phenols.

Recent integrative metabolomics and microbiota studies in different vinegar types and other oxidative fermentations show clear correlations between AAB community structure and key volatile profiles, including fruity esters and caramel, nutty or woody notes [58,52,51].

In addition to sensory effects, AAB activities can influence functional and nutritional properties. Oxidative metabolism may stabilize or even increase polyphenol retention and antioxidant activity in some fruit-based fermentations, and acetic fermentation products have been associated with antihyperglycemic, antimicrobial and other bioactivities [59].

1.2.3 Yeasts

Yeasts are eukaryotic microorganisms that thrive in various ecological environments. They colonize sugar-rich habitats such as plant surfaces, fruit exudates, nectars, cereal substrates, and insect-associated niches, which explains their central role in food and beverage fermentations based on grains, fruits, and

other carbohydrate-rich matrices [60.61]. Taxonomically, yeasts encompass a diverse set of ascomycetous and basidiomycetous genera. In food systems, Saccharomyces cerevisiae remains the dominant workhorse for bread, beer and wine, while numerous non-*Saccharomyces* genera (e.g. *Hanseniaspora, Metschnikowia, Torulaspora, Wickerhamomyces, Pichia*) increasingly attract interest for their contribution to aroma complexity, texture and functionality in fermented foods and beverages [62,63].

From a physiological perspective, yeasts are facultative anaerobes with highly flexible metabolism. Under aerobic, low-sugar conditions, carbon flows predominantly through glycolysis, the TCA cycle and oxidative phosphorylation, supporting high ATP yields and biomass formation. When sugars are abundant or oxygen is limiting, most food-relevant yeasts shift carbon flux toward fermentative pathways, converting hexoses to ethanol and CO_2 via pyruvate decarboxylase and alcohol dehydrogenase, while regenerating NAD^+ to sustain glycolysis [61, 64].

In *S. cerevisiae* and other Crabtree-positive species, high glucose availability triggers fermentative metabolism even in the presence of oxygen (the Crabtree effect), which is now understood as an adaptive strategy linked to overflow metabolism, proteome allocation and competitive fitness in sugar-rich environments [65]. This metabolic flexibility is crucial in food fermentations, where redox balance, ATP yield, and carbon partitioning between ethanol, organic acids and aroma compounds determine both process robustness and product quality.

Beyond primary ethanol fermentation, yeasts generate a wide spectrum of secondary metabolites that shape the sensory profile of fermented foods. Through the Ehrlich pathway, amino acids are catabolized to higher alcohols and corresponding esters, contributing fruity, floral and solvent-like notes. Lipid metabolism yields medium-chain fatty acids and their esters, while sulfur amino acid catabolism produces volatile sulfur compounds with low sensory thresholds [60,66].

Non-*Saccharomyces* yeasts often show higher activities of β-glucosidases, esterases and carbon–sulfur lyases than *S. cerevisiae*, allowing them to liberate bound terpenes and other glycoside linked aroma precursors or to modulate sulfur

volatiles. Mixed or sequential fermentations that combine *S. cerevisiae* with selected non-*Saccharomyces* strains are therefore increasingly used to tailor flavor, acidity and ethanol content in wines, ciders and other fermented matrices [62,63].

Stress tolerance is another key trait in food-associated yeasts. Many strains withstand low pH, high osmotic pressure, elevated ethanol levels, temperature fluctuations and oxidative stress, relying on conserved stress-response networks, membrane remodeling, compatible solute accumulation and antioxidant systems. In bread doughs, for example, tolerance to osmotic and mechanical stress is essential for consistent leavening and gas retention, whereas in fruit and cereal fermentations, resilience to organic acid stress and fluctuating oxygen conditions underpins process robustness. Systems-biology and genome-scale metabolic modeling have increasingly been used to dissect these stress responses and guide rational strain development for targeted applications [67,68,69].

In complex food ecosystems, yeasts rarely act alone. They typically coexist with LAB and, in some cases, with AAB or filamentous fungi. These multispecies consortia engage in nutrient cross-feeding, co-metabolism and modulation of environmental conditions. Yeasts provide growth factors such as amino acids, vitamins and oxygen consumption, while LAB contribute acidification and production of bacteriocins, and AAB further oxidize ethanol to organic acids in aerobic niches [70]. Such interactions are essential in traditional spontaneous fermentations of cereals, legumes and fruits, where native yeasts help initiate fermentation, detoxify substrates, and create ecological conditions that favor beneficial bacteria over spoilage or pathogenic microorganisms.

Yeast metabolism also has important nutritional implications. Cell biomass is rich in high-quality protein with a balanced essential amino acid profile, B-group vitamins, minerals, and bioactive components such as β-glucans and mannans. Recent reviews emphasize the potential of yeast biomass and yeast protein ingredients as sustainable alternative proteins for human nutrition, with emerging applications in snacks, meat analogues and bakery products [71,72,73].
Yeast extracts, obtained by autolysis or hydrolysis of yeast cells, are widely used as flavor enhancers and sources of nucleotides and peptides, while also valorizing by-products such as brewer's spent yeast. These developments align with circular-

economy strategies and are particularly relevant for organic systems that seek to minimize waste and add value to side streams.

In parallel, certain yeasts are being recognized for their probiotic and health-promoting potential. *S. cerevisiae* var. *boulardii* is already commercialized as a probiotic with documented benefits in preventing or mitigating antibiotic-associated and traveler's diarrhea, modulating immune responses, and supporting intestinal barrier function [74]. A growing body of work is exploring probiotic attributes—acid and bile tolerance, adhesion to intestinal epithelium, pathogen exclusion, and production of antimicrobial or antioxidant metabolites—in strains of *Debaryomyces, Kluyveromyces, Pichia, Torulaspora* and other genera, many of them originally isolated from traditional fermented foods [70,74]. Although clinical evidence is still limited for most non-*Saccharomyces* strains, these findings highlight yeasts as potential contributors not only to food safety and quality but also to the functional properties of fermented products.

Overall, yeasts combine a distinctive set of traits—fast growth on inexpensive substrates, robust stress tolerance, flexible primary metabolism, rich aroma-forming capacity, and the possibility of contributing to nutritional and health benefits. Classical strain selection, adaptive evolution and modern metabolic engineering are progressively expanding this diversity and tailoring yeast performance to specific process objectives. In the context of organic and sustainable food systems, these characteristics make yeasts indispensable partners for LAB and AAB in designing innovative fermented products, valorizing underused raw materials, and supporting more circular, resource-efficient processing strategies.

1.3 References

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From Ancient Fermentations to Modern Biotechnology: Historical Evolution, Microbial Mechanisms, and the Role of Natural and Commercial Starter Cultures in Shaping Organic and Sustainable Food Systems

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Abstract

Chapter 2 provides a comprehensive and integrative review of fermented foods and starter cultures, examining their historical evolution, microbial ecology, technological functions, and relevance within organic and sustainable food systems. The chapter traces the origins of fermentation from ancient, spontaneous practices based on natural starters to modern biotechnological approaches relying on standardized commercial cultures. A wide range of fermented food categories—including cereal- and legume-based products, dairy foods, fermented vegetables, beverages, meat, and seafood—are discussed to illustrate the diversity of fermentation ecosystems and microbial consortia involved. Particular emphasis is placed on the microbial mechanisms governing natural starters, with sourdough fermentation presented as a paradigmatic model to describe microbial succession, metabolic interactions, and their impact on food quality and safety. The chapter further compares natural and commercial starter cultures in terms of functionality, reproducibility, safety, sensory outcomes, and technological performance, highlighting advantages and limitations of each approach. Regulatory aspects specific to organic food processing are also addressed, with attention to compliance with EU organic legislation and constraints on additives and processing aids. Overall, this chapter establishes the theoretical and methodological foundation for the experimental work presented in subsequent chapters, emphasizing the strategic role of starter cultures in leveraging fermentation as a tool for innovation, sustainability, and value creation in organic food production.

2.1 Introduction

In recent times, there has been a noticeable change in consumer behaviour towards organic food consumption, primarily fueled by a growing consciousness surrounding sustainable and healthy dietary choices. The global market for organic produce is valued at 90 billion euros [1]. In 2022, Italy had over 2.3 million hectares of land destined to organic agriculture and a total of over 30,000 organic operators, as reported by the regional Biobank Open Project and the national information system for organic agriculture (Sistema d'Informazione Nazionale sull'Agricoltura Biologica). This resulted in an annual domestic market value for organic products of 3.55 billion euros. Consumers perceive food from organic agriculture as natural and minimally processed [2,3]. Organic food products usually contain fewer pesticide residues and lower levels of heavy metals when compared to food from conventional agriculture [4,5,6,7]. Amidst this burgeoning trend, fermentation has emerged as a prominent method of careful food processing epitomizing the fundamental rules of EU about organic food (Reg. EU 848/2018) and harnessing the power of natural starters to allow food transformation. Food fermentation, with a history dating back to at least 6000 years, offers remarkable sensory attributes [8,9] and, if well controlled, does not need additives for ensuring safety [10,11] and long shelf life with low risk of spoilage [12,13,14].

Fermentation exploits the enzymatic activities of beneficial microorganisms (named “starters”), to convert organic substrates in simpler compounds, such as ethanol and organic acids. These starters include yeast for alcoholic fermentation, *Bacillus* spp. for alkaline-fermented foods, and lactic acid bacteria (LAB) for fermentations yielding lactic acid as the main compound. Fermentation enabled our ancestors, across diverse regions, to overcome challenging seasons and environmental constraints by enhancing the preservability and, although not automatically, safety of food. Today, fermented foods and beverages encompass a diverse array of products enjoyed worldwide, including alcoholic beverages, dairy products, pickled vegetables, sauerkraut, cured sausages, and soy-based products [15,9]. Approximately one-third of the food produced globally is fermented [16].

Fermentation fits well to the EU regulations on organic food production, because it often allows to avoid the use of chemically synthesized additives, which are prohibited by those regulations. Embracing fermentation as a core processing

technique in the organic food industry not only provides consumers with an extensive range of organic food choices endowed with appreciable sensory traits, but also aligns perfectly with the EU's vision of promoting a holistic and environmentally conscious approach to food production and consumption. Traditional fermentation processes remain relevant as alternatives in those areas where modern preservation methods, like refrigeration are absent [17,18,19,20,21]. However, fermentation, differently from refrigeration, unavoidably changes composition, flavor, and texture, of food, and cannot be applied to matrices rich in lipids. Indeed, lipids may be oxidized by microorganisms to aldehydes, ketones, and peroxides, producing off-flavors, rancid odors, and texture changes [26].

Previous review papers compared natural starters, consisting of preparations that harbor mixed and mostly undefined populations of pro-technological microorganisms, with commercial starters, consisting of selected and standardized microbial strains [22]. However, they focused just on one [23] or two [24] types of fermented food. Corbo et al. [25] examined several fermented food items, questioning about opportunities of using microorganisms isolated from natural starters to replace commercial starters. However, they did not primarily focus on comparison between natural and commercial starters and considered all the fermented food items, regardless of the origin (whether from conventional or organic farming) of raw matter/food ingredients.

The main objective of the current review is to provide a comprehensive and integrative overview of the evolution, microbial ecology, and technological applications of natural and commercial starter cultures in food fermentation, with particular emphasis on their role in organic food production systems. Specifically, the review aims to (i) trace the historical development of fermented foods and starter cultures, (ii) describe the microbial mechanisms governing natural starters with sourdough as a model, (iii) compare the functionality and safety of natural versus commercial starters, (iv) examine regulatory frameworks and organic certification requirements, and (v) explore future directions for harnessing natural microbial diversity in sustainable food processing.

2.1.1 Methodology of Literature Search

This review was conducted following a structured narrative approach to summarize and critically evaluate current knowledge on natural and commercial starter cultures in organic food fermentation. Scientific articles were retrieved

from major databases, including Scopus, Web of Science, PubMed, and Google Scholar, using combinations of the following keywords: “fermented foods,” “starter cultures,” “natural starters,” “commercial starters,” “organic fermentation,” and “regulation.” Searches were limited to publications in English from 2000 to 2025, supplemented with earlier seminal works for historical context. Additional relevant documents were identified through the reference lists of selected papers and official regulatory sources (e.g., European Food Safety Authority, EU Commission, U.S. Department of Agriculture). Studies were included based on their relevance to the microbiology, technological aspects, and regulatory context of fermentation.

2.2 Fermented food items through the Ages: the cradles of Natural Starters

The history of natural starters can be traced back to the dawn of human civilization when the discovery and utilization of fermentation as a method of food preservation became an essential aspect of early practices of food processing [26,27,28]. Certain authors have proposed that fermentation techniques and their resulting products were likely developed approximately 10,000 years ago with the goals of preserving food during scarcity, while enhancing taste [29,30]. Natural starters have played a pivotal role in traditional fermentation techniques across diverse cultures, boasting a remarkable history spanning millennia. During ancient times, fermentation was initiated by the action of microorganisms naturally present in the environment [31]. These complex microbial communities, consisting of bacteria, yeasts and filamentous fungi (i.e., molds) conducted the fermentation, ultimately contributing to the unique flavors, aromas, and textures of the final products [32]. Early civilizations unknowingly noticed the power of microorganisms, allowing for the spontaneous fermentation of foods and beverages. As human knowledge advanced, intentional selection and cultivation of specific starter cultures emerged, leading to the deliberate use of well-adapted microorganisms in fermentation [33,34]. This deliberate selection and use of natural starters, influenced by indigenous knowledge and cultural practices, has shaped the development and refinement of fermented foods and beverages throughout history.

2.2.1 Fermented Cereals and Legumes

Fermentation of cereals and legumes has left an indelible mark on many cultures. Microorganisms that naturally contaminate these matrices are able to convert them into a diverse range of staple, delicious and healthy food products, such as bread and porridge [35]. The art of fermentation has also enriched the global culinary landscape with its diverse range of textures and flavors [36,37]. Examples of traditional fermented foods prepared from cereals and legumes in different parts of the world are listed in **Table 1.**

Table 1. Fermented cereal- and legume-based food products from various substrates, with indication of country of origin/common consumption and microorganisms involved in processing [38,39,35,40,41,42,43,44,45,46,47,48,49,50,51].

Country	**Product**	**Substrate**	**Microorganisms conducting fermentation**
Albania, Turkey, Bulgaria, and Romania	Boza	Wheat, millet, maize, and other cereals	Bacteria: *Lactobacillaceae (*formerly *Lactobacillus* species*), Leuconostoc mesenteroides*; yeasts: *Saccharomyces cerevisiae*
Benin, and Togo	Mawè	Maize	Bacteria: *Limosilactobacillus fermentum (*formerly *Lactobacillus fermentum), Limosilactobacillus reuteri (*formerly *Lactobacillus reuteri), Levilactobacillus brevis (*formerly *Lactobacillus brevis), Latilactobacillus curvatus (*formerly *Lactobacillus curvatus), Weissella confusa* (formerly *Lactobacillus confuses*), *Ligilactobacillus salivarius (*formerly *Lactobacillus salivarius), Lactococcus lactis,*

			Pediococcus pentosaceus, Pediococcus acidilactici, L. mesenteroides; yeasts: *Pichia kudriavzevii (*formerly *Candida krusei), Kluyveromyces marxianus (*formerly *Candida kefyr), Candida glabrata* and *S. cerevisiae*
Brazil	Cachiri	Maize	Bacteria: *Lactobacillaceae*; yeasts: *S. cerevisiae* and *Candida* spp.
	Fubá	Germinated and fermented maize grains	Bacteria: *Lactobacillaceae;* yeasts: *Saccharomyces* spp.
Botswana	Bogobe	Sorghum	Bacteria: *L. reuteri, L. fermentum, Lacticaseibacillus harbinensis (*formerly *Lactobacillus harbinensis), Lactiplantibacillus plantarum (*formerly *Lactobacillus plantarum), Lentilactobacillus parabuchneri (*formerly *Lactobacillus parabuchneri), Lacticaseibacillus casei (*formerly *Lactobacillus casei)* and *Loigolactobacillus coryniformis (*formerly *Lactobacillus coryniformis)*
Burkina Faso	Bikalga	Roselle (Hibiscus sabdariffa)	Bacteria: *Bacillus subtilis, Bacillus licheniformis, Bacillus cereus, Bacillus pumilus, Pseudobacillus badius (*formerly *Bacillus badius),*

			*Brevibacillus porteri (*formerly *Bacillus bortelensis), Lysinibacillus sphaericus (*formerly *Bacillus sphaericus)* and *Lysinibacillus fusiformis (*formerly *Bacillus fusiformis)*
	Soumbala	Locust bean	Bacteria: *B. subtilis, Bacillus pumilus, Priestia megaterium (*formerly *Bacillus megaterium)* and *Bacillus licheniformis*
China	Chee-fan	Soybean wheat curd	Molds: *Mucor* spp. and *Eurotium herbariorum (*Formerly *Aspergillus glaucus)*
	Furu/Lufu/Sufu	Soybean curd	Molds*: Actinomucor, Mucor*, *Rhizopus*,
	Lao-chao	Rice	*Rhizopus arrhizus (*formerly *Rhizopus oryzae), Rhizopus microsporus* var. *chinensis* and *Saccharomycopsis guttulate (*formerly *Cyniclomyces guttulatus)*
	Yandou	Soybean	*B. subtilis*
China and Taiwan	Douchi	Soybean	Molds: *Mucor racemosus (Chlamydomucor racemosus), Aspergillus oryzae, Aspergillus egyptiacus, Rhizopus delemar (*formerly *Rhizopus oryzae)* and *R. microsporus* var. *oligosporus*
	Meitauza	Soybean	Bacteria: *B. subtilis*
China,	Ang-kak	Red rice	Molds: *Monascus purpureus*

Taiwan, Thailand, and Philippines			
Congo	Poto poto	Maize	Bacteria: *L. mesenteroides*
Colombia	Champuz	Maize or rice	*Lactobacillus* spp. and *S. cerevisiae*
Cyprus, Greece, and Turkey	Tarhana	Sheep milk, wheat	Bacteria: *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus salivarius* subsp. *thermophilus, L. lactis, Lactobacillus acidophilus, L. mesenteroides subsp. cremoris , L. casei;* yeasts: *S. cerevisiae*
Egypt	Kishk	Oat, barley, bulgur, wheat	Bacteria: *L. plantarum, L. brevis, L. casei* and *B. subtilis*
	Sourdough	Rye, wheat	Bacteria: *Fructilactobacillus sanfranciscensis*, *L. plantarum, L. paracase, L. casei, Pediococcus pentosaceus, L. mesenteroides, Weissella cibaria and W. confusa;* yeasts: *S. cerevisiae, Kazachstania humilis, Kazachstania exigua, Wickerhamomyces anomalus, Torulaspora delbrueckii and Pichia kudriavzevii*
Ethiopia	Enjera/Injera	Tef flour, wheat	Bacteria: *L. delbrueckii subsp. Bulgaricus; yeasts:*

			*Meyerozyma guilliermondii (*formerly *Candida guilliermondii)* and
	Borde	Maize, sorghum, wheat, finger millet, and barley	Bacteria*: Lactobacillaceae, and Leuconostoc* spp.; yeasts: *S. cerevisiae* and *Candida* spp.,
Ghana	Banku	Maize, or maize and cassava	*Lactobacillus* spp.
Ghana	Kenkey	Maize	Bacteria: *L. fermentum, L. reuteri, L. plantarum, L. brevis, Pediococcus pentosaceus, L. mesenteroides,* and *Pediococcus acidilactici*; yeasts: *S. cerevisiae, Pichia kudriavzevii* and *Scytalidium candidum*
	Koko	Maize	Bacteria: *Klebsiella aerogenes* (formerly *Enterobacter cloacae), Acinetobacter* spp. , *L. plantarum, and L. brevis*; yeasts: *S. cerevisiae* and *Candida vini (*formerly *Candida mycoderma)*
Ghana, and Nigeria	Dawadawa	Locust bean	*Bacillus* spp.
India	Adai	Cereals/legume	Bacteria: *Pediococcus* spp., *Streptococcus* spp., *Leuconostoc* spp.
	Ambil	Ragi flour, cooked rice,	Bacteria: *Lactobacillus and Streptococcus* species

		and buttermilk	
	Anarshe	Rice	*Lactobacillus* spp.
	Bekang	Soybean	*Bacillus* spp.
	Bhallae	Black gram	Yeasts: *Candida famata* (formerly *Debaryomyces hansenii),* *Cutaneotrichosporon curvatum (*formerly *Candida curvata), Tausonia pullulans (*formerly *Trichosporon pullulans), Ogataea polymorpha (*formerly *Hansenula polymorpha),* and *Candida parapsilosis.* Bacteria: *L. fermentum* and *L. mesenteroides.*
	Dhokla	Bengal gram (*Cicer arietinum*), dehulled black gram (*Phaseolus mungo*), and milled rice (*Oryza sativa*)	Bacteria: *L. fermentum* and *L. mesenteroides*; mold: *Wynnella silvicola (*formerly *Helvella silvicola)*
	Hawaijar	Soybean	*Bacillus* spp.
	Tungrymbai	Soybean	*Bacillus* spp.
	Vada	Cereal/legume	Bacteria: *Pediococcus, Streptococcus* and *Leuconostoc* spp.
India, and Himalaya	Jaanr	Millet	Yeasts: *W. anomalus (*formerly *Hansenula anomala),* and

			*Mucor indicus (*formerly *Mucor rouxianus)*
India, Nepal, and Bhutan	Kinema	Soybean	Bacteria: *B. subtilis, B. licheniformis, Bacillus cereus, Niallia circulans (*formerly *Bacillus circulans), Bacillus thuringiensis, Lysinibacillus sphaericus,* and *Enterococcus faecium*; yeasts: *Candida parapsilosis* and *Scytalidium* cand*idum (*formerly *Geotrichum candidum)*
India, Nepal, and Pakistan	Jalebi	Wheat flour	Yeasts: *Saccharomyces uvarum (*formerly *Saccharomyces bayanus)*
India, and Pakistan	Rabadi	Buffalo or cow milk and cereals, pulses	Bacteria: *Bacillus* and *Micrococcus* spp.; molds: *Talaromyces acidilactici (*formerly *Penicillium acidilactici),*
India, and Sikkim	Bhattejaanr	Rice	Molds: *W. anomalus (anomala)* and *Rhizopus arrhizus*
India, Sri Lanka, Malaysia, Singapore	Dosa	Rice and black gram or other dehusked pulses	Bacteria: *L. mesenteroides, Streptococcus faecalis,*and *L. fermentum*; yeasts: *Tausonia pullulans (*formerly *Tricholsporon pullulans), Torulopsis* (now classified under *Candida* and *Cryptococcus),* and *Candida* spp.

	Idli	Rice, black gram or other dehusked pulses	Bacteria: *Bacillus amyloliquefaciens (*now recognized as *Bacillus velezensis), L. mesenteroides, L. plantarum* subsp. *plantarum, Lactiplantibacillus pentosus, L. lactis,* and *Lactiplantibacillus argentoratensis (*formerly *Lactobacillus plantarum* subsp. *argentoratensis*); yeasts: *Torulopsis*, *Candida,Tausonia pullulans, Pediococcus cerevisiae (*now named as *Pediococcus damnosus* or *Pediococcus acidilactici),*
Indonesia	Brem	Cassava, glutinous rice	Bacteria: *Acetobacter aceti*; yeasts: *S. cerevisiae;* molds: *Rhizopus delemar, Monilesaurus rouxii,* and *A. oryzae,*
	Brembali	Rice	Yeasts *and Molds: M. indicus* and *Candida* spp.
	Kecap	Soybean, wheat	Bacteria: *Tetragenococcus halophilus (*formerly *Pediococcus halophilus)*
	Oncom Hitam (Black Oncom) and Oncom Merah (Orange Oncom)	Peanut press cake, tapioca, soybean curd starter	Molds: *Chrysonilia sitophila (Neurospora sitophila)* and *Rhizopus microsporus* var. *oligosporus*

	Tape Ketan	Glutinous rice, Ragi	LAB; Yeasts: *S. cerevisiae, W. anomalus, C. glabrata, C. tropicalis, Hanseniaspora uvarum* and *Pichia kudriavzevii*; and molds: (from starter "ragi tape") *Amylomyces rouxii, Rhizopus oryzae, Mucor indicus, Aspergillus oryzae*.
	Tauco	Soybean	Bacteria: *L. delbrueckii*; yeasts and molds: *Hansenula* spp., *Zygosaccharomyces* spp.and *A. oryzae*
Japan	Miso	Soybean	Molds*: Aspergillus oryzae, Clavispora lustaniae,* and *Meyerozyma guilliermondii* Bacteria*: Bacillus velezensis, B. subtilis, Enterococcus durans, Rothia kristinae (*formerly *Kocuria kristinae), L. plantarum, Leuconstoc citreum, Leuconostoc pesudomesenteroids, Pediococcus acidolactici, Pediococcus pentosaceus, W. cibaria* and *W. confusa (*formerly *Lactobacillus coprophilus)*
	Natto	Soybean	Bacteria: *B. subtilis*
Kenya, Uganda, Tanzania	Uji	Maize, sorghum, millet, cassava flour	Bacteria: *L. mesenteriodes* and *L. plantarum*
Korea	Chongju	Rice	Yeasts: *S. cerevisiae*

	Chungkokjang (or jeonkukjang, cheonggukjang	Soybean	Bacteria: *B. subtilus*
	Doenjang	Soybean	Bacteria: *Bacillus, Tetragenococcus, Staphylococcus, Enterococcus, Pediococcus, Weissella, Hyphopichia, Debaryomyces,* and *Wickerhamomyces* spp.
	Gochujang	Soybean, red pepper	*Bacillus velezensis.*
	Ganjang	Soybean, meju, salt, water	Bacteria: *Cobetia, Bacillus* and *Chromohalobacter* spp.
Mexico	Pozol	Maize	LAB (mainly *L. plantarum*, *L. casei*, and *L. delbrueckii*)
Mongolia	Darassum	Millet	Bacteria (LAB)
Nepal, India	Maseura	Black gram	Bacteria: *L. fermentum, L. salivarius, Pediococcus pentosaceus, Enterococcus durans*; yeasts: *S. cerevisiae.*
	Kunu-zaki	Maize, sorghum, millet	Bacteria: *Lactobacillus, Pediococcus, Lactococcus, Leuconostoc* and *Bifidobacterium* spp.
Nigeria	Ogi	Maize, sorghum, millet	Bacteria: *L. plantarum*; yeasts and molds: *S. cerevisiae, Kregervanrija fluxuum (*formerly *Candida mycoderma), Corynebacterium, Aerobacter, Rhodotorula, Cephalosporium,*

			Fusarium, Aspergillus, and *Penicillium* spp.
	Okpehe	Seeds from Prosopis africana	Bacteria: *Bacillus* spp.
	Ugba	African oil bean (*Pentaclethra macrophylla*)	Bacteria: *Bacillus* spp.
Nigeria, Benin	Iru	Locust bean	Bacteria: *Staphylococcus* and *Bacillus* spp.
	Burukutu	Sorghum	Bacteria: *Leuconostoc* spp.; yeasts: *S. cerevisiae*
Nigeria, Ghana	Busaa	Maize	Bacteria: *Lactobacillus helveticus, L. salivarius, L. casei, L. brevis, L. plantarum, Lentilactobacillus buchneri (*formerly *Lactobacillus buchneri)*; yeasts and molds: *S. cerevisiae,* and *Penicillium damnosus*
Northern India	Dhokla	Rice or wheat and bengal gram	Bacteria: *L. mesenteroides, Enterococcus faecalis*; yeasts and molds: *candida* spp. and *Trichosporon pullulans (*recently known *as Guehomyces pullulans)*
Peru	Chicha	Maize	*Aspergillus* and *Penicillium* spp., yeasts and other bacteria *(*unspecified*)*
Sierra Leone	Kinda	Locust bean	*Bacillus* spp.
Southern Mexico	Atole	Maize	LAB (not specified)

South Africa	Mahewu	Maize meal and wheat flour	Bacteria: *L. plantarum, L. mesenteroides;* yeasts: *S. cerevisiae*
Sudan	Kawal	Leaves of legume (Cassia sp.)	Bacteria: *B. subtilis* and *Propionibacterium*
	Kisra	Sorghum	Bacteria: *Pediococcus pentosaceus, W. confusa, Lactobacillus cellobiosus (*recently as *Limosilactobacillus fermentum), L.s brevis, Lactobacillus amylovorus,* and *L. reuteri;* yeasts*: Sungouiella intermedia (*formerly *Candida intermedia), C. famata* and *S. cerevisiae*
	Merissa	Sorghum and millet	Yeasts: *Saccharomyces* spp.
Syria, Turkestan	Busa	Rice or millet	Bacteria: *Lactobacillus* spp.; yeasts: *Saccharomyces* spp.
Tanzania	Togwa	Cassava, maize, sorghum, millet	Bacteria: *L. plantarum, L. brevis,, L. fermentum, Pediococcus pentosaceus, W. confusa*; yeasts: *Issatchenkia orientalis (*now known as *Pichia kudriavzevii), S. cerevisiae, Candida pelliculosa* and *Candida tropicalis.*
Thailand	Khamak (Kao-mak)	Glutinous rice, Look-pang (starter)	Yeasts and molds: *Rhizopus, Mucor, Saccharomyces* and *Hansenula* spp.
	Thua nao	Soybean	Bacteria: *B. subtilis*

West Africa	Pito	Maize, sorghum	Bacteria: *Lactobacillus* spp.; yeasts: *Scytalidium candidum,* and *Candida* spp.
West, East and Central Africa	Ogiri/Ogili	Melon Seeds, castor oil seeds, pumpkin bean, sesame	Bacteria: *B. subtilis*
Western Ughanda	Bushera	Germinated sorghum and millet grains	Bacteria: *L. brevis, Lactobacillus, Lactococcus, Leuconostoc, Enterococcus,* and *Streptococcus* spp.
Zimbabwe	Chikokivana	Maize and millet	Yeasts: *S. cerevisiae*
	Doro	Finger millet malt	Yeasts: *S. cerevisiae*, *Issatchenkia occidentalis*, *Kluyveromyces marxianus*, *C. glabrata*, *Sporobolomyces holsaticus* and *Rhodotorula* sp p.

2.2.1.1 Sourdough bread

Production of sourdough bread is performed since ancient times [52]. The microorganisms that drive fermentation are harbored in starters known as "levain" or "mother doughs". Archaeologists unearthed the oldest known leavened bread, which dates back more than 5,000 years, during an excavation in Switzerland [53]. However, it was mentioned that it could be dated as far back as 10,000 years BC [52,54]. Egypt is believed to be the birthplace of sourdough bread, as people living there noticed an increase in volume when they left the mixture of water and wheat flour for some time and then, they deliberately added this mixture to a new dough, observing that the fermentation was thus enhanced. From Egypt, knowledge about sourdough bread was gradually spread throughout Greece and the Roman Empire. Egyptians also utilized beer foam in bread making [55], as beer foam contained

metabolically active microorganisms—primarily yeasts—that could initiate a new fermentation when added to dough.

In Greece, bread initially served as a food for home consumption in affluent households, with women handling its preparation. Later, evidence suggests the emergence of bakers, possibly organized in guilds, who produced bread for retail sale. The Greeks made remarkable improvements to the technology and baking equipment [56]. The adoption of sourdough in Greece is believed to have occurred around 800 BC [55]. Greek gastronomy boasted a rich variety of bread types, including both sweet and savory types, made with different cereals and prepared using various techniques. Bread held significant dietary importance during the Roman Empire, leading to the establishment of numerous public bakeries. Bakers themselves became public officials, employed by the State to produce substantial quantities of bread that were distributed freely among Roman citizens. The profession of being a baker became a family tradition, passed down from one generation to the next. Greeks were known to offer votive offerings consisting of flour, cereal grains, or toasted bread and cakes mixed with oil and wine. During rituals dedicated to Dionysus, the god of fertility, joy, and passion, large loaves of bread were presented by priestesses. The transition from sacrificial to curative bread occurred swiftly, as patients visiting temples dedicated to Asclepius would leave bread imbued with the healing power attributed to the god and would receive a portion of it upon leaving [53]. The history and social significance of sourdough finds rich documentation in Countries such as France, Italy, and Germany, where this traditional biotechnology remains widely practiced.

2.2.1.2 Miso and Natto

Miso and natto are original to Asia. Miso is a fermented soybean paste, whose roots trace back over a thousand years in Chinese cuisine. The earliest form of miso, known as "chiang" was initially made with fish, shellfish, and game. It dates back to before the Chau dynasty (722–481 BC). By the fourth century AD, chiang evolved into its soybean-based variant, miso, which became more common than chiang. Miso was then introduced to Japan between 540 and 552 AD, where it became a staple fermented food [57]. The Japanese adopted and adapted these techniques to suit their own tastes and ingredients. In Japan, it is called miso; in China, it remains known as chiang. In other Asian Countries it was named differently: tauco

in Indonesia, jang or doenjang in Korea, taochieo in Thailand, and tao-si in the Philippines [58].

Initially, miso was prepared by mashing soybeans and fermenting them with salt and other ingredients in wooden barrels or pots [59]. This early form of miso was known as "naan" or "isobe miso." However, it was the introduction of koji, a specific natural starter dominated by the filamentous fungus *A. oryzae*, that revolutionized the miso-making process. Koji mold had been used in China for centuries to ferment various foods [60], including soy sauce and sake. In Japan, the use of koji in miso production is attributed to the Zen Buddhist priest Kakushin, who is said to have brought the knowledge of koji from China in the 14th century [61]. The incorporation of koji into miso-making allowed for a more controlled and efficient fermentation. This innovation marked the beginning of what is now recognized as traditional Japanese miso. Over the centuries, miso has continued to evolve, with regional variations and diverse ingredients. The production of miso now primarily involves soybeans, rice, salt, and koji mold. Today, there are numerous types of miso, each with its unique flavor and character, depending on factors such as the type of koji mold used, the proportions of soybeans with rice and the duration of fermentation. Miso remains a cornerstone of Japanese cuisine, finding its usage into soups, marinades, sauces, and countless other dishes [62].

Natto, a distinctive Japanese fermented soybean dish, has a long history spanning centuries. It is believed to have originated in Japan around 2000 BC [63]. However, its exact origins are unclear, with various theories and historical accounts suggesting different developments. One theory posits that natto was accidentally discovered by Japanese villagers who left cooked soybeans in warm, humid conditions that favored fermentation by naturally contaminating microorganisms. Another theory suggests that Buddhist monks from China's Yunnan province introduced natto to Japan during the Nara period (710-794 AD) [64,65]. The most widely accepted theory is that natto was discovered accidentally around the 11th century during the Heian period (794-1185 AD). The fermentation of natto relies on the bacterium *B. subtilis*, that gives natto its characteristic slimy texture and strong aroma. Traditionally, natto was made by wrapping cooked soybeans in rice straw, which naturally contains the bacterium, and allowing them to ferment. Natto became a staple food in Japanese cuisine, especially during the Edo period (1603-1868 AD), and was commonly consumed in Tokyo [66]. Today, natto remains

a beloved and culturally significant food in Japan, often enjoyed as a breakfast dish over steamed rice with condiments like soy sauce, mustard, and green onions. Despite its strong flavor and slimy texture, natto's nutritional benefits, including high protein content and probiotic properties, have solidified its place in Japanese culinary heritage [67].

2.2.1.3 Tempeh

Tempeh's history is deeply rooted in Indonesian culture and dates back several centuries. This traditional fermented soybean product is believed to have originated in the island of Java [68], where it has been a dietary staple food for generations. The earliest recorded mention of tempeh dates to 1875. In the Serat Centini manuscript, which originates from Java in the early 19th century, the term "tempeh" appears, for instance, in dishes like "jae santen tempeh" (a dish featuring tempeh with coconut milk) and "kadhale tempe serundeng". This reference indicates that tempeh has been a well-established staple food among the Javanese people for centuries, particularly in regions like Yogyakarta and Surakarta [69]. Tempeh was accidentally "born" likely when cooked soybeans were left out in warm and humid conditions, allowing naturally occurring microorganisms to ferment the soybeans. Tempeh's production process involves a starter culture known as "ragi tempeh," which harbors different filamentous fungal species, among which *Rhizopus oligosporus* [70]. This fungus plays a crucial role in fermentation by binding the soybeans together into a cake-like form and producing enzymes that break down the soybean's proteins and carbohydrates. Tempeh's popularity gradually spread beyond Indonesia to other parts of Southeast Asia and eventually to the Western world, where it gained recognition for its nutritional value and usage in vegetarian and vegan diets. In the 20th century, tempeh was introduced to the United States, primarily by individuals interested in plant-based diets and alternative protein sources [71].

Today, tempeh is enjoyed worldwide and is celebrated for its nutty flavor, firm texture, and high protein content. It can be grilled, sautéed, stir-fried, or used in a variety of dishes, making it a beloved ingredient in both traditional Indonesian cuisine and modern global cuisine.

2.2.1.4 Fermented Tofu

It is commonly known as "stinky tofu/sofu" and dates back over a thousand years and has earned its place as a beloved and iconic dish in Chinese food culture. According to legend, tofu was discovered by a Chinese prince named Liu An around 164 BC while experimenting with the coagulation of soy milk. The earliest documented mention of tofu appears in the Han dynasty (206 BC-220 AD) writings. Another historical viewpoint mentioned the inception of tofu can be traced to the year 965 AD when it was initially documented by Tao Ku in China within the text "Anecdotes, Simple and Exotic" [72]. In the Ch'ing dynasty (1644-1912 AD), numerous historical accounts emerged regarding the widespread practice of producing fermented tofu using a molding process. Tofu's production involves soaking, grinding, and boiling soybeans to produce soy milk, which is then coagulated using either magnesium chloride or calcium sulfate to form curds, which are then pressed into solid blocks of tofu [73]. The process of making fermented tofu with this molding technique is detailed in the book "Shi Xian Hong Mi", which was written during the middle of the Kang-Xi period (1681-1706). Intriguingly, the author noted that red koji was used in its production [74]. Unlike traditional koji, which employs *A. oryzae* as the starter culture, red koji utilizes *Monascus purpureus*, responsible for its distinctive red pigmentation [75]. When applied in tofu fermentation, red koji imparts characteristic earthy, mildly sweet, and tangy flavor profiles, while its red pigments contribute to the unique coloration of the final product. Tofu's popularity spread from China to neighboring regions, notably reaching Japan and Korea. In Japan, tofu was introduced around the Nara period (710-794 AD). By the Edo period (1603-1868 AD), tofu had become a common food among the general population [68]. Similarly, it became known as "dubu" in Korea. The spread of tofu continued southward into Southeast Asia, where it became a staple in Countries such as Vietnam, Thailand, and Indonesia, adapting to local culinary traditions. Over centuries, tofu has evolved from a regional specialty to a global food, celebrated for its versatility and health benefits [76,77].

2.2.1.5 Douchi

It is a traditional fermented black soybean product, has its origins in China and has been produced for over 3000 years [78]. Its presence is evident from the discovery of douchi in Han Tomb No. 1 at the Mawangdui Han Tomb in South-

Central China, dating back to 165 BC However, the earliest documented reference to douchi can be found in Shi Ming (Explanations of Names), during the Han Dynasty period [79]. Douchi was developed as a method of preserving soybeans through fermentation, which not only extended their shelf life but also enhanced their flavor and nutritional value. The process involves fermenting soybeans, added with salt, with different microbial populations, such as *Aspergillus* spp., *Mucor* spp., and bacteria. Among these, the *Aspergillus*-type douchi is the most ancient and enjoys the broadest production; *Aspergillus* spp. imparts a distinctive umami taste and dark color to the beans [80]. While traditional methods of making douchi involved natural fermentation, modern production often incorporates commercial starters with controlled environments and standardized processes to meet commercial demand. This has led to variations in flavor and quality compared to artisanal douchi. Nowadays, it is available in many international markets and its influence extends beyond China's borders. It has been integrated into the cuisines of neighboring countries, including Thailand, where it is known as "tao jiow," and Vietnam, where it is called "tuong" [81].

2.2.1.6 Dosa and Idli

As mentioned by Tamang [57] , Dosa, also known as dosai, is a traditional South Indian dish made from a fermented mixture of rice and black gram. Its existence can be traced back to Tamil Nadu in the first century AD, as mentioned in Tamil Sangam literature. Idli, a round, steamed, spongy, savory staple food made from fermented rice and black lentils, was first documented in Kannada literature in 920 AD, in the work "Vaddaradhane" by Shivakotiacharya. Dosa is thought to have evolved from idli batter. It is believed that the leftover idli batter was spread thin on griddles and cooked to create dosa. Both idli and dosa are highly nutritious and easily digestible due to the fermentation [82], making them staples in South Indian cuisine.

2.2.2 Fermented Dairy Products

The historical roots of fermented dairy products are deeply embedded in diverse cultural traditions worldwide. Milk fermentation, with origins dating back over 10,000 years, likely emerged as a means to preserve dairy products prior to the advent of refrigeration. As noted by Leonardi *et al.* [83], the domestication of animals began in the Middle Euphrates valley around 11,000 BC for sheep and goats

and approximately 10,500 BC for cows. Archeozoological evidence from the mid-9th millennium BC illustrates shifts in the slaughtering practices of these animals [84], marking the onset of their domestication, with this transition occurring at varying times in different regions [85]. Furthermore, evidence of milk fermentation can be traced back to ancient Egypt's Ptolemaic era, as documented in depictions on stelae, hieroglyphics, and engravings. During this period, milk was stored in egg-shaped earthenware containers sealed with grass to protect it from insects and was typically consumed shortly after milking [86]. These historical developments led to diverse methods of fermenting milk, resulting in a wide array of fermented dairy products, such as kefir, cheese, and dahi. These products have played vital roles in regional cuisines, offering distinctive flavors and enhanced nutritional value.

2.2.2.1 Cheese

Cheese-making is believed to have originated around 7,000 years ago, likely as a way to store surplus milk and make it more palatable [87,88]. The earliest cheese production can be traced to Southwest Asia and parts of Europe by the late Neolithic, considering that an earlier origin is also possible [84]. Cheese evolved in the 'Fertile Crescent' between the Tigris and Euphratres rivers, in Iraq, some 8000 years ago during the "Agricultural Revolution", when certain plants and animals were domesticated [85]. Ancient evidence, including clay pots with small holes, suggests that early cheese-makers separated curds from whey by allowing fermentation to take place in these containers. The practice gradually spread across the Mediterranean, with the Egyptians, Greeks, and Romans all contributing to the development and refinement of cheese-making techniques. The Roman Empire played a crucial role in disseminating cheese throughout Europe [86], as soldiers and settlers carried the knowledge of cheese production with them. Afterwards, in medieval Europe monasteries became centers of cheese-making expertise. Cheese continued to evolve with the expansion of European exploration and colonization, as cheese-making practices adapted to local ingredients and conditions. In the New World, American colonists created their own cheeses, like cheddar, which gained international recognition. The industrial revolution in the 19th century made cheese production more efficient and cheese became accessible to a wider number of people. This era saw the birth of iconic cheese varieties like Swiss Emmental and Gouda. Today, cheese boasts an

astonishing variety of styles, flavors, and textures, with thousands different varieties produced worldwide. It holds a prominent place in global cuisine, from the rich and creamy Brie of France to the pungent blue cheeses of Roquefort, and from the crumbly feta of Greece to the sharp cheddars of England and the United States [84].

2.2.2.2 Fermented dairy beverages

The tradition of fermented dairy beverages that ultimately gave rise to today's yogurt extends back millennia, rooted in the domestication of milk-producing animals in the Near East and Central Asia. Archaeological and textual evidence indicates that sour milk-based food items were used by pastoral communities as early as the Neolithic period, when milk from goats, sheep, or cattle was unintentionally fermented during transport and storage in warm climates [89,90]. In this Neolithic context (10,000–15,000 years ago), the domestication of sheep, goats, and later cattle in the Middle East coincided with use of fermented milk as food [89, 91]. Early written references to fermented milk appear in Indo-Aryan Ayurvedic texts dating to approximately 6000 BCE and among Turkic nomads in Central Asia, where fermenting milk allowed people to benefit from nutrients in milk during transport [89]. The term "yogurt" itself derives from the Turkish verb *yoğurmak*, meaning "to thicken, curdle, or coagulate," underscoring the product's cultural origins in Turkish and surrounding societies [89,92]. With the development of microbiology in the late nineteenth and early twentieth centuries, the microbial basis of yogurt fermentation became better understood. In 1905, Stamen Grigorov isolated the bacterium now known as *Lactobacillus delbrueckii* subsp. *bulgaricus* from Bulgarian fermented milk, a discovery that paved the way for the industrial use of defined starter cultures [93].

The historical roots of kefir extend back to a time before written records. It is believed the original kefir grains were gifted by the prophet Muhammad to Orthodox Christians living in Georgia's Caucasus region [94], possibly in present-day Russia, Armenia, or Georgia. These grains, containing mixed microbial populations, passed down through generations, were used in homes to ferment either cow's or goat's milk, typically in leather sacks or oak barrels, and obtain kefir [94,95]. Much like the widespread popularity of yogurt, kefir gradually spread from the Georgian mountains and is now well-known in Eastern Europe and beyond [96,97,98].

Dahi holds great cultural and culinary significance in South Asia. Its history is closely intertwined with the broader history of dairy farming in the Indian subcontinent. The domestication of cows and buffaloes in ancient India provided a steady supply of milk, which, when fermented into dahi, could be stored and consumed over extended periods without spoiling. This made dahi an essential part of the diet, particularly in areas with hot and tropical climates. Throughout history, the art of dahi-making has been passed down through generations, with each region and community developing its unique methods and variations. Dahi is also a key component of religious rituals in Hinduism, often offered as prasad (sacred food) in temples. In recent years, dahi has gained international popularity for its probiotic properties and health benefits.

2.2.3 Fermented Horticultural Produce

Vegetable fermentations were described as early as the Song dynasty in the 10th to 13th centuries AD in China [54]. In Asia and the Far East, a wide range of fermented products were traditionally made using cabbage, turnips, radishes, and carrots. This technology gradually spread to Europe during the 16th century, where regional vegetables like European round cabbages were utilized as the primary raw materials. As European settlers migrated to the New World, they brought along cabbages and knowledge of sauerkraut production. Additionally, it is highly likely that other fermented vegetables, particularly pickles and olives, were produced and consumed in the Middle East since biblical times [8].

2.2.3.1 Kimchi

Kimchi is a Korean-style fermented cabbage that can be traced back to the primitive pottery age, where withered vegetables were naturally fermented in seawater [99]. According to historical records, kimchi was invented approximately 4,000 years ago, as documented in the Sikyung, a book of ancient Chinese poetry that was compiled during the Zhou dynasty (1046–256 BC). On the other hand, evidence from the Samkuksaki, a historical record of the three states of Korea (Goguryeo, Baekje, and Silla) that was written during the Goryeo dynasty (918–1392 AD), indicates that people were already consuming kimchi in the three states around 1,500 years ago. The ancestors discovered the fermentation of kimchi and realized that the presence of red pepper inhibits the growth of harmful microorganisms during fermentation, while promoting the growth of beneficial

bacteria, such as LAB [100]. It became closely tied to Korean identity and cuisine during this time. Its role extended beyond just a food preservation method, as it became a staple in Korean households and was incorporated into various dishes. Kimchi-making even became a communal activity, with families and neighbors coming together to prepare large batches for the winter months. In the modern era, kimchi has evolved further, with regional variations [101]. It has evolved beyond its traditional role as a side dish and is now featured as a primary ingredient in various Korean dishes. Examples include kimchi fried rice (kimchi bokkeumbap), kimchi noodles (kimchimari guksu), kimchi pancake and kimchi dumplings (kimchi mandu) [102].

2.2.4 Fermented beverages of vegetable and fruit origin

Fermented beverages have been crafted and consumed for millennia, with evidence dating back to ancient civilizations in Mesopotamia, China, and Egypt. These early brewers and fermenters discovered that allowing natural microorganisms, such as yeast and bacteria, to interact with ingredients like grains, fruits, and honey could transform them into flavorful liqueurs or alcoholic beverages. Over time, these discoveries gave rise to a rich tapestry of fermented beverages across the globe, from beer and wine to mead and various indigenous brews. These beverages have held cultural, religious, and social significance, and the techniques and recipes have been passed down through generations, shaping the diverse world of fermented drinks we enjoy today. Although beer and wine are significant examples of fermented beverages, they are not specifically addressed in this review. Readers interested in these products are referred to recent comprehensive reviews [103,104].

2.2.4.1 Kombucha

Kombucha is defined as 'a gelatinous mass of symbiotic bacteria (as *Acetobacter xylinum*) and yeasts (as of the genera *Brettanomyces* and *Saccharomyces*) grown to produce a fermented beverage held to confer health benefits' [105]. The exact origins of Kombucha in believed to be in Manchuria region, Northern China [106]. It was known during the Qin Dynasty, over 2,000 years ago for its detoxifying properties [107,108]. According to legend, Emperor Qin Shi Huang, the first emperor of unified China, was fascinated by the health benefits of tea and was keen to explore its potential further leading to the discovery of Kombucha [109]. In

traditional Chinese medicine it was believed that fermented teas possessed unique healing properties. Traditional Kombucha in China was made by fermenting sweetened tea with a unique Symbiotic Culture Of Bacteria and Yeast (SCOBY). The SCOBY is often referred to as the "tea fungus" or "tea mushroom" due to its appearance [110]. When the SCOBY is inoculated in sweetened tea, bacteria and yeasts start to grow on the surface, conferring a gelatinous aspect to kombucha. Over time, knowledge of Kombucha and its fermentation spread throughout China, and it became a staple in traditional Chinese medicine. In 414 AD, the physician Kombu introduced the tea fungus to Japan, using it to alleviate Emperor Inkyo's digestive diseases. Japanese warriors often carried the invigorating beverage with them onto the battlefield to stay refreshed and strong [111]. With the expansion of trade routes, kombucha, formerly known as "Mo-Gu," made its way into Russia under various names like Cainiigrib, Cainii kvass, Japonskigrib, Kambucha, and Jsakvasska. Subsequently, it entered other Eastern European regions, including Germany, where it became known as Heldenpilz and Kombuchaschwamm, around the early 20th century [108, 105]. In recent years, Kombucha has gained global popularity as a functional beverage, appreciated for its unique, tangy flavor [112].

2.2.4.2 Vinegar

Vinegar is an acidic solution produced through the transformation of ethanol and oxygen into acetic acid (also known as ethanoic acid) and water, by acetic acid bacteria (AAB) [113]. It is an essential ingredient in European, Asian, and various cuisines with a long history dating back to ancient times. Its name originates from the Old French term “vin agre”, signifying “sour wine”. For millennia, vinegar has been both produced and utilized, with archaeological evidence of its presence dating back to around 3000 BC in Egyptian artifacts [114]. The Babylonians were known to produce and trade vinegars infused with fruit, honey, and malt up until the 6th century B.C. Historical accounts, including references in the Old Testament and the writings of Hippocrates, suggest that vinegar was employed for medicinal purposes, particularly in treating and cleansing wounds. In 10th-century China, Sung Tse, regarded as a pioneer of forensic medicine, advocated the use of a mixture of sulfur and vinegar as a handwashing solution to prevent infection and maintain hygiene [115]. It has been an integral part of human diets serving various purposes as a preservative, condiment, aromatizer, and even a medicinal product

[116]. Traditionally, vinegar was primarily derived from cereals [117]. Typically, vinegar classification relies on the source material used in its production [118]. It is widely produced from a range of raw materials, such as fruits, grains, and vegetables. There are two main types: cider vinegar, derived from fruit juices, and regular vinegar, typically made from raw plant-based sources like grains, apples, grapes, or sugarcane [119]. Cider vinegar, in particular, is valued for its health-promoting properties and is believed have beneficial effects in controlling blood glucose indices and lipid profile in patients with type 2 diabetes with daily consumption [120]. The specific raw materials, fermentation methods, and microbial communities involved in production result in vinegars with distinct sensory attributes, including unique flavors and aromas.

2.2.5 Fermented seafood

Seafood fermentation can occur naturally (uncontrolled) or be a controlled process where starter cultures are introduced into the raw material, such as marine fish, shellfish, and crustaceans. Across different regions and cultures, the practice of fermenting seafood dates back thousands of years [121]. It was originated as a practical solution to preserve fish in the absence of refrigeration. Early societies discovered that allowing fish to naturally undergo microbial fermentation not only extended its shelf life but also transformed its texture and flavor. This process was especially vital in coastal and fishing communities, where access to fresh fish could be limited in some seasons of year. Archaeological evidence suggests that fish fermentation practices were already in use in ancient Egypt around 3000 BC, where salted and fermented fish were staples in the diet. Similarly, ancient Greeks and Romans employed fermentation techniques to produce garum, a fermented fish sauce highly prized for its savory flavor [122]. In Asia, particularly in Countries like China, Japan, Korea, and Southeast Asia, fish fermentation has been a cornerstone of culinary traditions for millennia. In Japan, for example, fermented fish products, like narezushi, date back to the Yayoi period (300 BC - 300 AD) [123]. Over time, various methods and techniques for fermenting fish were developed, often incorporating local ingredients and flavors.

2.2.5.1 Surströmming

Surströmming is a traditional Swedish fermented fish (herring), whose origin traced to the northern regions of Sweden where preserving fish was essential due

to long, harsh winters, causing scarce supply of fresh food. The name surströmming derives from "sur" (sour or acidic) and "strømming" (the local name for Baltic herring) [124]. This preservation method evolved from packing fresh herring in barrels with minimal salt to ferment. The characteristic pungent odor comes from the action of LAB and enzymes breaking down fish proteins and fats, producing compounds like butyric acid. Historically, the process became popular during periods of trade embargoes and limited salt supply in the 1520s and 1530s, as it allowed for the preservation of large catches using a low amount of salt. In some Swedish regions, surströmming was a staple food and even supplied as army rations in the 17th century [125].
In modern times, surströmming is served with thin bread, potatoes, onions, and sour cream. Fermentation may continue even after canning, causing cans to bulge due to gas production [126].

2.2.5.2 Feseekh

Feseekh is a traditional Egyptian salted-fermented fish (usual species are: Bouri (*Mugil cephlus*), Pebbly fish (*Alestes baremose*) or Tiger fish (*Hydrocynus* spp.) that dates back thousands of years [129]. Evidence of fermented fish consumption in the region dates back to at least the time of the Pharaohs. Fish, including mullet, held significant symbolism in ancient Egyptian culture, often associated with fertility and rebirth due to their connection with the Nile River, which was central to Egyptian life. Feseekh is typically made by layering fish with generous amounts of salt in a glass jar. Once layered, the jar is tightly sealed and left to ferment for approximately two months at room temperature, allowing naturally occurring LAB and enzymes to break down the proteins and preserve the fish [130]. Historically, feseekh was consumed during the springtime celebrations of Sham El Nessim, an Egyptian holiday that dates back to more than 4500 years ago [131,132]. This holiday marks the beginning of spring and the awakening of nature, and eating feseekh became a customary way to celebrate the season. Despite its health concerns associated with improper fermentation and the potential risk of foodborne illnesses, feseekh consumption has not declined in recent years. Today, feseekh remains a part of Egyptian culinary heritage with some modern adaptations and safety precautions.

2.2.6 Fermented meat

Meat fermentation was used by ancient civilizations of Northern Europe and Asia to use meat as source of nutrients also in periods (e.g. long winter) of scarcity of fresh meat. Evidence from ancient Egypt suggests that fermentation of meat was likely used early on [133].

The earliest records of sausage production trace back to the Sumerians, while written references to these techniques date back to around 600 BC in ancient Greece. Cato the Elder provided detailed descriptions of meat curing (involving, although unconsciously, fermentation) around 160 BC in his work "De Agri Cultura." [134]. The Romans possibly adopted these methods from the Gauls and Celts. Celtic roundhouses featured spaces for producing raw dry-cured ham and similar products. The Romans adapted these techniques, incorporating them into their own culinary traditions. Sausage-shaped fermented meats, for instance, are attributed to the Romans, who learned from the Lucanians (Southern Italy), originators of the Greek and Spanish names for dry-cured sausage ("loukaniko" and "longaniza"). The term "salami" likely originates from the Medieval Latin word "salumen" or possibly from the Cypriot city of Salamis. Fermented sausages were known as "brig" in the Roman Empire, a Celtic word meaning "hill," and possibly the origin of "Brianza" (as in "salame Brianza") [135]. The Romans laid the foundation for the wide variety of fermented meats in Italy and may have discovered the reddening effect of curing salt, attributed to nitrogen monoxide formation from nitrite, derived from bacterial reduction of nitrates in saltpeter. Their advancements in meat preservation and flavoring techniques contributed significantly to the development of cured and fermented meats in Europe and beyond. With the advent of modern food preservation techniques, such as refrigeration and vacuum sealing, the necessity of fermenting meat for preservation declined. However, fermented meat products remain popular in many culinary traditions around the world for their unique flavors and textures. Today, artisanal producers and chefs continue to explore and innovate with traditional fermentation techniques, creating a wide range of fermented meat products enjoyed by people globally.

2.3 Microbial mechanisms within natural starters affecting food quality: sourdough as a paradigm

The transformation of raw materials into fermented foods is underpinned by intricate processes whereby microorganisms, such as lactic acid bacteria (LAB) and yeasts, exert profound effects on food quality through acidification, proteolysis, and release of aroma compounds. Overall in those food fermentations driven by LAB—typically belonging to genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*—, those microorganisms rapidly metabolize available carbohydrates (with preference for mono- and di-saccharides) to lactic acid and, for those that display hetero-fermentative metabolism, also acetic acid or ethanol and carbon dioxide, thereby lowering pH, which influences texture and flavour, may increase the activity of some food enzymes (e.g., endogenous proteases) and selects for acid-tolerant microorganisms, such as yeasts [136, 137]. By reducing pH and generating organic acids, LAB limit growth of spoilage and pathogenic microbes [138]. At the same time, many LAB possess a more or less complete proteolytic system. It consists of extracellular or cell-wall associated proteinases that degrade large proteins into peptides; specific transport systems that bring peptides into the cell; and intracellular peptidases that further hydrolyzes them into smaller peptides and free amino acids [139,140]. This proteolytic activity contributes not only to microbial growth (providing essential amino acids also in substrates that are poor in free amino acids) but also to flavor and texture development. Indeed, peptides and free amino acids released upon proteolysis act as precursors for volatile organic compounds; in addition, the protein matrix of food is modified, resulting in its softening [141,142]. Furthermore, secondary pathways of LAB metabolism can liberate compounds such as diacetyl/acetoin, hydrogen peroxide, bacteriocins and exopolysaccharides, which affect sensory aspects, texture, aroma and shelf-life of food.

Sourdough represents a paradigmatic natural starter culture wherein interactions between populations of LAB and yeasts greatly impact on the quality of food items, namely leavened baked goods. Although different types of sourdough are available, the most intriguing interactions occur in type 1 (*alias* traditional) sourdough, produced and propagated through backslopping [23]. In food items fermented with natural starters, yeasts—commonly from genera such as *Saccharomyces* and *Kazachstania*— may modulate redox and sugar flows and carry out alcoholic

fermentations of carbohydrates, producing ethanol and carbon dioxide; in addition, they release a rich suite of flavour-active volatiles via amino acid catabolism (e.g., Ehrlich pathway), ester synthesis, and conversion of phenolic acids [143,144]. While carbon dioxide results in leavening (for baked goods) or fizz (for beverages), the generated flavor-active volatiles complement the acidifying and proteolytic functions of LAB. In food items fermented with natural starters dominated by LAB and yeasts, those populations may act in sequence or contemporarily.

2.4 The Shift towards Commercial Starters

Traditionally, food fermentation relied on spontaneous processes, initiated by naturally occurring microorganisms or was driven by (unconsciously) selected microorganisms inhabiting natural starter preparations, such as sourdough. However, in modern commercial production, many fermented foods have transitioned to fermentation driven by specific starter cultures for ensuring standardized quality and minimize the risk of microbiological hazards. Overall, while artisanal, small-scale processes often utilize natural starters, industrial-scale fermentation predominantly employs commercial starters [145]. The transition towards employing commercial starters in food fermentation processes can be traced back to the late 19th and early 20th centuries [33]. Since the initial advancements made by Emil Christian Hansen at Carlsberg Brewery, who later founded the Christian Hansen company specializing in dairy starter cultures, the starter cultures sector has expanded significantly into a thriving global industry [146]. During this period, several key factors, including population growth, and the demand for consistent and varied food products, drove the industrialization of food production [147]. Concurrently, significant advancements in scientific and technological understanding, particularly in microbiology, played a crucial role in the development of commercial starters. In 1857 Louis Pasteur found that lactic fermentation in food was due to bacteria. Milk acidification was found to be caused by a pure culture of *Bacterium lactis*, isolated by Joseph Lister in 1878 [148]. The capacity to obtain pure cultures was a turning point in the production and distribution of starter cultures. Noteworthy figures such as Joseph Harding played a seminal role in advocating for the adoption of standardized starter cultures, catalyzing significant transformations within industries such as dairies [149]. The

shift from reliance on natural starters to fermentation methods driven by commercial starters marked a paradigm shift in food production methodologies. Prior to the development of defined starter cultures, fermentation was commonly initiated using the back-slopping technique, i.e., inoculation of the raw material with a small quantity of a previous successfully fermented batch [150]. This practice ensured microbial continuity but posed challenges in standardization and microbial stability. In the 1890s, some food manufacturers began to replace back-slopping with powdered starter cultures [151]. Later, advances in biotechnology enabled the direct use of concentrated starter cultures in form of frozen or freeze-dried formulations [152]. Furthermore, Hansen's pioneering work at the Carlsberg Laboratory in Denmark, which led to the isolation and cultivation of pure yeast strains suitable for brewing, epitomized the pivotal role played by scientific innovation in the shift from natural to commercial starters [153]. The adoption of commercial starters accelerated significantly in the mid-20th century, particularly as food production became more industrialized and mass-produced.

Nowadays, many microorganisms are component of commercial starter preparations, marketed to produce various fermented products (**Table 2**). For example, in cheese-making, commercial starters frequently include LAB like *L. lactis* subsp. *lactis* and *Streptococcus salivarius* subsp. *thermophilus*, while yogurt production relies on strains of *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* Similarly, fermented vegetables and beverages like sauerkraut, kimchi, beer, wine, and kombucha have shifted to fermentation driven by commercial starters. Leavened baked goods can be produced using either commercial baker's yeast, a preparation mostly composed of *S. cerevisiae* biomass, characterized by rapid and consistent leavening, or sourdough [154] (**Table 2**). Although, many sourdoughs are produced and propagated at artisanal level and so their microbial composition is undefined, nowadays sourdough starters are also commercially available for professional and home use [23].

Table 2. Examples of microbial species included in commercial starter preparations used to produce fermented products

Microbial Group	Application	Examples	References

LAB and staphylococci	Dairy Fermentation, Dairy-based Beverages, Kefir	*L. acidophilus*, *L. casei*, *L.plantarum*, *L. lactis*, *S.s cremoris*, *L. lactis* subsp. *lactis biovar diacetylact*, and *L. mesenteroides* subsp. *Cremoris*	[155,156,157,158]
	Cheese Making	*L. lactis* subsp. *lactis*, *S. cremoris* and *S. thermophilus*	[159,160,161]
	Dry Sausages, Cured Meats	*Latilactobacillus sakei*, *L. pentosus*, *L.* casei, *L. curvatus*, *L. plantarum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Kocuria varians* (Formerly known as *Micrococcus varians*), *Staphylococcus carnosus*, *Staphylococcus equorum* and *Staphylococcus xylosus*	[162,163,164,165,166]
	Sauerkraut, Kimchi, Pickles	*L. plantarum*, *L.mesenteroides*, *Pediococcus pentosaceus*, and *L. sakei*	[167,168]

	Plant-based Beverages, Kombucha	*L. plantarum*, *Liquorilactobacillus nagelii*, and *Oenococcus oeni*	[169,170]
	Fermented Vegetable Juices	*L. plantarum*, *L. brevis*, and *L. sakei*	[171,172]
	Wine making	*L. brevis*	[173,174]
Cutibacterium (formerly known as Propionibacterium)	Cheese Making	*Propionibacterium freudenreichii*, and *Acidipropionibacterium acidipropionici*	[175,176,177]
Bifidobacteria	Various Dairy and Non-Dairy Products (Health promoting role)	*Bifidobacterium animalis*, and *Bifidobacterium bifidum*	[178]
Yeasts	Bread Making, pastry, sourdough starters	*S. cerevisiae* (baker's yeast)	[179]
	Brewing (Beer, Wine)	*S. cerevisiae*, *S. pastorianus,* and *S. uvarum*	[180]
	Cheese making	*C. famata*, and *Yarrowia lipolytica*	[181]
	Fermented sausages	*C. famata,* and *Y. lipolytica*	[182]
	Soy Sauce, Miso Beer	*Brettanomyces bruxellensis*	[183]

	(Sour/Farmhouse Ales)		
Molds	Cheese Making	*Penicillium roqueforti*, and *Penicillium camemberti* (Known as *Penicillium candidum*)	[184,185]
	Soy Sauce, Miso	*Aspergillus oryzae*	[186]

In response to increasing consumer awareness and demand for organic and sustainably produced foods, the food industry developed organic starter cultures produced in accordance with organic farming and production standards. Today, organic starters represent a significant niche within the fermentation landscape, offering consumers a choice aligned with their preferences for organic products. The Italian market offers organic starters for fermented foods, such as leavened baked goods (e.g., sourdough), dairy products (e.g., kefir grains), and fermented vegetables (**Figure 1**). Some of these starters (e.g., "Kefirko organic kombucha starter") do not report the microbial species, resulting in a hybrid category of "commercial starters with undefined composition".

The global starter cultures market is projected to reach a value of 1.3 billion USD by 2025 [187]. Key contributing countries include China, the United States, South Korea, and Japan. According to the same analysis, the market is expected to grow at a compound annual growth rate (CAGR) of 6.3%, reaching approximately 2.5 billion USD by 2035. Furthermore, bacteria-based starter cultures are anticipated to dominate the market, accounting for 68% of the total revenue share in 2025. These bacterial cultures are primarily composed of LAB, notably species within the genera *Lactobacillus* and *Streptococcus*.

(a) **(b)** **(c)** **(d)** **(e)**

Figure 1. Organic commercial starters in the Italian market; a) Bionova organic yogurt starter (*S. termophilus* and *L. bulgaricus*); b) Bionova organic kefir starter (Genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Saccharomyces*); c) Bakerdale organic sourdough starter (microorganisms not specified); d) Yalacta organic vegetable starter (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum*, *L. acidophilus*, and *Bifidobacterium lactis*); e) Kefirko organic kombucha starter (microorganisms not specified)

2.5 Starter production: A Comparative Analysis of Natural and Commercial Starters

2.5.1 Natural starters

Natural starters, also known as wild or indigenous starters, harbor microorganisms recruited from various sources (environment, raw materials, processing tools) [188]. Their microbial composition is a reflection of the unique ecological niches which they originate from, encompassing a diverse array of bacteria, yeasts, and molds. The composition of natural starters is influenced by factors such as geographical location, climate, raw materials, and processing techniques, resulting in a vast microbial diversity.

The production of natural starters originates from the traditional method of back-slopping. This process intrinsically exerts selective pressures towards microbial community, caused for instance by heat treatment (e.g., natural whey milk cultures obtained after cooking cheese curd at 45-55 °C), specific incubation temperatures, and low pH. Unlike more controlled methods, no special precautions are taken to prevent environmental contamination of natural starters, and there is minimal control during their reproduction (also referred to as "propagation"). Consequently, natural starters consist of undefined mixtures of multiple microbial strains and/or species and their microbial community often shows time-dependent and almost unpredictable variations (sometimes even from

one month to the following month) [189]. Acidification process, driven by naturally occurring bacteria (such as LAB), is often an important driver of the microbial community of natural starters [190]. Acidifying bacteria are predominantly found within the phylum Firmicutes, which includes key LAB genera such as *Lactobacillus*, *Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Enterococcus, Tetragenococcus, Aerococcus, Carnobacterium, Weissella, Alloiococcus, Symbiobacterium*, and *Vagococcus* [191]. In addition, some acid-producing bacteria belong to the phylum *Actinobacteria*, notably *Bifidobacterium* and *Atopobium*, which, while not classified as LAB, also contribute to fermentation through carbohydrate metabolism and organic acid production. Sourdough used as a natural starter for bread making harbors LAB (typically rod-shaped and originating from flour and bakery environment) that lower the pH of a wheat/rye-based dough to values ranging from 3.7 to 4.8 [192]. As a result of the inherent variability of natural starters, different flavor profiles and sensory attributes could make them prized assets in artisanal food production [193].

The unique and complex flavor profiles of fermented food obtained through natural starters results from the intricate metabolic activities of the diverse microbial populations harbored in the starter preparation (**Table 3**). These activities produce a myriad of compounds during fermentation, including organic acids, alcohols, esters, and aromatic compounds, all of which contribute to the sensory characteristics of the final product [194]. Social, economic, and environmental trends are leading to an increasing reliance on fermentations driven by natural starters, particularly within the realm of traditional, regional, and artisanal foods, including those with either Protected Designation of Origin (PDO) or the Protected Geographical Indication (PGI). This shift also extends to organic and biodynamic production methods [195]. Finally, the high bacterial diversity characterizing many natural starters protects against fermentation failure caused by bacteriophages (**Table 3**).

Table 3: Features distinguishing Natural from Commercial Starters

Feature	**Natural Starters**	**Commercial Starters**
Microbial Diversity	High	Moderate to low
Consistency	Variable	High
Production Scale	Small-scale	From Small- to Large-scale

Production Environment	In the same place where they are used	Controlled Facilities/ Laboratories
Certification	Not Typically Certified	Can be Certified (e.g., Organic)
Easiness of use	Depending on the Starter	Yes
Most common Application	Traditional/ Artisanal Foods	Industrial Food Production
Risk of pathogenic or spoilage microorganisms	From low to moderate	Tending to zero
Fermentation failure to due bacteriophages	Low risk	Moderate risk
Customization	Theoretically infinite (provided that operators with high education adapt the starter)	Relatively High

However, natural starters display some issues. One of those consists in that, in most cases, natural starters are produced and propagated in the same place (e.g., bakery, dairy farm) where they are used. This implies the presence of some well-educated (especially for traditional sourdough) professionals have to care for the correct propagation and performances of the natural starter. In addition, even when in presence of well-educated professionals, some variables cannot be timely controlled (e.g. nutrients and microorganisms in the ingredients used for propagation). This implies both a higher risk of undesired microorganisms, detrimental to fermented food safety and shelf-life, as well as variability of consistency. The latter aspect is particularly critical in the context of large-scale commercial operations, because the inherent variability of natural starters can make it difficult to achieve consistent product quality and time-reproducible fermentation outcomes (**Table 3**). The variability of consistency inherent to natural starter preparations is well known by those bakers who use traditional sourdough for their leavening baked goods. **Figure 2** exemplifies different

outcomes of reproducibility of food quality, due to use of natural starter vs. commercial starter. In most cases people appreciate butter aroma, related to diacetyl, in leavened baked goods. If the microbial community of a type-I sourdough (the natural starter "par excellence" in the field of baked goods) is dominated by *S. cerevisiae* and two LAB species, one of which is a diacetyl producer (such as some strains of L. lactis), the resulting final product will have a buttery note. After several back-slopping steps, by which this starter is propagated at bakery, using water and flour (intrinsically not sterile), a change may occur in the dominant populations: another species (e.g., *L. plantarum*), possibly originating from the flour used for propagation, may replace the diacetyl producer, resulting in a leavened baked good perceived as less pleasant than before. On the opposite, if the baker uses commercial cultures of *S. cerevisiae* and diacetyl producing *L. lactis*, the resulting baked goods will be characterized, along time, by a buttery note, as long as the baker will use those commercial starters (**Figure 2**).

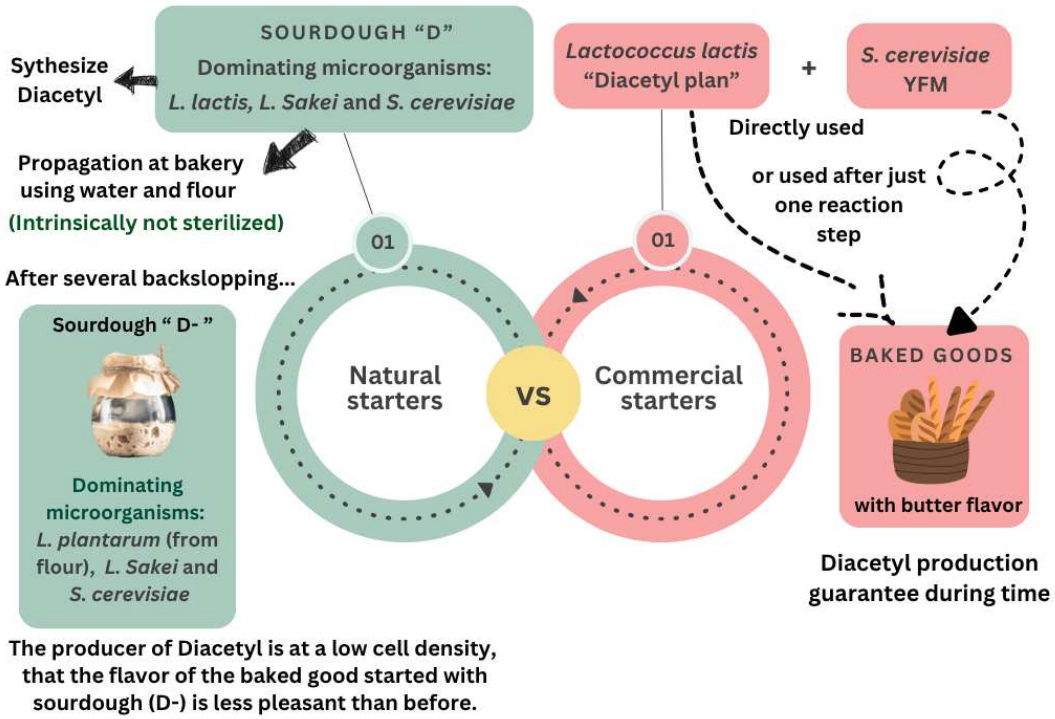

Figure 2. Example of the differences between a natural and a commercial starter, in terms of consistency and change of food quality attributed to the change of microbial community of sourdough

2.5.2 Commercial starters

In contrast to natural starters, commercial starters are intentionally selected microbial strains, produced under controlled conditions and designed to provide consistent and predictable fermentation outcomes. The latter trait makes them invaluable tools in large-scale food production [165]. The production of commercial starters involves a series of carefully orchestrated steps, from strain isolation and selection to propagation, formulation, and quality control. The journey of a commercial starter begins in the laboratory, where strains are isolated, identified, and selected based on their fermentative capabilities, flavor-producing abilities, and stress tolerance [196]. Selected microbial strains intended for use as starter cultures in the European Union must undergo thorough evaluation and be included on the Qualified Presumption of Safety (QPS) list maintained by the European Food Safety Authority (EFSA). The QPS framework serves as a pre-market safety assessment tool for biological agents used in food and feed, streamlining regulatory approval while ensuring a high safety standard. Assessments are conducted primarily at the species level, based on four main criteria: defined taxonomic identity, a comprehensive body of knowledge supporting safe use (familiarity), intended use, and absence of pathogenicity or harmful metabolite production, including biogenic amines and toxins [197,198]. The presence of acquired antimicrobial resistance genes is also critically evaluated. The QPS list, updated annually, includes a range of LAB (e.g., *Lactobacillus*, and *Lactococcus*), certain *Bacillus* species with specific safety qualifications, and yeasts such as *S. cerevisiae*, though some subtypes require caution in vulnerable populations. Filamentous fungi remain excluded due to ongoing taxonomic revisions and limited toxicological data. Only microbial strains meeting these stringent criteria are recommended as food ingredients, thereby ensuring consumer safety and consistency in fermentation processes. Once selected, the strain is propagated (cultivated) under sterile conditions in controlled tanks filled with nutrient-rich media optimized for obtaining the highest yield in biomass. Large scale production of a strain that will be part of a commercial starter is obtained using bioreactors equipped with precise control systems for temperature, pH, and oxygen levels [199]. The microbial biomass obtained after cultivation is harvested, concentrated, and formulated into various forms, such as freeze-dried powders, liquid cultures, or granules, which are easy to handle and store. The

facilities where commercial starter cultures are produced adhere to Good Manufacturing Practices (GMP) and other regulatory standards to ensure the safety, purity, and efficacy of the starter cultures. This ensures that the production of commercial starters adheres to the Hazard Analysis and Critical Control Point (HACCP) system. Quality assurance procedures, including microbial testing, potency assays, and stability studies, are conducted to verify the performance and shelf-life of the commercial starters before they are released to the market [200]. A given commercial starter may harbor one or more microbial species, each one individually propagated and then combined to give a defined single (one species) or mixed (two or more species/strains) starter culture.

Besides being particularly precious in large-scale food process plants, defined starter cultures enhance the predictability of small-scale fermentation processes, improve the aroma of traditional products, and increase product safety [146]. Differently from many natural starters, the commercial ones are produced by industries, so that the food manufacturer does not have to care about starter propagation. In addition, most of them can be used by operators with a low to moderate level of education. Their inclusion prevents interruptions in fermentation and the formation of unwanted metabolites [189] . By using specific strains of bacteria and fungi, commercial starters ensure a uniform outcome in terms of flavor and texture. Additionally, they minimize the risk of contamination by undesirable microorganisms, significantly enhancing food safety. Moreover, commercial starters typically accelerate the fermentation. For instance, bread leavening with commercial starters consisting of *S. cerevisiae* are much faster (1-2 h) compared to traditional sourdough (4-8 h). Notwithstanding the level of customization of commercial starter cannot be as high as that of natural starters, commercial starters can be tailored to specific applications, to meet the requirements of different food products and processing conditions.

Commercial starter cultures also display some disadvantages. One of them consists in that in most products with PDO or PGI designation their use is not envisaged in the production disciplinary, because they would cause a loss of typicity and traditionality. Furthermore, those commercial starters based on one or few bacterial strains are inherently exposed to infections of bacteriophages, which would cause a rapid loss of metabolic activity, thus impeding the onset of fermentation and, consequently, the obtainment of the fermented food/beverage.

2.6 Fermented food and beverages from organic agriculture: how to respect Regulation about organic food using microbial starters

The production of organic starters involves cultivating microorganisms under strict conditions to meet organic certification standards. Organic starters are essential in various fermentation processes, including dairy products like organic yogurt and cheese, as well as sourdough bread and fermented vegetables. EC Regulation 834/2007 specifies certain products and substances permitted for use in the production of processed organic foods. Some of these substances, like potato starch and vegetable oils, must be of organic origin. However, other substances can derive from conventional farming, provided their use does not exceed 5% of the total production. The Regulation specifies that "For the production of organic yeast, only organically produced substrates shall be used. Additionally, organic yeast must not be included in organic food or feed alongside non-organic yeast. Despite this, there are no explicit standards or requirements for the production of organic starters, with certification criteria currently only encompassing yeast.

For other microorganisms usable as starters, the future key aspects of organic starters production may include adhering to organic certification requirements, which mandate the use of organic ingredients throughout the propagation. This may involve employing organic substrates/media for microbial growth, such as organic milk for dairy starters or organic flour for sourdough starters. The selection of specific microbial strains is critical, focusing on those naturally occurring or isolated from organic sources to ensure they thrive in organic environments and contribute to desired fermentation characteristics. Organic starter production has the potential to emphasize environmental sustainability by reducing synthetic inputs and supporting ecological balance within production systems, aligning with organic farming principles to minimize the ecological footprint of fermentation. Overall, organic starter production aims to meet consumer demand for high-quality, organic-certified foods.

Within the context of organic agriculture, the production of fermented products holds particular significance. However, ensuring compliance with regulatory standards related to organic food production presents unique challenges, particularly regarding the use of microbial starter cultures. At the heart of organic fermentation lies a delicate balance between tradition and regulation, where the artistry of fermentation meets the rigor of certification requirements. Organic

agriculture is founded on management practices that prioritize the preservation, restoration, maintenance, and enhancement of ecological balance. It adheres to sustainability principles, thereby contributing to environmental, economic, and social sustainability goals [201]. These goals mandate strict adherence to principles such as soil health, biodiversity conservation, and the exclusion of synthetic inputs, including pesticides, fertilizers, and genetically modified organisms (GMOs). The principles extend to all stages of food production and the use of microbial starters in organic fermentation must also align with them. As such, the sourcing and cultivation of microbial starters must adhere to organic certification standards, ensuring that they are derived from organic-certified sources and cultivated using organic-compliant practices. Microbial starters used in organic fermentation must of course meet stringent criteria for purity, safety, functionality, as well as compatibility with organic substrates and processing conditions.

While organic fermentation prioritizes natural processes and microbial diversity, ensuring microbial safety within these systems remains a regulatory and technological challenge. As mentioned above, organic certification frameworks, such as EU Regulation 2018/848, only mandate that all microbial cultures used in organic food processing be non-GMO, produced without synthetic growth media, and free from antibiotics or chemical preservatives. However, these regulations primarily emphasize input integrity rather than detailed microbiological risk assessment. As a result, when natural or undefined starters are used, variability in microbial composition can increase the risk of contamination with pathogenic species. To mitigate that risk, EFSA recommends the use of QPS species and periodic verification of microbial identity, purity, and absence of virulence or antimicrobial resistance genes [198]. In practice, producers of organic fermented foods are encouraged to implement GMP, HACCP systems, and whole-genome-based microbial traceability to ensure both compliance and consumer safety. Integrating these safety evaluations into organic certification schemes would help bridge the current gap between traditional artisanal practices and modern food safety requirements, thereby strengthening the integrity and market credibility of organic fermented products.

2.7 Embracing Nature: Harnessing the Potential of Natural Starters in Organic Food Processing

Organic food processing is increasingly focusing on the use of natural starters. Alike commercial starters, they may benefit nutrition and health, for instance by increasing the bioavailability of vitamins and minerals; additionally, natural starters may include microorganisms with probiotic traits [202].
Natural starters, as well as commercial one, can play a crucial role in preserving organic foods and ensuring their safety, without synthetic preservatives, which aligns with the principles of organic food processing [203]. However, differently from commercial starters, the use of natural starters for organic food production targets one of the broader goals of organic agriculture, namely promotion of biodiversity, because, as discussed above, natural starters are characterized by higher microbial diversity than commercial starters. Finally, natural starters, unlike commercial starters, do not require specialized laboratory equipment. Although no generalization can be made, it may be hypothesized that a fermented food obtained by using a natural starter produced at the same place could be more environmentally sustainable than its counterpart, produced with a commercial starter.

2.8 Challenges and Future Perspectives

2.8.1 Overcoming Hurdles: Identifying Limitations and Addressing Challenges in the Use of Natural Starters in Organic Food Processing

Despite the numerous advantages of natural starters, several challenges must be addressed to fully realize their potential in organic food processing. One significant challenge is the variability and unpredictability inherent in natural starter cultures [204,205]. It may be faced with application of standardized protocols for their production and with selection of microbial strains that are robust, i.e. capable of controlling over contaminant microorganisms that may become dominant in natural starter cultures, thus causing deviations from the standard quality of a given fermented food/beverage. In practice, robust lactic acid bacteria (LAB) and yeast strains in starter cultures can exert competitive exclusion and secrete antimicrobial compounds, helping to inhibit spoilage organisms and pathogens [206]. Among contaminating microorganisms, some can increase the risk of either foodborne disease or spoilage before the end of shelf-life. This challenge must be faced through selection of bacteriocinogenic microorganisms

as components of natural starters and through application of stringent hygiene practices [207].

In addition to technical challenges, there are some logistical and economic issues that must be considered. Cultivation and maintenance of natural starter cultures may require adequate infrastructure and expertise, which may pose barriers to entry for small-scale producers. Furthermore, the scalability of natural starters production may be limited compared to commercial counterparts, which are produced on an industrial scale using automated processes and standardized protocols [208]. Finding ways to overcome these barriers will be crucial for expanding their adoption in organic food processing. Nevertheless, addressing these challenges requires collaboration between researchers, producers, and regulatory authorities to develop standardized protocols, quality control measures, and safety guidelines for the use of natural starters in organic food processing. Investing in research and innovation to improve our understanding of natural starters application and how to optimize their performance will be essential for overcoming hurdles and unlocking their full potential in organic food processing.

2.8.2 Innovating for the Future: Advancements and Future

Recent innovations in starter culture production leverage advanced screening techniques and omics technologies to enhance fermentation. High-throughput screening, along with genomics, proteomics, and metabolomics, provides deep insights into microbial strains, enabling the selection of those with enhanced fermentation performance, flavor development, and probiotic traits [209,210,211,212,213,214]. Synthetic biology is emerging as a promising field for future starter culture production. It enables precise manipulation of microbial genomes, allowing the engineering of strains with tailored traits and functionalities. These technologies facilitate the identification of specific genes or pathways responsible for beneficial traits, streamlining the selection process. CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9) and other genetic engineering tools have revolutionized starter culture development by allowing precise editing of microbial genomes. CRISPR-Cas9 has also become a standard practice in many laboratories, including those that perform manipulations with yeast [215]. This technique leverages the natural "adaptive immunity" mechanism observed in bacteria and archaea to create a tool capable of precise genome editing in any

organism [216]. Consequently, this enables the enhancement or introduction of traits such as stress tolerance, improved metabolic pathways, and bacteriophage resistance, ensuring consistent and reliable fermentation outcomes.

Under current EU law, most designer microbes intended for food fermentations are treated as GMOs, imposing stringent approval and labeling requirements. In 2018 the Court of Justice of the EU confirmed that organisms modified by new techniques (e.g. CRISPR) qualify as GMOs under Directive 2001/18/EC [217,218]. As a result, any food containing or produced *from* an engineered microorganism falls under Regulation (EC) No. 1829/2003 and requires full EFSA safety assessment and pre-market authorization [219,220]. Only if an engineered microbe is used as a *processing aid* (i.e. completely removed from the final product with no DNA or cells detectable) can the food be considered "produced with" a GMO and thus escape GMO labeling [221,222]. In that case no separate GMO authorization is needed, though applicants must still demonstrate the absence of viable modified cells or DNA in the finished food. In practice, this means that purified additives (enzymes, vitamins, flavorings, etc.) made by genetically modified microorganism (GMM) fermentation can reach the market without GMO labeling, but live or DNA-containing engineered cultures in foods cannot. For example, the EU now requires that any food enzyme, flavoring or additive derived from a GMO be authorized through EFSA's evaluation. Together with lengthy approval timelines, these rules make new engineered starter cultures hard to deploy under today's framework: regulatory agencies in the EU (as elsewhere) investigate both the modified microbe and its product for safety.

On the other hand, under current EU organic regulations, the use of genetically modified organisms (GMOs), including genetically modified microorganisms (GMMs) or organisms altered by new genomic techniques, is strictly prohibited. EU Regulation 2018/848 (which replaced earlier rules) expressly bans the use of "GMOs and products produced from or by GMOs" in organic production. This prohibition covers not only plants but also microorganisms used in any part of the organic food chain (e.g. processing aids, enzymes, starter cultures) since microorganisms fall under the category of "microorganism or animal material" derived from or by GMOs. Organic rules also require that for processed organic foods, only a limited set of additives, processing aids, enzymes, microorganisms etc., are permitted—but only if they are authorized under organic rules, which

excludes those derived from or through GMOs. Thus even if a microorganism engineered via synthetic biology or CRISPR offers desirable traits (flavor, stress tolerance, etc.), its engineered status disqualifies it for use in organic-certified products. Until the legislation is revised or new guidance is issued, the feasibility of using gene-edited microbes in food remains very limited under EU law.

There are notably some sustainable practices that are being integrated into starter culture production. The use of agro-industrial byproducts as substrates is gaining popularity, reducing production costs and minimizing waste. For instance, whey, a byproduct of cheese production, can be used to cultivate LAB, contributing to a circular economy [223]. Additionally, innovations in starter cultures for plant-based food items cater to the increasing demand for vegan products. These cultures are developed to effectively ferment plant-based substrates, producing high-quality plant-based cheeses, yogurts, and other fermented food items with enhanced texture, flavor, and nutritional profiles. They could also potentially exhibit enhanced growth and metabolism in plant-based milk products, in comparison to dairy strains, due to their broader metabolic capacities [224].

Automation in fermentation has led to the development of efficient and scalable starter culture production systems. Automated systems can monitor and control environmental conditions such as temperature, pH, and oxygen levels in real-time, optimizing growth kinetics and biomass yields. Digital twins and predictive modeling further enhance efficiency by simulating fermentation and allowing producers to adjust parameters for optimal outcomes, reducing the need for trial-and-error [225]. Lastly, advancements in downstream processing technologies, such as freeze-drying, spray drying, and encapsulation, have improved the stability, shelf life, and functionality of commercial starter cultures. These techniques allow for the production of starter cultures in convenient, user-friendly formats that are easy to store, transport, and use. Formulation and delivery system innovations, such as microencapsulation and protective coatings, further enhance the viability and survivability of starter cultures during storage.

Innovating for the future involves leveraging advancements in technology, science, and sustainability to enhance the utilization of natural starters in organic food processing. Future advancements may include the development of novel techniques for microbial isolation, characterization, and selection to identify microbial strains with desirable fermentation properties and functional attributes.

Additionally, advances in fermentation science and biotechnology may enable the engineering of custom-tailored starter cultures with enhanced functionalities and performance characteristics.

Moreover, advancements in food safety technologies and quality assurance systems can help mitigate food safety risks associated with natural starter cultures, ensuring stability of fermented products. Implementing blockchain technology and traceability systems can enhance transparency and accountability throughout the supply chain, enabling consumers to make informed choices about the products they purchase.

2.9 Conclusions

The rich microbial diversity of natural starter cultures offers a major advantage for fermented foods and beverages, as microbial interactions during fermentation generate products with distinct—and often unique—sensory traits. This is particularly relevant for organic fermented foods, whose consumers typically value not only more sustainable production systems, but also sensory profiles clearly differentiated from those of large-scale, industrial products. However, the high biodiversity of natural starters can also lead to time-dependent variations in sensory quality. By contrast, commercial starters facilitate process control and ensure consistent product quality over time, with minimal risk of contamination by undesired microorganisms. Yet they are usually unsuitable for traditional or typical products because they tend to "flatten" sensory profiles, undermining the features that define product typicity.

For producers of fermented foods and beverages, two complementary strategies can help maximize the benefits of natural starters while limiting their inherent risks: (i) implementing control measures and rapid assays during starter propagation to verify the absence of undesired microorganisms, and (ii) improving the training and education of operators responsible for managing natural starters. Enhanced knowledge in this area not only increases awareness of practical measures to reduce variability but also strengthens the ability of operators to adapt starter cultures to evolving consumer expectations.

For starter-culture manufacturers, we recommend collaborating with research institutions to develop natural starter cultures that combine high viability with robustness. Once available on the market, such cultures would provide producers

with an alternative to self-propagated natural starters and would allow the use of commercial preparations capable of preserving the distinctive sensory qualities of traditional and typical fermented foods and beverages. These recommendations apply to both conventional and organic production systems, but they are particularly relevant in the organic sector, where biodiversity—and especially microbial diversity—plays a central role.

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Development of Organic Sourdough Bread with Paste from Germinated Seeds

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Abstract

Chapter 3 investigates the development of an innovative organic sourdough bread formulated with pastes obtained from germinated seeds, with the aim of enhancing nutritional value, sensory quality, and technological performance through fermentation. Organic sourdoughs were prepared using different water sources (tap water and grape water) and subsequently applied in bread-making, while germinated lentil and bread wheat seeds were selected as ingredients based on their technological and nutritional potential. The study evaluated microbial dynamics, bread physicochemical properties, sensory attributes, nutritional composition, and microbiological safety. Results showed that sourdough fermentation ensured stable populations of lactic acid bacteria and yeasts and produced breads free from total coliforms, confirming the safety of the process. The inclusion of germinated seed pastes influenced specific sensory attributes, color parameters, and nutritional composition without negatively affecting loaf volume or overall acceptability. Additionally, drying and subsequent revitalization of sourdoughs demonstrated that microbial functionality could be preserved, offering a practical solution for sourdough management in small-scale organic bakeries. Overall, this chapter shows that fermentation can be effectively exploited to develop value-added organic bakery products using simple, low-input technologies compatible with organic regulations and the operational needs of small and medium-sized enterprises.

3.1 Introduction

The number of people who pay special attention to the overall quality of food, especially staple food items, is continuously on the rise [1]. Consumer demand for

local organic food, in particular, is also rising due to its perceived health and sustainability benefits. So making use of new strategies such as foods with 'marketed patrimonies' for alternative sustainable products is nowadays essential [2]. Health-centric attributes are the second most significant factor, after taste, that consumers consider when choosing plant-based food items [3]. Bread is one of the most widespread staple foods [4,5]. Although baker's yeast is frequently used to obtain bread in a short time [6], sourdough is used as an alternative leavening agent that improves bread quality at different layers: (i) it reinforces dough structure [7]; (ii) enriches aromatic profile [8]; (iii) improves nutritional profile [9]; and (iv) extends shelf-life [10]. Indeed, in Europe, at least 30% of bread production incorporates sourdough as either a leavening agent or a genuine improver [11]. Albeit different commercial preparations of sourdough are available [12], traditional sourdough (also known as type I sourdough) is usually obtained upon an initial "spontaneous" fermentation of flour and water, relying on lactic acid bacteria (LAB) and yeasts contaminating flour and the environment of production. Additional ingredients, such as yogurt, grapes, apples [13], water from macerated fruits, grape must [14], and others may also be used because they enrich the dough ecosystem with pro-technological microorganisms (e.g., yeasts) and readily fermentable nutrients [15]. After initial fermentation, a back-slopping process is applied: a portion of the fermented dough is used to inoculate a fresh batch of flour and water ("refreshment"), which undergoes a second fermentation. Repeating these refreshment-fermentation steps daily, while maintaining constant fermentation conditions, the microbial com-munity evolves primarily through dough acidification (attributed to lactic acid fermentation), resulting in a relatively stable ecosystem known as "mature sourdough," which is dominated by LAB and yeasts [16]. The use of different additional ingredients could either affect the time to reach the low values of pH that characterize mature sourdough [13,14] or boost the technological performances of sourdough [17].

In the last decade, the incorporation of sprouted grains into cereal-based food products has garnered attention due to their potential to enhance minerals and vita-mins bioavailability, while reducing anti-nutritional factors [18]. Numerous studies have shown that the incorporation of flour from germinated seeds (sorghum, quinoa, chickpea, wheat) in sourdough bread enhanced its nutritional

content, flavor, and texture [19,20,21,22]. Recently, Bresciani et al. [23] compared germinated chickpea flour and grits (coarsely ground hulled seeds) to evaluate their effect in bread contain-ing 25% of these additional ingredients. They found that, compared to the use of flour from germinated chickpeas, the use of grits led to an increase in dough development time and stability; at the same time, bread enriched with grits had higher specific volume and softer crumb than bread enriched with chickpea flour [23]. Incorporation of germinated seeds (not milled to flour) could emerge as a viable strategy for improving the nutritional and functional attributes of sourdough bread. In addition, since consumption of fresh sprouts has been associated with foodborne diseases [24], we hypothesize that incorporating them into bread could fulfill food safety requirements, because oven baking could inactivate pathogenic bacteria. Furthermore, the use of germinated seeds could be more convenient than "germinated" flour, because in this way, two steps (drying and milling) would be skipped. To our knowledge, no study has so far investigated the effects of incorporating germinated seeds on sourdough bread quality.

This study aimed to: (i) obtain sourdough made from wheat flour from organic agriculture and to use it as starter/improver of baked goods; ii) develop organic sourdough that can be oven-dried, trying to limit the decrease of viability of sourdough microorganisms; iii) provide feasible processing technology that is adapted for small/medium scale farmers and processors; iv) Involve small/ medium enterprises (SMEs) in the food product development and the design process by collaboration, building network, sharing knowledge and promotion of products; v) investigate the effect of using water that had been in contact with raisins in the production of traditional sourdough; (vi) select seeds for use in laboratory-scale sourdough bread production; and (vii) assess the effect of incorporating fresh germinated seeds into recipe of sourdough bread on nutritional, technological, and sensory properties.

All ingredients used in this study were from organic farming. Although previous studies considered the use of additional ingredients in the production of traditional sourdough [13-15,17], no study so far compared the effect from addition of water from raisins on sourdough with that of tap water.

3.2 Material and methods

3.2.1 Materials

Seeds from lentils (*Lens culinaris* ssp. *culinaris*), einkorn wheat (*Triticum monococcum*), barley (*Hordeum vulgare*), chickpeas (*Cicer arietinum*) and bread wheat (*Triticum aestivum* L., cultivar Gentil rosso) were provided by Perniola Alimenti (Rutigliano, Bari, Italy). Bread wheat (cultivar Maiorca) wholemeal flour was produced by Perniola Alimenti. Dried Corinthian raisins and commercial baker's yeast were purchased from the local market. All raw materials originated from organic farming. Seeds and dried grapes were vacuum-packaged and stored at 25 °C.

3.2.2 Sourdough preparation

Two kinds of type-I (traditional) sourdough (classified according to De Vuyst *et al.* [25] were prepared starting from bread wheat flour (150 g): (i) control (CSD), produced by hand-kneading flour with tap water (150 ml); (ii) "boosted" (BSD), produced by hand-kneading flour with 150 ml of "grape water". Dough Yield (DY) was 200. Grape water had been previously obtained upon soaking 50 g of dried grapes in 250 ml of tap water and discarding the grapes after incubation (at room temperature, 48 h). Both doughs were inserted in a plastic jar, covered by a lid, and incubated at 25 °C (relative humidity: 80%) for 24 h. Subsequently, they were back-slopped, inoculating 50 g of the spontaneously fermented dough into a fresh dough, composed of 50 g of flour and 50 ml of tap water (inoculum ratio: about 33%), and then incubated (25 °C, 24 h) (**Figure 1**).

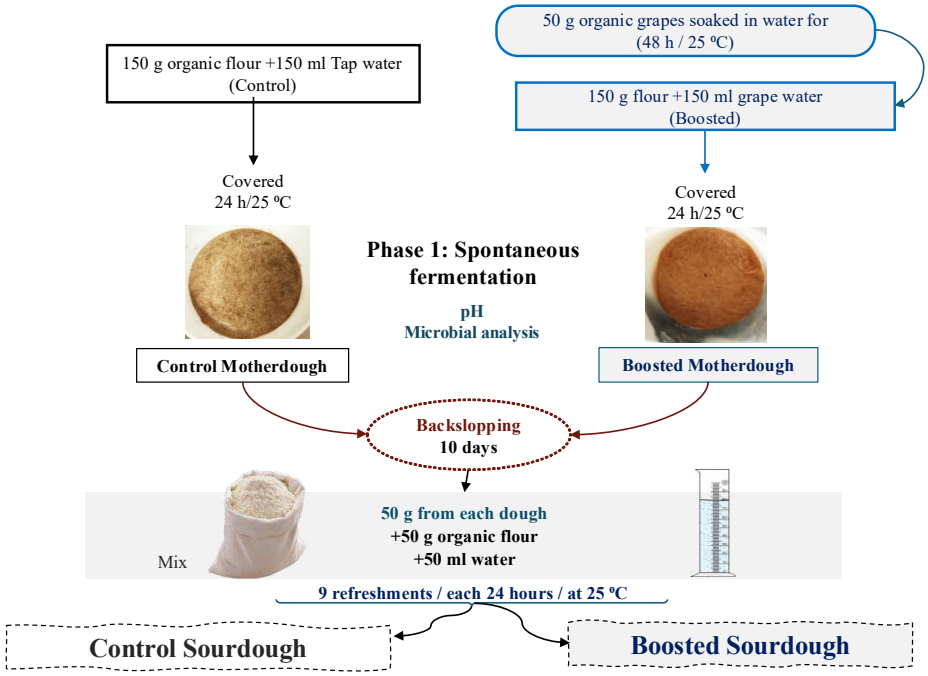
This back-slopping and fermentation step was repeated for at least nine days. BSD corresponded to the protocol usually adopted by Perniola Alimenti. pH of doughs was measured every day (except for the first spontaneous fermentation) after incubation by directly inserting a Foodtrode electrode (Denver Instrument, USA). Acidification rate was calculated using the following formula: $\Delta pH/\Delta T$, whereas ΔpH represents the difference between the pH value measured after one day and the pH value measured after 10 days of fermentation (pH_1- pH_{10}), and ΔT represents the difference in fermentation times (T_{10}-T_1). Microbiological analyses

were performed on fermented doughs, as detailed below, after one, three, five, seven, and ten days.

Figure 1. Production of control and boosted sourdough.

3.2.3 Seed germination

The germination protocol was adapted from that described by Yang et al. [26], with modifications tailored to optimize sprouting. The seeds were first thoroughly



washed under tap water at approximately 15 °C for 20 to 30 min to remove impurities. Afterwards, seeds (25 g) were inserted in a jar with 50 ml of water and soaked separately for 5 h at 25 °C. After soaking, excess water was drained, and the seeds were collected and put on tissue paper. Then, seeds were incubated in a thermostat set at 25 °C in the dark. Relative humidity inside the thermostat during incubation was not controlled; however, seeds were regularly sprinkled with tap water every 12-h intervals to maintain moisture. Sprouting was interrupted when primary roots reached ~5 mm (24 h) to limit exposure to warm, humid conditions that can significantly increase pathogenic bacteria and to align with EFSA guidelines recommending collecting sprouts before the development of leaves [27]. The percentage of germination (% G) was calculated using Equation (1):

(1) % G = germinated seeds · 100/total seeds.

3.2.4 Bread-making

Sourdough bread was made using the BSD and germinated wheat and lentils, according to the recipe provided by Perniola Alimenti, with some modifications. Before bread-making, germinated seeds were transferred into a meat blender machine (Trita Express) to form a homogenous paste. The blending process commenced by starting at a low speed and gradually increasing the speed. Seed paste was used fresh in bread-making. To prepare one loaf of 500 g, 246.7 g of bread wheat flour (49.34%), 5.05 g of sodium chloride (1.01%), and 25 g (5%) of germinated (lentils or wheat) paste were mixed in a clean and sterilized bowl of a planetary mixer (Electrolux Orbital stand mixer, model EKM4000). Subsequently, 74.1 g (14.82%) of BSD, 0.95 g (0.19%) of baker's yeast, and 148.2 ml of water were added to the bowl, and the mixture was kneaded (5 min) to create dough. In parallel, a control sourdough bread was prepared, using 271.7 g of flour and the other ingredients listed above, except for germinated seed paste, which was not added. Each dough was then divided into four loaves and carefully placed onto baking trays lined with parchment paper. Following this, the trays were allowed to undergo a 2-h fermentation at 38 °C (relative humidity: 80%). The fermented loaves were baked in a preheated (to 220°C) oven for 20 min. Upon completion of the baking process, the bread samples were taken out of the oven and cooled at room temperature for 3 h. In total 12 loaves were prepared, corresponding to four loaves of each type: control, germinated lentils (GL), and germinated wheat (GW) bread.

3.2.5 Microbiological analyses

Viable populations of LAB and yeasts were estimated on tap water, grape water, and dough/sourdough (fresh, dried and revitalized), using plate count with selective culture media. Before inoculating plates, 10 g of dough/sourdough samples were homogenized with 90 mL of sterile saline solution (0.9% NaCl) using a stomacher for 3 min. Homogenized samples, as well as tap water and grape water, were serially diluted, and aliquots (1 ml) were pour-plated in Sabouraud Dextrose Agar (SDA), supplemented with chloramphenicol (0.1 g/l) for enumeration of yeasts and molds, M17 agar supplemented with glucose (5 g/l w/v), for coccus-shaped LAB, and modified de Man, Rogosa, and Sharpe (mMRS) agar, supplemented with maltose (5 g/l), fresh yeast extract (50 ml/l), adjusted to pH

5.6, for rod-shaped LAB. M17 and mMRS had also been supplemented with 0.1 g/l cycloheximide after sterilization. Colonies of presumptive yeasts and LAB were counted after incubation at 30 °C for 48 h [28]. In addition, yeast extract agar (YE agar: yeast extract 3 g/l, tryptone 6 g/l, agar 15 g/l, pH 7.2) was employed to enumerate psychrophilic and mesophilic heterotrophic microorganisms in both tap water and grape water, after incubation at 22 °C for 72 h (psychrophilic) and at 37 °C for 48 h (mesophilic) [28]. The detection limit for plate count was 1 log CFU/ml (or g). Calibration standards for each agar medium are reported in **Table 1**. Germinated seeds and bread underwent microbiological analyses, after homogenization and serial dilution, as described for doughs/sourdough samples. Total aerobic mesophilic microorganisms were enumerated on Plate Count Agar (PCA), incubated at 30 °C. Germinated seeds and bread underwent microbiological analyses, after homoge-nization and serial dilution, as described for doughs/sourdough samples. Total aerobic mesophilic microorganisms were enumerated on Plate Count Agar (PCA), incubated at 30 °C for 48 h. Total coliforms were estimated using Violet Red Bile Glucose (VRBG) agar, incubated at 37 °C for 24 h [20].

Table 1. Calibration standards for each agar medium used in microbiological analyses

Agar medium	Calibration standard
Sabouraud Dextrose	*Candida albicans* ATCC 10231
M17	*Streptococcus thermophilus* ATCC 14485
Modified MRS	*Lactobacillus gasseri* ATCC 19992
Yeast Extract	*Escherichia coli* ATCC 25922
Plate Count	Not available
Violet Red Bile Glucose	*Escherichia coli* ATCC 25922

3.2.6 Descriptive sensorial analysis of bread

A panel test was conducted at the Department of Plant, Soil, and Food Sciences at the University of Bari Aldo Moro to qualitatively describe sensory traits of bread samples. The panel consisted of 10 assessors (6 females and 4 males) with ages spanning from 27 to 47 years. The panelists underwent two training sessions, which encompassed tasting bread samples that differed from those used in the

panel test, and familiarization with sourdough bread enriched with germinated seeds, aimed at establishing a standardized sensory vocabulary. A total of 22 sensory attributes, covering aspects such as appearance (crumb and crust color, crust thickness, crust appearance, and crumb structure), odor (yeasty, fermentation, toasted, vegetables, nutty, off-odor), texture (elasticity, dryness, hardness, softness, graininess, compactness, and crust crunchiness), and taste (bitter, salty, legume-like, acidic and overall taste) were considered [29]. Furthermore, panelists were encouraged to suggest novel descriptive terms to augment the existing attributes. During the assessment, the bread samples were sliced to a 1.5 cm thickness and served at 22 °C under standard lighting conditions. To mitigate any potential bias, samples were coded with unique 3-digit identifiers and presented in a randomized sequence [30]. Each panelist had three different samples of each type of bread and rated the sensory attributes on a non-structured 15 cm line scale using sheets describing the vocabulary for each attribute, with scores ranging from 0 (not detected) to 15 (extremely strong), adhering to ISO 13299:2010 standard for sensory analysis.

3.2.7 Specific volume, image, and color analysis of bread samples

Specific volume of bread samples was measured through the rapeseed displacement method AACC 10-05.01 [31]. In addition, bread crumb was examined utilizing image analysis technology. Pictures of the sliced bread were scanned (at 300 dpi) through an Image Scanner (Amersham Pharmacia Biotech, Uppsala, Sweden). Full-scale images were gray-scaled, and two sub-images (1,001 × 1,393 pixels, field of view) were analyzed using the UTHSCSA ImageTool application (version 2.0, University of Texas Health Science Centre, San Antonio, Texas, United States). Gas cells and non-cells were distinguished using a threshold approach, using a threshold value of 130 [32]. Gas cell area in the bread crumb was calculated by the software as a percentage ratio between the area filled by gas cells (black pixels) and the total area. A Minolta CR-10 camera was used to assess color in three separate locations on the bread crust and crumb [33]. It was done using the L^*, a^*, and b^* color space analysis approach, where L^* stands for lightness (white to black) and a^* (red-green) and b^* (yellow-blue) for chromaticity coordinates.

3.2.8 Nutritional analysis of bread

Nutritional analysis of breads included determination of proteins (total nitrogen · 6.25), lipids, ashes, and water content, according to the AOAC methods 978.04, 2003.06, 930.05, and 930.04, respectively. Total available carbohydrates were calculat-ed as the difference [100 – (proteins + ashes + lipids + water)]. Total dietary fibers were determined using the Total Dietary Fiber Kit (Megazyme-Ireland), following the AACC 32-05.01 method. Energy value was calculated based on the amount of lipids, proteins, and total carbohydrates, multiplied by the corresponding energy value of each nutrient [34].

3.2.9 Drying and revitalization of sourdoughs

The drying process of the organic sourdough starters were done considering a careful processing method which is oven drying, to test the influence of drying and rehydration on the microbial viability. This was done to understand the possibility of producing organic sourdough dried starter as a product to preserve the microbiota and facilitate the transport process to potential consumers.

Both control and boosted sourdough starters were prepared by transferring active sourdough cultures into sterile trays, allowing air circulation. The starters were dried in an oven at 48°C for 20 hours. After cooling, they were stored airtight. Some samples were retained for microbiological analysis. Dried sourdoughs were rehydrated with a 1:1 ratio of dried sourdough and water, stirred, and left for 2 hours at 25°C until the dried starter dissolves. Then, organic flour and water were added at a ratio of 1:1:1 to the rehydrated sourdough. After 24 hours at room temperature, pH and microbial count were analyzed. This process was repeated for three days, and if pH reached around 3.6, sourdough was considered successfully revitalized. This process was done to test the effect of drying on the technological characteristics of sourdough and on the viability of LAB and yeasts.

3.2.10 Triangle test

Sensory evaluation was conducted on two types of sourdough bread sample: one prepared with the revitalized BSD and the other with the fresh BSD. The Triangle test involved three samples, with two being identical and one different (Sample C). Samples were randomized and coded (Sample A and Sample B were identical, whereas sample C differed from the two other samples) to reduce bias, and panelists were unaware of sample composition. Panelists were instructed to

identify the different sample (Sample C) by taste. The ISO standard methodology 4120:2004 for Triangle test sensory analysis was followed. A panel of ten volunteers (5 females and 5 males, aged 25 to 50) from the laboratory staff participated. Slices of bread were presented at 22°C in normal lighting conditions, with a 3-hour cooling period before evaluation. To assess the statistical significance of the results, a binomial probability test using R software was employed to determine the likelihood of the observed responses occurring by chance. The resulting p-value provided a quantitative measure of the statistical significance of the sensory differences detected.

3.2.11 Statistical analysis

All experiments were conducted in triplicate, and results were expressed as mean ± standard deviation. Statistical analyses were performed using SPSS Statistics version 28. Data were subjected to one-way analysis of variance (ANOVA) to assess significant differences among samples. When ANOVA indicated significant effects ($P < 0.05$), means were compared using Tukey's Honestly Significant Difference (HSD) post hoc test. Spearman correlations for color indices and scores attributed to bread crust and crumb were computed using Statistica v. 7.0.

3.3 Results

3.3.1 Microbial cell densities differed between tap water and grape water

The microbiological analysis revealed that grape water exhibited significantly higher ($P < 0.05$) counts of all the targeted microbial groups, except for heterotrophic mesophilic microorganisms. The latter were found at higher ($P < 0.05$) cell density in tap water (**Figure 2**). Grape water was dominated (at an order of magnitude of 6 log CFU/ml) by psychrophilic heterotrophic microorganisms, presumptive yeasts, and presumptive coccus-shaped LAB.

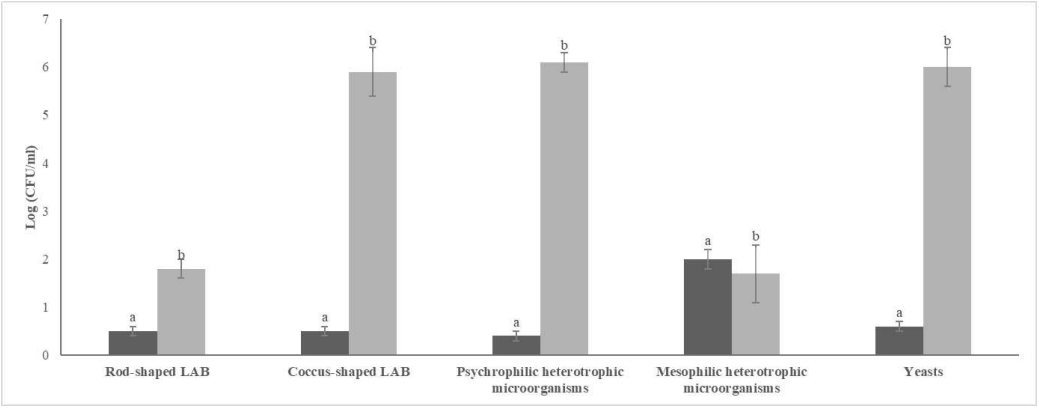


Figure 2. Cell densities (expressed in log CFU/ml) of presumptive LAB, yeasts, psychrophilic and mesophilic heterotrophic microorganisms found in tap water (dark grey bars) and grape water (light grey bars) used in the first sourdough fermentation. All experiments were conducted in triplicate, and results were expressed as mean ± standard deviation. For a given microbial group, bars showing different letters are significantly different ($P < 0.05$).

3.3.2 Boosted sourdough gave similar performances to control sourdough

The pH of both CSD and BSD sourdoughs steadily declined throughout the 10-day fermentation period (**Figure 3**), with both falling below 4.5 on day 3. pH reached about 3.8 on day 7 in both sourdough samples, and then it remained constant. The acidification rate was slightly higher in the CSD (0.24 pH units/day) than in the boosted sour-dough (0.22 pH units/day).

From the beginning of fermentation until day 3, BSD exhibited significantly ($P < 0.05$) higher counts of presumptive coccus-shaped and rod-shaped LAB than the control (Figure 4). After the first fermentation, presumptive yeasts were detected at very low cell density in both sourdough samples, with no significant ($P > 0.05$) between BSD and CSD. At day 3, CSD harbored a higher ($P < 0.05$) cell density of yeast than BSD, although in both sourdough samples, yeasts were in the order of magnitude of 2 log CFU/g. Yeasts increased in both sourdough samples after 7 and 10 days to 5 and 6 log CFU/g, respectively, with CSD characterized by higher ($P < 0.05$) cell density than BSD (**Figure 4**). Presumptive LAB increased until day 7 up to 9 log CFU/g; afterwards, they did not increase. Both on days 7 and 10, no significant ($P > 0.05$) difference in cell densities of LAB was found between BSD and CSD.

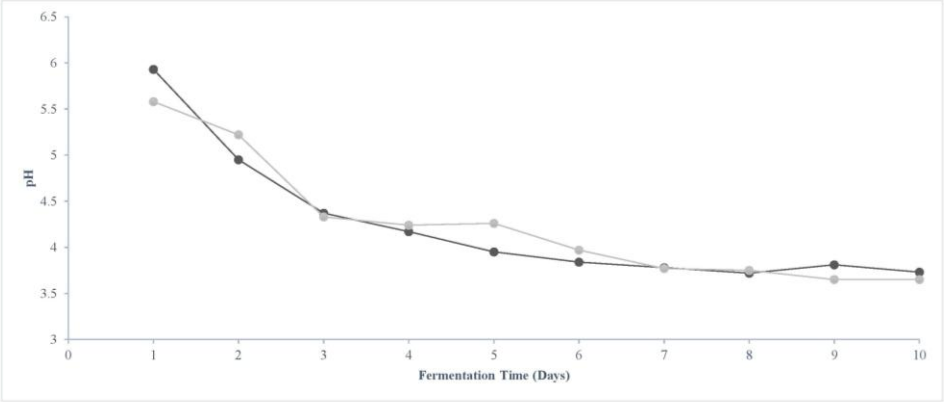


Figure 3. Values of pH of control (dark grey plot) and boosted (light grey plot) sourdoughs after each daily fermentation. Error bars are not visible because error values were below 0.07.

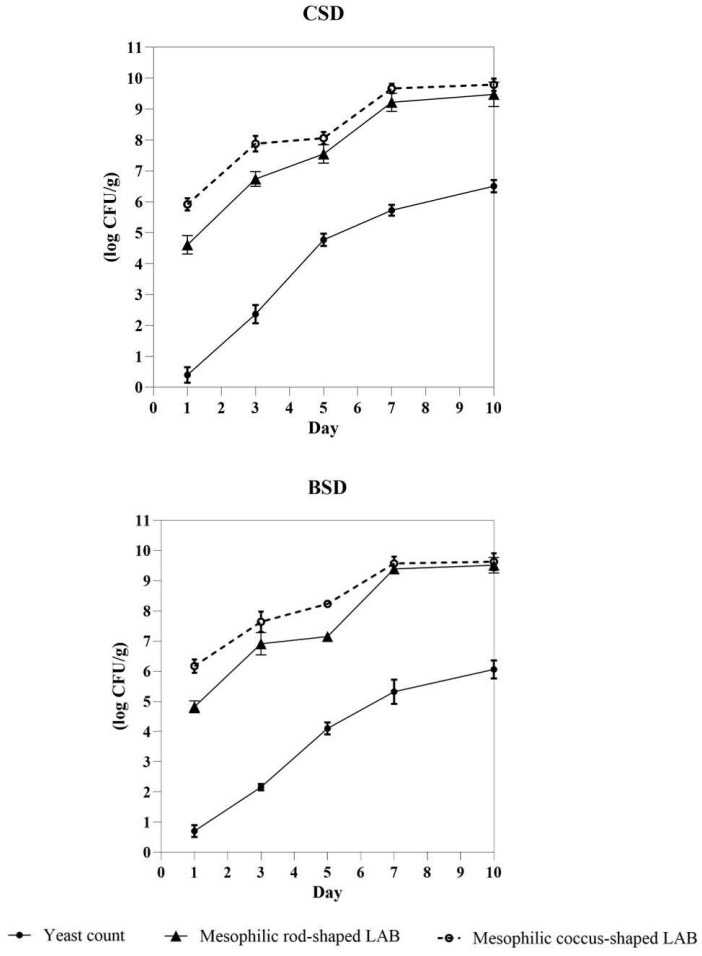


Figure 4. Cell densities (log CFU/g) of presumptive yeasts, mesophilic rod-shaped LAB, and mesophilic coccus-shaped LAB in the control sourdough (CSD) and boosted sourdough (BSD) after each daily fermentation. All experiments were conducted in triplicate, and results were expressed as mean ± standard deviation.

3.3.3 Germinated lentil and bread wheat seeds were selected as additional ingredients for bread

Germination tests showed that, after 24 h at 25 °C, lentils and bread wheat exhibited the highest germination percentages (**Figure 5** and **Figure 6**). Einkorn wheat and barley, showing germination percentages lower than 20%, were discarded from further experiments. Microbiological analyses showed that germinated lentils and bread wheat harbored total coliforms at an order of magnitude of 8 log CFU/g, with no significant difference between them (data not shown). Total mesophilic aerobic bacteria were found at higher ($P < 0.05$) cell density in germinated lentils (8.2 log CFU/g) than in germinated bread wheat (7.8 log CFU/g).

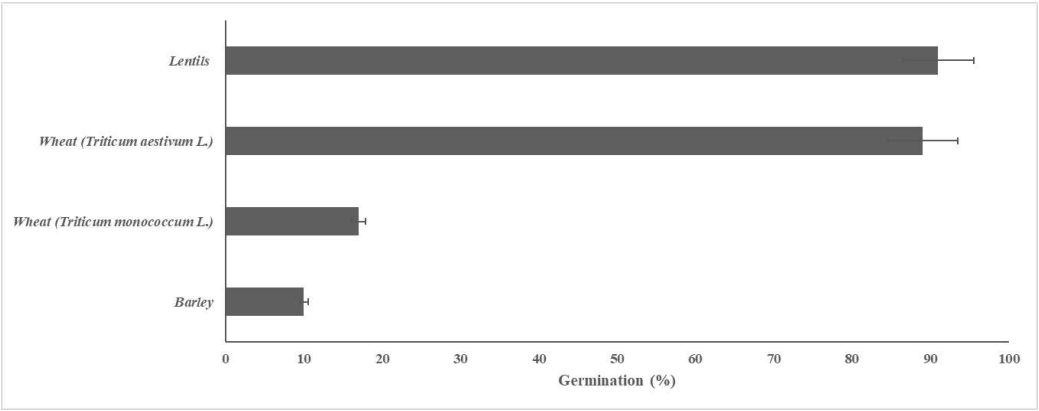


Figure 5. Percentage of germination, determined after 24 h at 25 °C, of lentil, bread wheat, einkorn wheat, and barley seeds. Results are shown as mean of 3 replicates, and were expressed as mean ± standard deviation.

Lentils *(Lens culinaris* **ssp.** *Culinaris)*

Wheat *(Triticum aestivum* **L.)**

Wheat *(Triticum monococcum)*

Barley *(Hordeum vulgare)*

Figure 6. Germinated lentils, wheat, chickpeas, and barley seeds.

3.3.4 Sourdough bread was free from total coliforms

Preliminary results of specific volume (data not shown) suggested using, besides sourdough, baker's yeast as the main leavening agent for bread-making. Three types of bread were manufactured using BSD: (i) with paste from germinated lentils (GL); (ii) with paste from germinated bread wheat (GW); (iii) control. Microbiological analyses performed on bread with paste from either germinated lentils or wheat showed that baking reduced total aerobic mesophilic microorganisms and total coliforms to undetectable levels (<1.0 log CFU/g).

3.3.5 Bread types differed in terms of specific sensory attributes

The descriptive sensory analysis showed that some attributes received very low scores, with no significant difference between the three types of bread (**Table 2**). For instance, no off-odors were reported in any bread, and this attribute, as well as legume-like taste, was not graphically shown. Control bread showed the highest values for elasticity (3.4±0.5), crust crunchiness (11.2±0.6), and compactness (11.7±0.4). GL received the highest average scores for crumb color (11.5±0.7), crust color (10.4±0.9), and overall taste (8.6±0.8), while also exhibiting higher bitterness (4.5±0.3) and legume-like flavor (3.9±0.2) (**Figure 6**). GW had the highest softness (5.6±0.5) and the lowest bitterness scores (3.5±0.2).

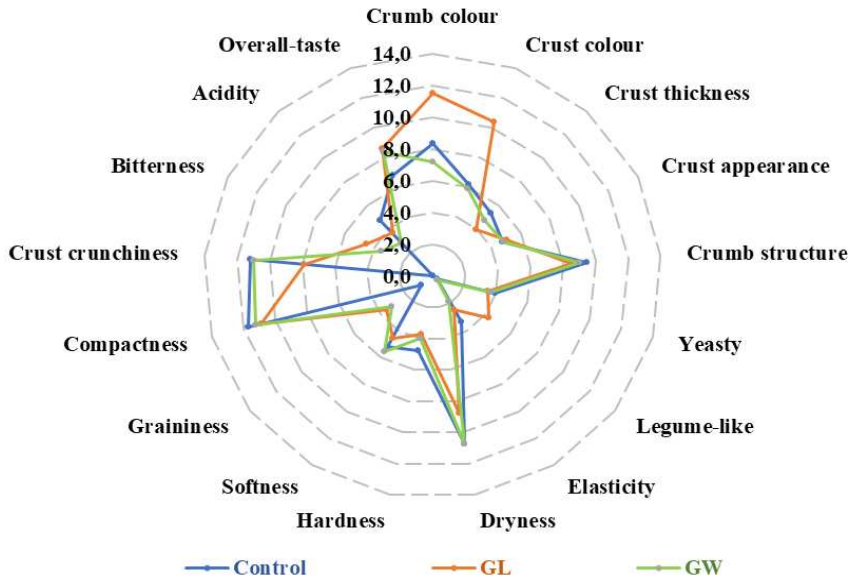


Figure 6. Descriptive sensory analysis of organic sourdough bread, manufactured with germinated lentils (GL) or germinated wheat (GW) paste or without germinated seed paste (Control). Data are the means of scores attributed by ten trained panelists.

Table 2. Sensory analysis of organic sourdough bread, manufactured with germinated lentils (GL) or germinated wheat (GW) paste or without germinated seed paste (Control). Data values are the means of scores attributed by ten trained panelists. Values in the same column with different superscript letters (a–b) are significantly different ($P < 0.05$).

	Descriptors	Control	GL	GW
Visual	Crumb color	8.3±0.9b	11.5±0.3a	7.1±0.4b
	Crust color	6.2±0.4b	10.4±0.3a	5.9±0.6b
	Crust thickness	5.3±0.4a	4.0±0.5b	4.7±0.5a

	Crust appearance	4.8±0.6a	5.1±0.2a	4.9±0.3a
	Crumb structure	9.5±0.5a	8.5±0.6a	9.0±0.9a
Olfactory	Yeasty	9.5±0.5a	3.5±0.5a	3.7±0.3a
	Legume-like	0.3±0.2b	4.3±0.2a	0.4±0.3b
Structure	Elasticity	3.4±0.5a	2.4±0.3b	1.9±0.3b
	Dryness	10.8±0.4a	8.8±0.5b	10.7±0.9a
	Hardness	4.8±0.7a	3.7±0.4a	4.0±0.4a
	Softness	5.2±0.5a	4.6±0.3a	5.6±0.5a
	Graininess	0.9±0.5b	3.5±0.4a	3.2±0.8a
	Graininess	11.7±0.4a	10.9±0.5a	11.2±0.5a
	Crust crunchiness	11.2±0.6a	7.9±0.5b	11.0±0.5a
Taste	Bitterness	0.0	4.5±0.1a	3.5±0.9a
	Acidity	4.8±0.8a	3.7±0.5a	2.9±0.4b
	Overall-taste	6.8±0.8b	8.6±0.3a	8.4±0.3a

3.3.6 Image analysis and color indexes differed among the bread types

The use of germinated lentils and wheat did not affect the specific volume of sourdough bread (**Table 3** and **Figure 7**). The highest gas cell area was found for GL, whereas control bread was characterized by the lowest values of this crumb parameter. The crumb of GL bread was less light ($P < 0.05$) than control bread, but not significantly different from GW bread.

The lowest value for L^* index was found for crust of GL bread. In addition, this bread showed the highest a^* value (**Table 3**). Correlation analysis between color indexes and scores attributed by the panelists to crumb and crust color showed that the crust color was highly correlated and inversely correlated to a^* index and L^* index, respectively (**Table 4**).

Table 3. Specific volume, gas cell areas, and values of color indexes of organic sourdough bread, manufactured with germinated lentils (GL), or germinated wheat (GW) paste, or without germinated seed paste (Control). Results are shown as mean of 3 replicates, and were expressed as mean ± standard deviation.

Bread	**Specific volume (cm3/g)**	**Gas cells area (%)**	***Crust color***			***Crumb color***		
			L*	***a****	***b****	***L****	***a****	***b****

Control	2.2±0.2a	14.1±0.5c	59.6±1.0a	9±0.5b	23.7±0.8a	62.3±0.1a	4.4±0.2a	16.9±0.2a
GL	2.2±0.1a	28.6±0.8a	52.1±0.4b	11.0±0.6a	24.2±0.5a	56.4±1.4b	4.5±0.1a	17±0.4a
GW	2.2±0.1a	18.1±0.8b	58.4±0.9a	8.5±0.5b	22.5±0.9a	58.9±2.1ab	4.5±0.4a	18±1a

[a-c] Values in the same column with the different superscript letters are significantly different ($P < 0.05$).

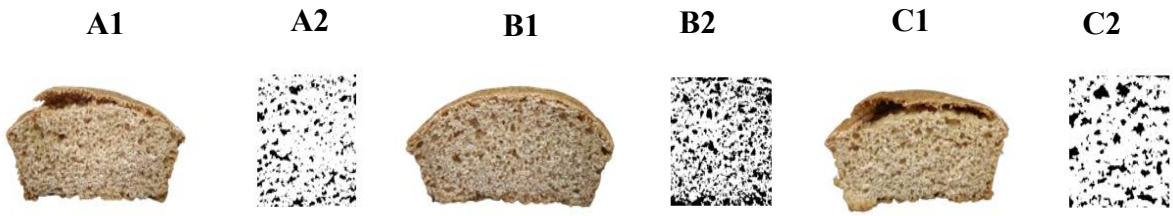


Figure 7. Sections of organic sourdough bread, manufactured without germinated seed paste (control, A1), or with germinated lentils (GL, B1) or germinated wheat (GW, C1) paste. A2, B2, and C2 show crumb subsections, after thresholding to calculate the black pixel area, of control, GL, and GW bread, respectively.

Table 4. Correlation coefficients between values of color indexes (L^*, a^*, b^*) of crust and crumb and the sensory panel evaluation scores of organic sourdough bread (GL, GW and control).

	R
L^* crust vs. sensory score	-0.978
a^* crust vs. sensory score	0.992
b^* crust vs. sensory score	0.767
L^* crumb vs. sensory score	-0.638
a^* crumb vs. sensory score	0.254
b^* crumb vs. sensory score	-0.651

3.3.7 Nutritional analysis of bread

GL bread was characterized by the lowest and highest values of lipids and proteins, respectively (**Table 5**). In addition, this bread showed the lowest concentration of carbohydrates. GW showed lipid and protein content that were not significantly different ($P > 0.05$) from the control. GW and control bread showed the highest concentration in total carbohydrates. Total dietary fibers, as well as water content and energy values, were not significantly ($P > 0.05$) different among the three types of bread (**Table 5**).

Table 5. Nutritional analysis of organic sourdough bread, manufactured without germinated seeds (control), with germinated lentils (GL), or germinated wheat (GW).

Bread	Lipids (%)	Proteins (%)	Water (%)	Total carbohydrates (%)	Dietary Fibers (%)	Energy value (kcal/100 g)
Control	0.4±0.1^{a}	8.6±0.1^{b}	37.9±0.1^{a}	44.8±0.1^{a}	6.6±1.2^{a}	243±1.0^{a}
GL	0.3±0.1^{b}	9.9±0.1^{a}	37.4±0.2^{ab}	43.3±0.1^{b}	5.7±0.5^{a}	245±1.0^{a}
GW	0.4±0.1^{a}	8.8±0.1^{b}	37.0±0.1^{b}	45.5±0.1^{a}	6.2±0.6^{a}	247±1.0^{a}

Results are shown as mean of 3 replicates, and were expressed as mean ± standard deviation. $^{a-b}$ Values in the same column with the different superscript letters are significantly different ($P < 0.05$).

3.3.8 Drying, rehydration and revitalization of sourdoughs

During the 4-day feeding period, both control sourdough (CSD) and boosted sourdough (BSD) exhibited dynamic changes in pH levels and microbial populations (Table 3). Initially, both sourdoughs with addition of water only started with an acidic pH, with CSD at 4.1±0.1 and BSD at 4.0±0.02. As the feeding progressed, the pH increased reaching 5.1±0.1 for CSD and 5.2±0.1 for BSD on day 1, followed by a gradual decrease to 3.6-3.8 at day 4. This pH evolution reflects the active sourdough fermentation process influenced by microbial metabolic activities for 3 days as it remained at the same level after subsequent feeding for an extra day. Yeast populations, initially below the limit of detection, as they were almost totally inactivated, increased during the feeding, reaching 6.4±0.1 log cfu/g for CSD and 6.0±0.2 log cfu/g for BSD on day 3. The drying process typically results in a reduction of moisture content, which is a key factor for yeast activity and growth. Yeast cells require a certain level of moisture to remain active and multiply. When sourdough is dried, the reduced moisture content can lead to a state where yeast cells become dormant or less active, resulting in a lower yeast cell density. Therefore, rehydration of the dried sourdough is a particularly important step to recover dormant or less active microbial cells

Mesophilic coccus-shaped LAB were consistently higher than yeasts, with both sourdough types showing increasing LAB counts over time, and BSD generally exhibiting higher counts compared to CSD. Similarly, mesophilic rod-shaped LAB counts increased during feeding, with BSD consistently surpassing CSD. LAB counts remained at constant levels after 3rd feeding, in accordance with the results

of pH. These findings imply that a successful sourdough revitalization period of three days is sufficient, with BSD potentially offering a more conducive environment for the proliferation of LAB. This enhanced LAB growth in BSD could be attributed to the incorporation of grape water during processing, which exhibited a more visible effect in the dried and revitalized samples compared to freshly prepared ones (**Table 4**).

Table 4. pH and microbiological analysis (expressed in log10 cfu/gm) of dried and revitalized sourdoughs during the feeding of 4 days. SD: Sourdough; CSD: Control sourdough; BSD: Boosted sourdough; LAB: Lactic acid bacteria. Data are shown as the average value obtained from 3 replicates ±standard deviation. Values with different letters within the column are significantly different (P<0.05) Tukey's test for one-way ANOVA.

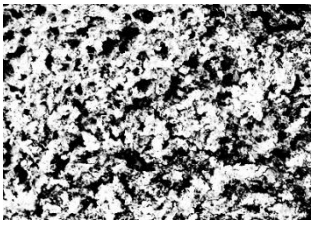
Feeding	SD Type	pH	Yeasts	Mesophilic coccus-shaped LAB	Mesophilic rod-shaped LAB
0	CSD	4.1 ± 0.1^{cd}	$<1.0^{a}$	3.88 ± 0.09^{a}	3.91 ± 0.06^{a}
	BSD	4.0 ± 0.1^{c}	$<1.0^{a}$	4.41 ± 0.09^{b}	5.42 ± 0.08^{b}
1	CSD	5.1 ± 0.1^{d}	5.71 ± 0.09^{cd}	8.04 ± 0.04^{c}	8.57 ± 0.09^{c}
	BSD	5.2 ± 0.1^{de}	5.42 ± 0.1^{b}	9.08 ± 0.04^{d}	8.90 ± 0.05^{e}
2	CSD	4.0 ± 0.1^{c}	5.92 ± 0.06^{e}	9.38 ± 0.03^{e}	8.73 ± 0.03^{d}
	BSD	3.9 ± 0.1^{b}	5.69 ± 0.09^{c}	10.12 ± 0.15^{g}	9.26 ± 0.02^{f}
3	CSD	3.6 ± 0.1^{a}	6.49 ± 0.12^{g}	9.68 ± 0.06^{f}	9.21 ± 0.07^{f}
	BSD	3.6 ± 0.1^{a}	6.04 ± 0.04^{f}	10.37 ± 0.17^{h}	9.55 ± 0.08^{g}
4	CSD	3.7 ± 0.1^{ab}	6.65 ± 0.05^{h}	9.65 ± 0.06^{f}	9.22 ± 0.06^{f}
	BSD	3.6 ± 0.1^{a}	6.43 ± 0.13^{g}	10.32 ± 0.13^{h}	9.52 ± 0.07^{g}

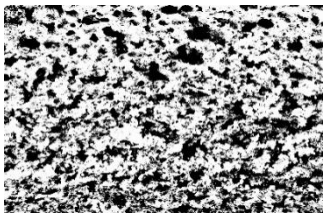
The triangle test was conducted to evaluate the panelists' ability to detect a perceptible difference between two types of sourdough bread samples. We selected the boosted sourdough (both fresh boosted and dried-revitalized) and developed a bread with it (FBSD and DBSD). Out of the ten panelists, one panelist (10%) correctly identified the different sample (Sample C) in the triangle test, while the remaining panelists did not discriminate between the samples. The distribution of responses indicated that 50% selected Sample A, 40% selected Sample B, and 10% correctly selected Sample C as the different one out. The binomial probability test revealed that the results were not statistically significant, with a probability value (p-value) of 0.128. This shows that panelists were unable

to perceive differences among the sourdough bread samples and were randomly guessing, suggesting that the samples likely had very similar sensory characteristics.

Fresh boosted sourdough bread (FBSD) and dried and revitalized sourdough bread (DBSD) exhibited comparable specific volume, with FSD having a slightly higher specific volume (**Table 5**). However, the difference between the two is not substantial, suggesting that the drying and revitalization process did not significantly alter the overall structure and texture of the bread in terms of its volume per unit weight. Additionally, the results of image analysis indicate that both FSD and DSD have similar black pixel ratios. These findings suggest that the drying and revitalization of sourdough did not notably impact the visual characteristics of the bread, and both types maintain a similar appearance in terms of black pixel percentage.

Table 6. Specific volume, black pixel percentage and image analysis of bread types where: Fresh boosted sourdough bread (FSD, made with fresh sourdough) and Dried and revitalized sourdough (DSD). Results are shown as mean of 3 replicates, and were expressed as mean ± standard deviation. Values with different letters within the column are significantly different (P<0.05) Tukey's test for one-way ANOVA.

Bread types	Specific volume (cm3 g)	Black pixel percentage	Image analysis	
FSD	1.81±0.22[a]	63.06%[a]		

DSD	1.79±0.21[a]	63.79%[a]		

3.4 Discussion

In collaboration with a small-medium food enterprise, we developed a novel organic sourdough bread, enriched with paste of freshly germinated, aiming to diversify the local bakery food products, in particular bread, that have the potential of targeting consumers interested in products: (i) being entirely manufactured with ingredients from organic farming, including type-I sourdough as genuine improver; (ii) containing paste from germinated seeds. The experimental plan envisaged the selection of the type of sourdough (produced using either tap water or "grape water"), as well as the selection of seeds among four: lentil, einkorn wheat, barley, and bread wheat. Raisins were chosen to produce "grape water" as an additional ingredient of sourdough, based on the protocol commonly adopted by the food enterprise. Raisins have been recently used to produce a sourdough starter with scarce leavening capacity [35]. This sourdough was dominated by *Pichia kudriavzevii* (heterotypic synonym: *Candida krusei*) and *Torulaspora delbrueckii*, two yeast species that, compared to *Saccharomyces cerevisiae*, are much less frequently used in breadmaking [35]. Under our experimental conditions, we found that "grape water" was enriched in psychrophilic heterotrophic microorganisms. Values of yeast cell density in grape water suggest that those psychrophilic microorganisms could be represented by yeasts, originating from raisins and transferred into water, in accordance with this finding, grapes putatively transferred a relatively high cell density of yeasts to dough [13]. When analyzing tap water used for producing sourdough, we found that the cell density of mesophilic heterotrophic microorganisms fell in the range (0.8 – 3.5 log CFU/ml) previously reported [28]. On the contrary, psychrophilic heterotrophic microorganisms in the tap water were lower than the range previously reported [28], although in that study, three out of ten water samples collected in different

Italian regions showed counts of psychrophilic heterotrophic microorganisms below the detection level. While we expected that use of grape water could have boosted sourdough during its early steps of production, we did not find any difference in yeast cell density between BSD and CSD. On the contrary, BSD was characterized by a higher cell density of LAB in the early production steps, compared to CSD. This result could be explained by hypothesizing that in the sourdough samples subjected to study, yeasts transferred from raisins to water could be less competitive than LAB. It is well known that the competitiveness of microorganisms in sourdough is an essential driver during its preparation [15]. Furthermore, we speculate that the increased LAB cell density in BSD might result from the increased content of fermentable carbohydrates derived from raisins during maceration in water, which might have promoted LAB growth over yeasts. Oshiro et al. [36] also hypothesized that different affinity to different carbohydrates shown by strains of LAB and *S. cerevisi*ae could be one of the bases for competition between LAB and yeasts in early steps of sourdough preparation. Under our experimental conditions, a pH less than 4.5 was reached at the end of the third fermentation, regardless of the use of grape water. On the contrary, we had previously reported that the use of water from pear maceration had favored a pH decrease to 4 already after the second fermentation [14]. This could be explained considering that raisins and pears may differ in terms of microbial community and concentration of sugars. Additionally, in the previous research, 100 g of pears were subjected to maceration [14] vs. 50 g of raisins processed in the present study. After 10 fermentation steps, we found that the use of grape water instead of tap water did not affect LAB cell density and their main metabolic activity, resulting in dough acidification. On the contrary, we found slightly (although significantly different) higher yeast cell density in CSD than in BSD, which could be due to the different composition (in terms of species and strains) of the microbial community, driven by the two different water types. Both sourdough types showed values of pH, cell densities of LAB and yeasts, falling in the typical range for traditional sourdough [15]. Further experiments focused on use of BSD for bread-making, because this kind of sourdough had been produced according to the protocol commonly used by the food enterprise that participated in this study. In addition, use of grape water for preparing sourdough could be helpful in all cases wherein flour does not harbor pro-technological

microorganisms [37]; in such cases, indeed, a longer time would be needed to obtain mature sourdough.

In parallel with comparison between BSD and CSD, we considered four seeds that, after germination, could be used as a paste and as additional ingredients of organic sourdough bread. We selected lentils and bread wheat, based on their higher percentage of germination, compared to barley and einkorn. Germination percentage could be affected by various factors, such as time and conditions of storage, eventual treatments of seeds, such as decortication, as well as parameters (e.g., temperature) applied during germination protocol [38]. Use of germinated seeds in food is consolidated in many African and Asian populations, because they improve the sensory properties [39] and texture of food items [40], besides enhancing the nutritional value [40,41]. Wheat bread based on 20% replacement with flour from germinated oat was characterized by increased bioactive compounds (gamma aminobutyric acid, total polyphenols, free phenolic acids) and decreased starch digestibility [42]. Bread entirely based on sprouted wheat flour showed higher *in vitro* protein digestibility than bread made of regular flour [43]. Chemical modifications occurring during seed germination typically cause an increase in microbial load [44,45].

Therefore, in the current study, germinated lentils and bread wheat grains were subjected to microbiological analyses, which showed high cell density of presumptive *Enterobacteriaceae* in accordance with previous studies [45,46,47]. However, the use of fresh germinated lentils/wheat, as a paste, as additional ingredients of organic sourdough bread did not raise any safety concerns. Indeed, after baking, *Enterobacteriaceae* were not detected, possibly because of the high temperature reached by bread during baking, in accordance with previous studies [48,49]. Seeds subjected to germination for being used as ready-to-eat food are usually contaminated, especially by *Salmonella* spp. and Shiga-toxin producing *Escherichia coli*, which may survive for long periods during seed storage [27]. In this study, we demonstrated that bread-making is a viable option for consuming germinated seeds, ensuring the food is free from microbiological hazards.

The three sourdough bread types (GL, GW, and control) considered in this study were subjected to a panel test, since the food enterprise collaborating with researchers was interested in understanding the potential of bread with germinated seeds in view of scaling up the process. Indeed, previous studies

reported that the use of flour from germinated seeds was able to modify the sensory attributes of bread. Atudorei et al. [50] found that adding 5–7.5% germinated lentil flour improved bread porosity, crumb color, and sensory appeal. Badawy et al. [51] reported that 6–9% germinated wheat flour enhanced aroma and crumb color. In the current study, we found that, compared to the control, sourdough bread incorporating 5% of germinated lentils/wheat grains received a higher score for overall taste and softness, respectively. In addition, panelists perceived the crumb and especially the crust of sourdough GL bread as more colored than the two other bread types. Higher scores of graininess and overall taste attributed to GL and GW breads could be explained by the use of 5% germinated seeds for two main reasons: (i) proteins, lipids, and minerals contained in the additional ingredient; (ii) the germination process itself leads to increased concentrations of enzymes and chemical compounds affecting sensory/texture properties [50]. The color analysis showed that GL bread had the lowest value of *L* index (lightness) and the highest value of *a** index (meaning color of crust approaching red). Overall, considering bread crust, the score attributed by the panelists to color was strongly correlated with *a** index, but highly and inversely correlated with *L* index.

Use of germinated lentils/wheat grains did not affect the specific volume of bread. Yet, image analysis showed that sourdough bread with paste from germinated wheat and, mainly, lentils, was characterized by a higher percentage of gas cells area than the control. These results are in agreement with a previous study that reported that the addition of 5% flour from 48-72 h germinated bread wheat did not affect the specific volume. However, the resulting bread appeared with a different size and distribution of gas cells, with respect to the control bread [52].

From a nutritional perspective, control bread and GW could be labelled as "rich in fiber" (at least 6% of fiber), whereas GL could be labelled as "source of fiber" (at least 3% of fiber) [53]. However, the use of germinated lentils as additional ingredients in sourdough bread resulted in providing more proteins and less lipids than the two other bread types. These findings are consistent with those reported by Hernández-Aguilar et al. [54], who developed wheat breads with 5% and 10% germinated lentil flour. They observed that incorporating 5% lentil flour increased the protein content to approximately 11.8% compared to 10.5% in the control, while the lipid content decreased from 12.16% to 10.3%. Another study also found that

the addition of 5–10% roasted-sprouted lentil flour to gluten-free bread (corn/rice based) had 77–90% higher protein than the control bread [55].

3.5 Conclusions

This study showed that the addition of paste from germinated lentils into organic sourdough bread enhanced nutritional quality and overall taste, while maintaining desirable technological characteristics. On-going research aims to evaluate the feasibility of scaling up the protocol (sourdough production, seed germination and blending, bread-making to small-medium enterprises and consumers' acceptance of the novel bread. Further studies may be conceived aiming to: (i) adapt the protocol to other underutilized seeds; (ii) assess the most appropriate storage conditions for paste from germinated seeds; and (iii) evaluate the impact of using germinated seeds paste on the shelf-life of bread.

3.6 References

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Valorization of organic third-category fruits through vinegar fermentation: A laboratory-scale evaluation of apples, peaches, and clementines

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Abstract

Chapter 4 explores the valorization of third-category organic fruits through vinegar production, using fermentation as a strategy to reduce waste and generate value-added products within organic supply chains. Organic apples, peaches, and clementines were processed into juices and subjected to sequential alcoholic and acetic fermentations under laboratory-scale conditions. The fermentation process was monitored through microbial growth kinetics, ethanol consumption, and acetic acid production. The resulting vinegars were characterized in terms of color attributes, volatile organic compound profiles, sensory properties, and antioxidant activity. All fruit matrices supported efficient yeast-driven alcoholic fermentation followed by successful acetic fermentation, yielding vinegars with distinct chemical and sensory profiles. Differences among fruit types significantly influenced volatile composition, color parameters, and sensory perception, while antioxidant activity was retained or enhanced after fermentation. The results showed that fermentation is an effective tool for converting low-market-value organic fruits into high-quality vinegars with differentiated characteristics. This study highlighted the potential of vinegar fermentation as a sustainable, low-input processing strategy for small and medium-sized enterprises, contributing to waste reduction, circular economy practices, and product diversification in the organic food sector.

4.1 Introduction

Food waste is a global challenge, with roughly one-third of all food produced – around 1.3 to 1.6 billion tons – lost or wasted annually [1]. Fruits and vegetables are among the categories most affected by the issue of food waste, with recent FAO

estimates indicating roughly 31–45% of fruit and vegetable output is lost or wasted along the supply chain [2]. Much of this waste occurs at farms and retailers due to stringent quality and aesthetic standards. So-called "second" or "third-category" fruits – those with aesthetic imperfections or irregular sizes – are often rejected despite being perfectly edible [3]. For example, in a study of apple suppliers, around 17–19% of production was culled for purely aesthetic reasons, such as blemishes or non-uniform appearance, yielding no financial benefit to growers [4]. In Europe, it is estimated that over one-third of fresh produce grown may never reach consumers because of aesthetic grading standards [5]. This practice not only represents a major economic loss for farmers and distributors but also entails significant environmental costs (greenhouse gas emissions, resource waste) associated with producing food that is ultimately never eaten. Therefore, it is a clear need for innovative strategies to re-utilize on-farm and retail fruit waste – particularly for "third-category" fruits that currently end up as waste – to improve food system sustainability.

Fermentation offers a promising pathway to valorize third category fruits and thereby mitigate food loss. In particular, vinegar production has emerged as an attractive waste-to-value strategy in the food industry [3]. Vinegar has been produced and utilized by humans since antiquity as a preservative and condiment, and it can be made from any sugary or starchy biomass via two successive fermentations: an alcoholic fermentation by yeasts, followed by an acetic acid fermentation by acetic acid bacteria (AAB) [6–8]. This process converts fruit sugars into a shelf-stable product, reducing fruit waste and creating a value-added food item. Notably, the acetic fermentation imparts strong preservative and organoleptic properties to the final product, meaning that even fruits of marginal fresh-market quality can yield acceptable vine-gar. Typically, vinegar classification relies on the source material used in its production [6]. It is widely produced from a range of raw materials, such as fruits, grains, and vegetables. Major categories are fruit vinegars, grain-based vinegars, alcohol-derived vinegars, and sugar or vegetable-based vinegars, produced either through traditional surface fermentation or modern submerged fermentation systems [9–11]. Fruits that are aesthetically unfit for sale can often be fermented into high-quality vinegars. Some case studies underscore the potential of this approach; for instance, citrus

processing byproducts (like bergamot orange peels and pulp) have been converted into vinegars rich in bioactive compounds [12].

Vinegars produced from fruits also retain many bioactive constituents of the raw fruit, such as organic acids (e.g. acetic, malic, citric), phenolic compounds, vitamins, and minerals, which together impart notable functional properties [13]. Studies have indicated that vinegars may have beneficial impacts on health, such as improving digestion, stimulating appetite, aiding in recovery from fatigue, reducing lipid levels, and regulating blood pressure [14–17]. A broad analysis of 23 different fruit vinegars found they contained diverse profiles of phenolics (e.g. gallic, chlorogenic, caffeic ac-ids) and organic acids, and exhibited considerable antioxidant capacities [18,19].

The choice of raw material used in vinegar production significantly influences the sensory and chemical characteristics of the final product [3]. Studies have been carried out on the production of vinegars from conventional fruits such as blueberry [20], strawberry [21], pomegranate [22], apple [23] and mango [24]. However, research is needed to evaluate vinegars from organic fruits. Italy is known for its robust organic market [25], where consumers have great enthusiasm for both locally sourced organic products and those with organic certification, showing a high level of awareness regarding organic standards [26]. This growth phase is indicative of the increasing demand for organic products, which has been attributed to various factors such as the rise in health and environmental awareness among consumers [27,28]. Organic farming has been recognized as a tool to reach the goals of sustainable production of food, as well as of improving livelihoods [29]. Despite extensive research on vinegar production from conventional fruits, limited studies have investigated the valorization of organic third category fruits, particularly peaches and clementines, through controlled fermentation processes.

Therefore, the aim of this study was to evaluate the feasibility of producing vinegar from organic third category apple, peach, and clementine fruits at laboratory scale. We sought to determine whether standard submerged fermentation processes (a yeast-driven alcoholic fermentation followed by AAB-driven acetic acid fermentation) can successfully convert these three fruits into vinegar, and to evaluate the fermentation dynamics, volatile organic compounds (VOCs), and sensory attributes of the produced fruit-based vinegars.

4.2 Materials and methods

4.2.1 Raw Materials and preparation of juices

Fresh organic apples (*Malus domestica*), peaches (*Prunus persica*), and clementine (*Citrus clementina*) fruits were purchased from supermarket located in Bari, Italy (NaturaSi) considering having a full physiological ripeness. The fruits were characterized by full color development and absence of microbial spoilage, but showed significant shape defects (e.g. bruised, undersized, or irregularly shaped). However, only fruits that were firm, aromatic, and free from mechanical damage were selected for vinegar production. Organic sugar and commercial unpasteurized organic apple vinegar ("Aceto di mele biologico", Alce Nero S.p.A., Bolzano, Italy) were also purchased from the same supermarket.

Fruits were cleaned with tap water, peeled and juices were prepared and filtered using a juice extractor (J80 Ultra, Robot coupe, France). pH and °Brix of juices were measured using pH meter (Denver Instrument, Denver, CO, USA) and Handheld refractometer (Mettler-Toledo GmbH, Switzerland), respectively. If needed, the juices were sweetened with organic sugar to achieve a standard concentration of 18 °Brix. Experimental plan is shown in **Figure 1**.

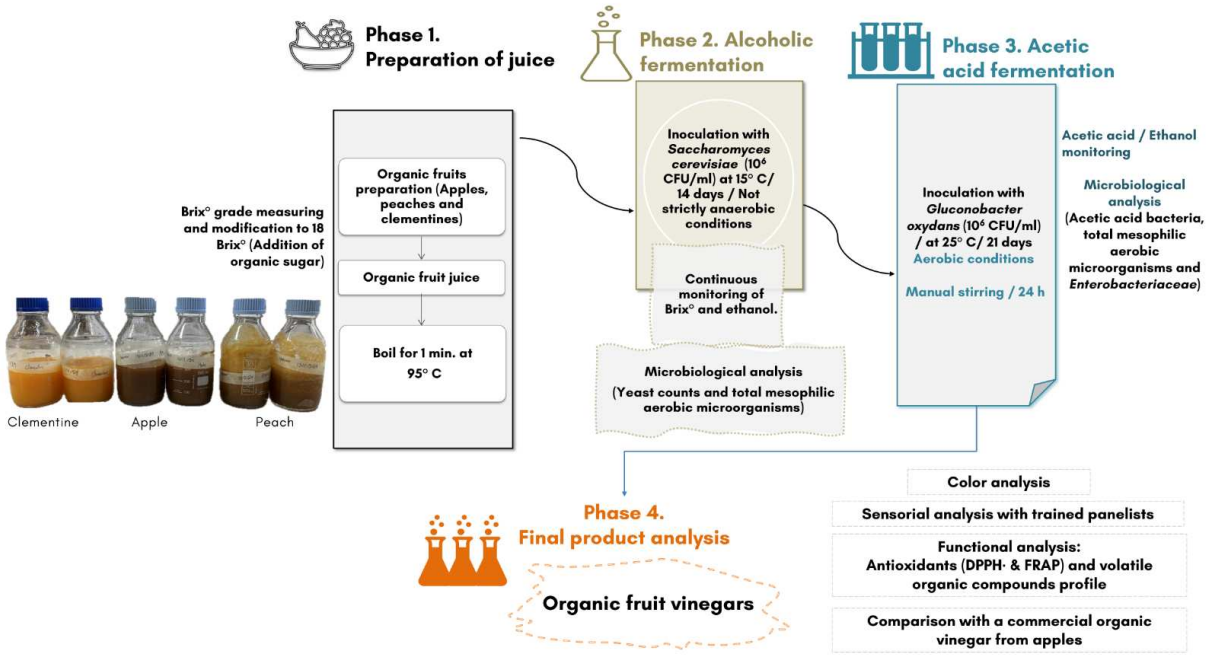


Figure 1. Experimental design for production of organic fruit vinegars from apples, peaches and clementines.

4.2.2 Microorganisms and culture conditions

Freeze dried *Saccharomyces cerevisiae* DSM 1848 and *Gluconobacter oxydans* DSM 7145 (both isolated previously from beer) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ), Science Campus Braunschweig-Süd, Germany.

S. cerevisiae DSM 1848 was routinely cultured in Yeast Malt (YM) medium (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L, pH 5.0±0.1) at 20 °C for 48 h. *G. oxydans* DSM 7145 was routinely cultured in Yeast Peptone Mannitol (YPM) medium (yeast extract 5 g/L, peptone 3 g/L, mannitol 25 g/L) at 25 °C for 48 h.

Prior to inoculation, colonies from each commercial strain were subjected to fermentation in each specific media to understand the growth kinetics. Microbiological analysis was performed for the yeast strain at 0, 3, 18, 21, 24, 27, 42, 45, and 48 hours of incubation and for bacterial strain at 0, 3, 6, 9, 24, 27, 30, 33, 48, 52 and 54 h of incubation. Growth kinetics were assessed also through spectrophotometry measuring absorbance values at a wave length of 620 nm.

4.2.3 Fermentation of fruit juices to vinegar

Juices (350 mL) from apples, clementines, and peaches were individually poured into sterilized 500 mL bottles and pasteurized at 100 °C for 1 min to reduce the native load of undesired microorganisms. After cooling to 20 °C, the juices were inoculated with *S. cerevisiae* at an initial cell density of 6 $\log_{10}$ CFU/mL. All fermentations were performed in triplicate.

The alcoholic fermentation was conducted at 20 °C for 14 days, a duration selected based on preliminary observations of ethanol stabilization typically occurring within this period in fruit-based fermentations. This ensured that an ethanol concentration of approximately 5% (v/v) was achieved, providing a sufficient substrate for the subsequent acetic fermentation while accounting for potential ethanol losses over time. Sugar and ethanol concentrations were monitored immediately after inoculation and then every 24 h. Additionally, samples were collected daily to determine yeast cell density and total mesophilic aerobic microorganisms.

For the secondary (acetous) fermentation, *G. oxydans* was added to the fermented juice at an initial cell density of 6 $\log_{10}$ CFU/mL. The acetous fermentation was carried out at 25 °C for 21 days. During this stage, ethanol and acetic acid concentrations were measured every 24 h, and the cell densities of AAB and total mesophilic aerobic microorganisms were determined daily as described below.

4.2.4 Determination of ethanol and acetic acid

Ethanol concentration in the fermenting juices was estimated through the Ethanol Assay Kit (K-ETOH, Megazyme Ltd., Wicklow, Ireland), according to the manufacturer instructions. Carbon dioxide was removed from juices through addition of 2 M NaOH, until the pH was adjusted to approximately 9. Juices were kept at room temperature for 30 min, then pH was measured again and corrected, if necessary. Afterwards, samples were diluted at a ratio of 1:50 using distilled water and used in the enzyme-based assay. Acetic acid concentration in the juices was determined using the Acetic Acid Assay Kit (K-ACET, Megazyme), following the manufacturer's instructions. Prior to analysis, samples were diluted with distilled water as necessary to ensure that acetic acid concentrations fell within the assay's linear detection range. The absorbance of the reaction mixtures prepared for both enzyme-based assays was measured at 340 nm using an Ultrospec 3000,

(Pharmacia Biotech, Uppsala, Sweden) spectrophotometer. Concentrations of ethanol and acetic acid were calculated using Megazyme Mega-Calc™ (www.megazyme.com), and expressed as % (v/v) for ethanol and % (w/v) for acetic acid.

4.2.5 Microbiological analyses

Presumptive yeasts and AAB, as well as total mesophilic aerobic microorganisms (TMA) in fruit juices, during and at end of fermentation, were enumerated through plate counts. Before plating, samples were serially diluted with saline solution (NaCl 9 g/L), and 1 mL aliquots were inoculated in Sabouraud Dextrose Agar (SDA) for yeasts, Glucose-Yeast Extract-Calcium Carbonate (GYC) agar (50 g/L glucose, 10 g/L yeast extract, 5 g/L calcium carbonate, and 20 g/L agar) for AAB, and Plate Count Agar for TMA. SDA and Plate Count Agar were inoculated through pour-plate, whereas GYC through spread plate technique. Plates were incubated at 30 °C for 48 h (yeasts and total aerobic microorganisms) or for 72 h (AAB). Additionally, total coliforms were enumerated at the beginning and end of acetous fermentation, to ensure the microbiological safety of the vinegar samples for consumption. Fermented juices and vinegars were pour-plated (as such and diluted) on Violet Red Bile Glucose Agar (VRBGA). Plates were examined after incubation at 37 °C for 24 h. SDA, Plate Count Agar, and VRBGA were purchased from Oxoid, whereas GYC was prepared at laboratory.

4.2.6 Color analysis

Instrumental color measurements of vinegar (experimental vinegars, as well as the commercial vinegar) were taken using a Konica Minolta CR-10 reader (Chiyoda, Tokyo, Japan) as described by Limongelli et al. [30]. The calibration of the instrument was performed by placing the tip of the color reader against a white blank paper. The analysis was performed according to the CIELab standard, namely using the L*, a*, and b* color space analysis approach, where L* stands for lightness (white to black) and a* (red to green) and b* (yellow to blue) for chromaticity coordinates.

4.2.7 Volatile organic compounds profiling

Experimental vinegars, namely juices from apples (AV), clementine (CV), and peaches (PV) at the end of acetous fermentation, were subjected to analysis of

Volatile Organic Compounds (VOCs). The commercial vinegar was used as reference. VOCs were profiled according to the method described by Hu et al. (2024) [31], with modifications reported by Limongelli et al. [30]. Briefly, 5 mL of vinegar, 0.5 g of NaCl, and 10 µL of internal standard solution (4-methyl-2-pentanol, final concentration 33 mg/L) were added to 20 mL glass vials, sealed with magnetic screw caps equipped with PTFE/silicone septa. VOCs were extracted through headspace solid-phase microextraction (HS-SPME) using a PAL autosampler system (CTC Analytics, Zwingen, Switzerland) equipped with a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, USA). Samples were equilibrated at 45 °C for 15 min, after which the fiber was exposed to the vial headspace at 45 °C for 45 min under continuous stirring. The extracted VOCs were thermally desorbed in the injection port of an Agilent 8890 GC system (Agilent Technologies, Santa Clara, USA) operated in split-less mode at 250 °C for 3 min. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The chromatographic separation was achieved using a DB-WAX capillary column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies) under the following oven temperature program: initial temperature 35 °C (8 min), increased to 60 °C at 4 °C/min, then to 160 °C at 6 °C/min, and finally to 200 °C at 20 °C/min, held for 15 min. Detection was performed with an Agilent 5977C GC/MSD quadrupole mass spectrometer, with the ionization source set at 250 °C, the MS transfer line at 230 °C, and electron ionization at 70 eV. Mass spectra were recorded over an m/z range of 34–350. Chromatograms were processed using the NIST 2008 mass spectral library for compound identification. Only peaks with an area greater than 1,000,000 and a match probability ≥ 85% were accepted, and manual verification of fragment patterns was performed when necessary. The relative concentration of each VOC was expressed as µg/g of 4-methyl-2-pentanol, calculated from the ratio between the peak area of each compound and that of the internal standard. All analyses were performed in triplicate.

4.2.8 Descriptive sensory analysis of vinegar samples

The experimental vinegar samples, as well as the commercial vinegar, were evaluated through descriptive sensory analysis by a panel of 10 trained assessors (aged 22–50) under controlled lighting and temperature conditions described by Wieczyńska and Cavoski (2018) [32] with some modifications. Each sample (20 mL)

was presented in randomized order in coded, odorless plastic transparent cups at room temperature (20 °C). Panelists assessed a total of 16 sensory attributes: visual (color intensity, clarity and viscosity), odor (intensity, alcoholic, floral, cider and citrus zest), taste (sweetness, acidity, bitterness, balance and astringency), mouthfeel (persistence and quality of taste) and overall acceptability. For each attribute, an unstructured 15 cm line scale was used, anchored at "low" (0 cm) and "high" (15 cm) intensity. Panelists were instructed to cleanse their palates with water between tasting different samples. The evaluation involved marking the scale to reflect the perceived intensity of each attribute, and the distance from the left end of the scale to the mark was recorded as the score. Panelists were given a lexicon to have a reference for the meaning of each attribute (**Table 1**).

Table 1. Lexicon developed for describing the sensorial attributes of the vinegar samples during the descriptive analysis

Aspect	Description
1.Visual Appearance	
a. Color Intensity	Varies from light to dark color.
b. Clarity	From clear to extremely cloudy.
c. Viscosity	Thickness of the vinegar, from light to viscous.
2. Aroma	
a. Intensity	Strength of the smell, from moderate to very intense.
b. Alcoholic	Alcohol smell, from absent to extremely alcoholic.
c. Floral	Related to peach, from slightly perceptible to extremely floral.
d. Cider	Related to apple, with a light acetone smell, from low to high intensity.
e. Citrus Zest	Spice linked to clementine, from absent to very intense.
3. Taste and Mouthfeel	
a. Sweetness	From low to high.
b. Acidity	From absent to extremely acidic.

c. Bitterness	From not bitter to extremely bitter.
d. Balance	Balance of ingredient flavors on the tongue, from absent to extremely balanced.
e. Astringency	From mild to moderate.
4. Aftertaste	
a. Persistence	Duration and intensity of the sensory impression, from short to long.
b. Final Quality	Evaluation of the impression left in the mouth after swallowing the vinegar.

4.2.9 Determination of antioxidant activity

In-vitro antioxidant activity of the vinegar samples (experimental and commercial) was assessed through two complementary assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radical scavenging assay and the ferric reducing antioxidant power (FRAP) assay. The DPPH· radical scavenging activity was determined as described by Caponio et al. [33], with modifications reported by Limongelli et al. [34]. Briefly, a 0.08 mM DPPH· solution was prepared in ethanol. An aliquot of 50 µL of each sample was added to 950 µL of the DPPH· solution in spectrophotometric cuvettes. After incubation in the dark for 30 min at 25 °C, the absorbance was measured at 517 nm using a spectrophotometer. A mixture containing 950 µL of DPPH· solution and 50 µL of 80% ethanol was used as the blank, while 50 µL of butylated hydroxytoluene (BHT) solution (0.45 g/L in 80% ethanol) served as the positive control. The free radical scavenging activity was calculated as follows:

DPPH· radical scavenging activity (%) = $[(A_{DPPH\cdot} - A_{sample}) / A_{DPPH\cdot}] \times 100$

where $A_{DPPH\cdot}$ is the absorbance of the control DPPH· solution and A_{sample} is the absorbance after reaction with the sample.

The FRAP assay was performed according to the procedure described by Son et al. [35], with modifications by Minervini et al. [36]. In brief, 200 µL of each sample were mixed with 200 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 200 µL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, followed by the addition of 200 µL of 10% (w/v) trichloroacetic acid to stop the reaction. After centrifugation (8000 × g, 10 min, 4 °C), 500 µL of the resulting

supernatant were combined with 400 µL of demineralized water and 100 µL of 0.1% (w/v) ferric chloride solution. After incubation for 10 min at 25 °C, the absorbance was measured at 700 nm. Ascorbic acid (1% w/v) was used as a positive control.

4.2.10 Statistical analysis

Analyses evaluating microbiological parameters, chemical composition (including acetic acid and ethanol concentrations), sensory attributes, color, and antioxidant activity were conducted in triplicate, and results are reported as mean ± standard deviation. Statistical analyses were performed using SPSS Statistics version 28. Data was subjected to one-way analysis of variance (ANOVA) to identify significant differences among samples. When ANOVA revealed significant effects ($p < 0.05$), means were compared using Tukey's Honestly Significant Difference (HSD) post hoc test. Data from HS-SPME-GC/MS were analyzed through principal component analysis (PCA) to evaluate differences in VOCs profiles among vinegar samples. Additionally, a heatmap with cluster analysis was generated using the "pheatmap" package in R [37].

4.3 Results

4.3.1 Growth kinetics of yeasts and bacteria

The initial yeast density for *s. cerevisiae* DSM 1848 was 6.17 log10 CFU/ml (**Figure 2**). A steady increase in yeast count was observed over time. After 18 h, DSM 1848 reached 7.71 log10 CFU/ml, indicating the overcome of the exponential growth phase. DSM 1848 reached its highest cell density (8 log10 CFU/ml) after 42 h. A slight decline was observed. The results were useful to get information for producing an adequate concentration of microbial starter for ethanol fermentation of juices.

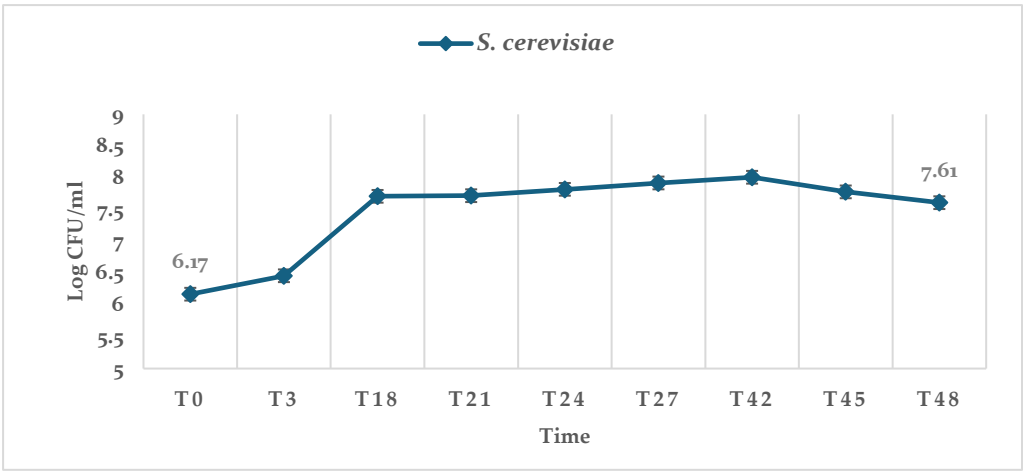


Figure 2. Growth kinetics of *S. cerevisiae* DSM 1848 during fermentation of 2 days. Results are shown as mean of 3 replicates, and were expressed as mean ± standard deviation.

The growth of *Gluconobacter oxydans* DSM 7145 was monitored over a 54-hour period **(Figure 3).** Initially, the bacterial load was 8.11 $\log_{10}$ CFU/ml. A gradual increase followed, with the bacterial count reaching 8.31 $\log_{10}$ cfu/ml after 6 h and 8.44 $\log_{10}$ CFU/ml after 9 h. A notable increase in microbial density was observed after 24 h with 8.91 $\log_{10}$ CFU/ml and 9.14 $\log_{10}$ CFU/ml after 27 h. The highest bacterial load was recorded after 33 h of cultivation, where the count peaked at 9.42 $\log_{10}$ CFU/ml indicating the overcome of the exponential growth phase. After 33 h, a slight decline occurred, with values decreasing to 9 $\log_{10}$ CFU/ml until 8.62 $\log_{10}$ CFU/ml at 54 h. These results indicate that *G. oxydans* exhibited steady growth up until 33 h, after which a decline occurred, suggesting entry into a stationary or death phase.

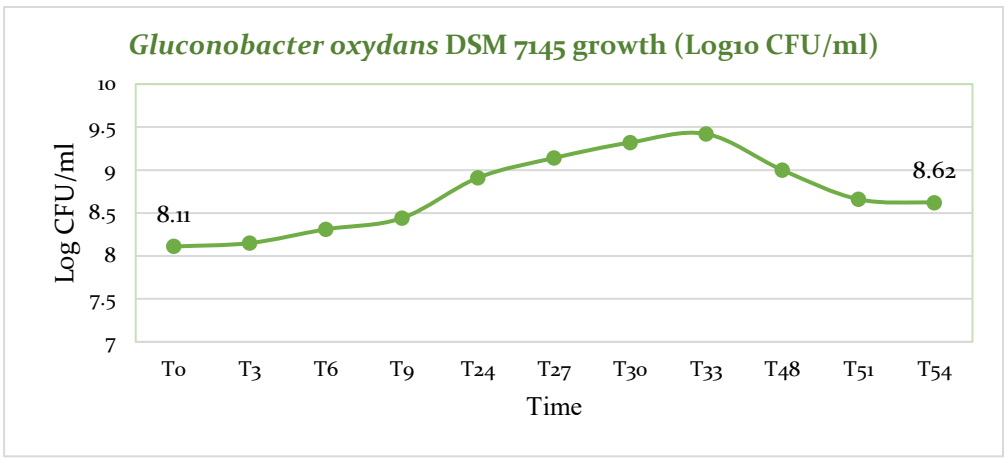


Figure 3. Growth kinetics of *G. oxydans* DSM 7145 during fermentation. Results are shown as mean of 3 replicates, and were expressed as mean ± standard deviation.

The absorbance data for DSM 1848 and DSM 7145 strains measured at 620 nm during fermentation correlate with the previous yeast and bacterial growth data in CFU (**Table 2 and 3**). For yeast strain, DSM 1848 rapid increase in absorbance was observed during the early phase of fermentation, indicating exponential growth, which mirrors the CFU data where yeast counts rose significantly between T3 and T27. The strain reached a peak absorbance by T42-T45, after which a slight decline was observed, corresponding to the stabilization in CFU values and suggesting the onset of the stationary phase. Similarly, DSM 7145 displayed a slower, more gradual increase in. The absorbance peaked at T48-T54, consistent with its CFU data, where the bacterial strain continued growing until approximately 48 hours before plateauing.

Table 2. Absorbance of *S. cerevisiae* DSM 1848 during fermentation for 2 days.

Time	Absorbance
To	0.214
T3	0.352
T18	1.329
T21	1.163
T24	1.345
T27	1.41
T42	1.702
T45	1.699
T48	1.68

Table 3. Absorbance of bacteria *G. oxydans* DSM 7145 during fermentation for 54 hours at 620 nm.

Time	Absorbance
To	0.113
T3	0.157
T6	0.30
T9	0.35
T24	0.46

T27	0.50
T30	0.80
T33	1.03
T48	1.09
T51	1.11
T54	1.11

4.3.2 Alcoholic fermentation occurred for all the fruit juices

Variations in °Brix, ethanol concentration, yeasts and TMA counts were used to monitor the alcoholic fermentation of apple (AJ), peach (PJ), and clementine (CJ) juices (**Figure 4**). On day four, sugar concentrations already declined from 18.0 °Brix to 16.1, 12.2, and 14.0 °Brix for AJ, PJ and CJ, respectively. Afterwards, sugar continued to decline and on day 14, the values decreased to 6 - 7.4 °Brix, with PJ showing the lowest value.

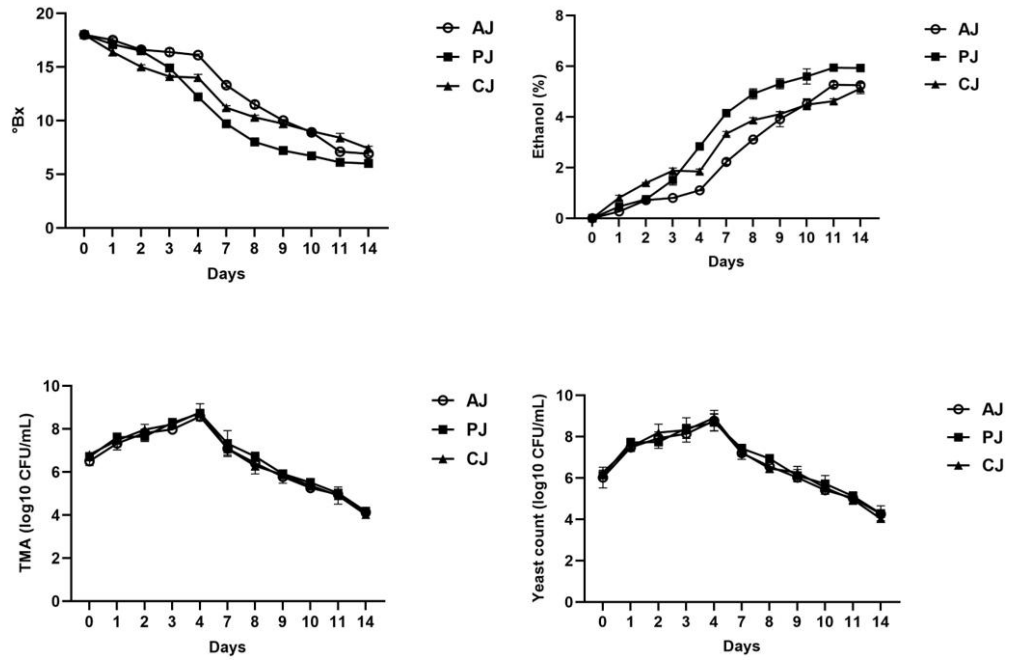


Figure 4. Alcoholic fermentation kinetics, expressed as °B, concentration of ethanol and microbial cell densities, expressed as log10 CFU/mL, of fruit juices from clementine (A), apple (B), and peach (C) during 14 days of alcoholic fermentation. Results are shown as mean values of 3 replicates ±standard deviation.

Ethanol concentration increased steadily, reaching 5.25 ± 0.11 % (AJ), 5.94 ± 0.08 % (PJ), and 5.12 ± 0.15 % (CJ), on day 14 (**Figure 4**). Yeasts increased sharply during the initial fermentation phase, reaching their maximum at day four (8.7 – 8.9 $\log_{10}$ CFU/mL). Subsequently, they declined gradually to 4.0 – 4.2 $\log_{10}$ CFU/mL on day 14. TMA counts followed the same trend as yeasts (**Figure 4**).

4.3.3 Acetic fermentation showed similar trends for all the juices

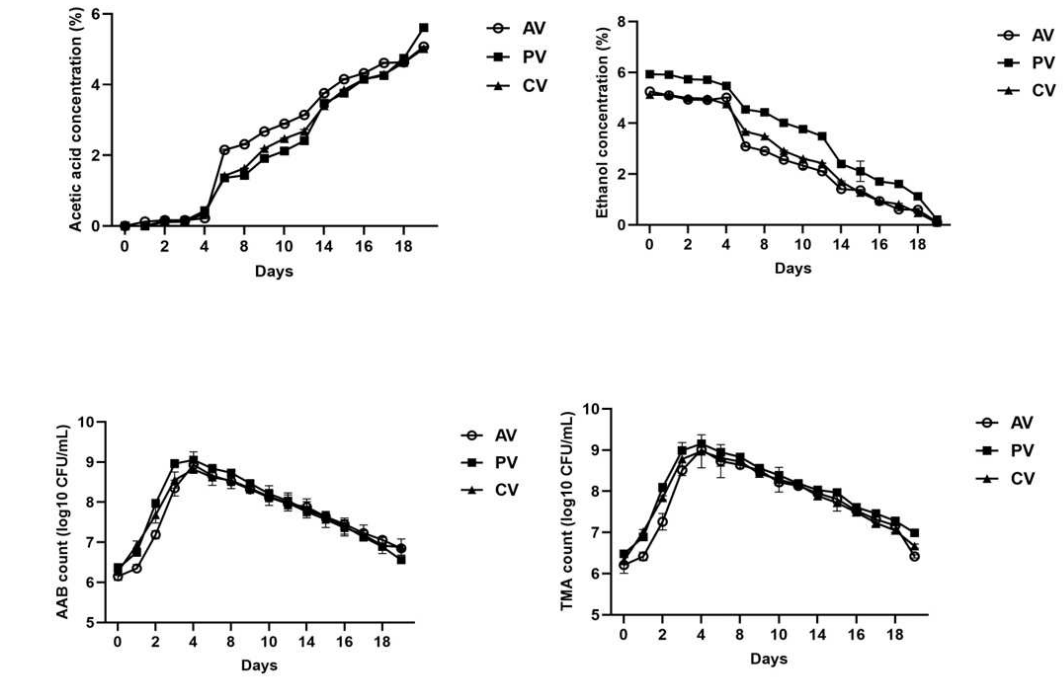


Figure 5. Acetic acid fermentation dynamics, expressed as concentrations of ethanol and acetic acid, and microbial cell densities, expressed as log10 CFU/mL, in vinegars from clementine (A), apple (B), and peach (C) during 21 days of acetous fermentation. Results are shown as mean values of 3 replicates ±standard deviation.

After inoculation with AAB, ethanol concentrations decreased progressively throughout fermentation ($p < 0.05$), confirming the oxidative activity of *G. oxydans* on that substrate (**Figure 5**). While in CV and AV we found that ethanol concentration overall stood quite constant during the first four days, in PV ethanol started to decrease in quite a linear trend from the first day of acetous

fermentation. At the end of fermentation (day 21), ethanol reached values ranging from 0.09 (CV) to 0.21 (PV) %.

After inoculation of *G. oxydans*, we found that in PV, on day three, AAB population reached a cell density in the order of 8 $\log_{10}$ CFU/mL (**Figure 5**), in line with the more rapid decrease of ethanol and increase of acetic acid observed for this vinegar. Then, in all the vinegars the highest values of cell density of AAB were reached on day four, ranging from 8.8± 0.2 $\log_{10}$ CFU/mL (CV) to 9.5 ± 0.2 $\log_{10}$ CFU/mL (PV). Afterwards, gradual declines were observed, with final counts ranging from 6.6 ± 0.2 (PV) to 6.9 ± 0.1 (CV) $\log_{10}$ CFU/mL. TMA counts followed similar kinetics to AAB population (**Figure 5**). Total coliforms were below the detection limit at the beginning of fermentation and at its end (21 days) in any of the vinegars.

4.3.4 Color indexes greatly varied depending on the fruit matrix

Instrumental color analysis was performed for all the three experimental vinegars (CV, AV, PV), as well as for the commercial organic apple vinegar. The latter exhibited the highest ($p < 0.05$) L^* value, corresponding to the brightest appearance (**Table 4**). The lowest values of L^* index were found for PV and CV. The a^* parameter, representing the red–green axis, significantly ($p < 0.05$) varied across vinegars. PV showed the highest redness, followed by CV and AV. For the b^* parameter, which reflects the yellow–blue component, PV also exhibited the highest yellowness, followed by AV and commercial vinegar (**Table 4**).

Table 4. Values of color indexes found for clementine (CV), apple (AV), peach (PV), and commercial organic apple vinegars. Values are expressed as mean ± standard deviation (n = 3). Different superscript letters a-d within the same column indicate significant differences ($p < 0.05$).

Vinegar type	*L**	*a**	*b**
CV	54.17 ± 0.34^{c}	4.33 ± 0.28^{b}	19.85 ± 0.53^{d}
AV	55.49 ± 0.25^{b}	2.94 ± 0.54^{c}	24.65 ± 0.48^{b}
PV	53.15 ± 0.53^{c}	6.73 ± 0.36^{a}	26.16 ± 0.22^{a}
Commercial	58.46 ± 0.65^{a}	1.83 ± 0.71^{d}	21.6±0.16c

4.3.5 VOCs profiles were affected by fruit matrix

A total of 61 VOCs were identified across the vinegar samples (the three experimental vinegars and the commercial one), encompassing alcohols, aldehydes, carboxylic acids, esters, hydrocarbons, ketones, phenols, terpenes, and miscellaneous compounds (**Table 5**). Quantitatively, AV showed a VOC profile dominated by branched-chain higher alcohols such as 3-methyl-1-butanol (3.22 ± 1.07 μg/g) and 2-methyl-1-butanol (2.49 ± 0.55 μg/g), together with elevated medium-chain fatty acids including hexanoic (2.90 ± 0.35 μg/g), octanoic (4.32 ± 0.31 μg/g), decanoic (4.73 ± 0.33 μg/g), and dodecanoic acids (1.73 ± 0.10 μg/g). Corresponding ethyl esters such as ethyl hexanoate (0.85 ± 0.09 μg/g), ethyl tetradecanoate (0.29 ± 0.01 μg/g), and ethyl hexadecanoate (0.42 ± 0.06 μg/g) were also significantly ($p < 0.05$) higher in AV compared with the other vinegars (**Table 5**). CV displayed a markedly different VOCs profile, with high levels of monoterpenes and phenolic compounds including alpha-Terpineol (4.47 ± 345 0.24 μg/mL), linalool (0.23 ± 0.03 μg/mL), phenylethyl alcohol (5.33 ± 2.01 μg/mL) and 2,4- 346 bis(1,1-dimethylethyl)-phenol (4.25 ± 3.69 μg/mL). These volatiles, which contribute to citrus and floral aromatic characteristics, were largely absent or present at trace levels in the other vinegars. PV exhibited an aldehyde- and acetate-ester-dominated pattern. Benzaldehyde (4.58 ± 0.30 μg/g), furfural (1.46 ± 0.09 μg/g), ethyl acetate (2.06 ± 0.12 μg/g), and benzoic acid (1.56 ± 0.29 μg/g) were among its most abundant VOCs. The commercial apple vinegar displayed the simplest VOCs profile, dominated by acetic acid (5.70 ± 1.42 μg/g). Phenylethyl

alcohol (4.14 ± 2.01 μg/g) was the only higher alcohol present at notable levels (**Table 5**).

Table 5. Concentrations (in μg/mL of 4-methyl-2-pentanol) of Volatile Organic Compounds (VOCs), in clementine (CV), apple (AV), peach (PV), and commercial organic apple vinegars. Values are expressed as mean ± standard deviation (n = 3). Different superscript letters a-d within the same column indicate significant differences ($p < 0.05$).

Class	Compound	AV	CV	PV	Commercial
Alcohols (10)	Benzyl Alcohol	0.052 ± 0.019^{a}	0.168 ± 0.054^{a}	1.214 ± 0.101^{a}	0.001 ± 0.002^{b}
	1-Dodecanol	0.051 ± 0.005^{a}	0.075 ± 0.008^{a}	0.17 ± 0.04^{a}	0.103 ± 0.034^{a}
	1-Butanol, 3-methyl-	3.222 ± 1.067^{a}	n.d.*	1.529 ± 1.044^{b}	0.912 ± 0.712^{b}
	1-Butanol, 3-methyl-, acetate	3.931 ± 2.845^{a}	0.526 ± 0.109^{c}	1.716 ± 0.239^{b}	1.831 ± 0.544^{b}
	1-Butanol, 2-methyl-	2.492 ± 0.546^{a}	2.131 ± 0.417^{a}	n.d.	1.451 ± 0.169^{b}
	Phenylethyl Alcohol	1.76 ± 0.92^{c}	5.332 ± 2.011^{a}	2.091 ± 0.234^{c}	4.143 ± 2.012^{b}
	Ethanol	0.713 ± 0.182^{a}	n.d.	0.127 ± 0.01^{a}	0.558 ± 0.375^{a}
	1-Propanol, 2-methyl-	0.341 ± 0.047^{a}	0.198 ± 0.032^{a}	0.321 ± 0.076^{a}	0.169 ± 0.083^{a}
	2-Butanol	0.006 ± 0.009^{a}	n.d.	0.015 ± 0.002^{a}	n.d.
	Alpha-Terpineol	0.128 ± 0.009^{b}	4.474 ± 0.24^{a}	0.134 ± 0.022^{b}	0.042 ± 0.038^{b}
Aldehydes (6)	Benzaldehyde	0.03 ± 0.002^{b}	0.032 ± 0.004^{b}	4.58 ± 0.299^{a}	1.029 ± 0.104^{b}
	Nonanal	0.161 ± 0.032^{a}	0.154 ± 0.019^{a}	0.59 ± 0.011^{a}	0.15 ± 0.022^{a}
	Butanal, 3-methyl-	n.d.	0.009 ± 0.002^{a}	0.069 ± 0.011^{a}	0.006 ± 0.006^{a}
	Hexanal	0.005 ± 0.001^{a}	n.d.	0.109 ± 0.013^{a}	n.d.
	Acetaldehyde	n.d.	n.d.	0.001 ± 0.001^{a}	0.001 ± 0.002^{a}
	Furfural	n.d.	n.d.	1.461 ± 0.092^{a}	0.821 ± 0.017^{a}
Carboxylic Acids (18)	Benzeneacetic acid	0.695 ± 0.153^{a}	0.355 ± 0.069^{a}	0.359 ± 0.095^{a}	0.154 ± 0.014^{a}
	Butanoic acid	0.218 ± 0.021^{a}	0.362 ± 0.028^{a}	0.087 ± 0.009^{a}	0.055 ± 0.006^{a}
	Pentanoic acid	0.018 ± 0.009^{a}	0.328 ± 0.016^{a}	0.016 ± 0.001^{a}	0.009 ± 0.002^{a}
	Acetic acid, 2-methylpropyl ester	2.362 ± 0.329^{b}	0.541 ± 0.03^{a}	1.713 ± 0.158^{b}	2.225 ± 0.22^{b}

	Heptanoic acid	0.038 ± 0.004[a]	0.123 ± 0.013[a]	0.052 ± 0.009[a]	0.013 ± 0.012[a]
	Nonanoic acid	0.126 ± 0.023[a]	0.367 ± 0.025[a]	0.097 ± 0.029[a]	0.159 ± 0.036[a]
	Octanoic Acid	4.319 ± 0.306[a]	4.604 ± 0.301[a]	3.976 ± 0.502[a]	4.217 ± 0.795[a]
	Ethyl Acetate	0.228 ± 0.016[b]	0.016 ± 0.027[b]	2.056 ± 0.119[a]	0.489 ± 0.055[b]
	Hexanoic acid	2.901 ± 0.348[a]	1.811 ± 0.123[b]	3.421 ± 0.396[a]	2.119 ± 0.545[b]
	Dodecanoic acid	1.728 ± 0.101[a]	0.114 ± 0.016[b]	0.119 ± 0.047[b]	0.145 ± 0.025[b]
	n-Decanoic acid	4.728 ± 0.33[a]	0.488 ± 0.006[b]	1.573 ± 0.206[b]	0.447 ± 0.063[b]
	Acetic acid	3.461 ± 2.346[b]	2.299 ± 1.448[c]	3.816 ± 2.529[b]	5.698 ± 1.42[a]
	Benzoic Acid	0.604 ± 0.037[a]	0.145 ± 0.02[b]	1.557 ± 0.288[a]	0.057 ± 0.02[b]
	Propanoic acid, 2-methyl-, 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.994 ± 0.107[a]	1.18 ± 0.033[a]	1.389 ± 0.059[a]	1.424 ± 0.083[a]
		0.329 ± 0.104[a]	0.594 ± 0.053[a]	0.493 ± 0.046[a]	0.315 ± 0.05[a]
	Dibutyl phthalate	0.313 ± 0.172[a]	0.307 ± 0.049[a]	0.393 ± 0.046[a]	0.245 ± 0.064[a]
	Benzoic acid, methyl ester	0.004 ± 0.003[a]	0.005 ± 0.004[a]	0.188 ± 0.016[a]	n.d.
	Eugenol	0.5 ± 0.035[a]	0.013 ± 0.003[a]	0.863 ± 0.143[a]	0.01 ± 0.003[a]
Carboxylic Ester (9)	Propanoic acid, ethyl ester	0.079 ± 0.008[a]	n.d.	0.009 ± 0.001[a]	0.018 ± 0.007[a]
	Octanoic acid, ethyl ester	0.168 ± 0.019[a]	0.166 ± 0.013[a]	0.031 ± 0.01[a]	0.207 ± 0.106[a]
	Hexanoic acid, ethyl ester	0.849 ± 0.086[a]	0.253 ± 0.006[a]	0.049 ± 0.004[a]	0.456 ± 0.02[a]
	Acetic acid, butyl ester	0.053 ± 0.008[a]	n.d.	0.003 ± 0.001[a]	n.d.
	Tetradecanoic acid, ethyl ester	0.29 ± 0.013[a]	n.d.	n.d.	n.d.
	Acetic acid, phenylmethyl ester	0.141 ± 0.015[b]	0.041 ± 0.003[c]	1.85 ± 0.246[a]	0.034 ± 0.003[c]

	Acetic acid, hexyl ester	0.385 ± 0.039[a]	n.d.	0.044 ± 0.004[a]	0.009 ± 0.008[a]
	Hexadecanoic acid, ethyl ester	0.419 ± 0.062[a]	n.d.	n.d.	n.d.
	Benzoic acid, ethyl ester	0.284 ± 0.024[a]	0.063 ± 0.005[a]	0.078 ± 0.008[a]	0.004 ± 0.002[a]
Hydrocarbons (6)	Styrene	0.027 ± 0.01[a]	0.031 ± 0.007[a]	0.03 ± 0.006[a]	n.d.
	Benzene, 1,3,5-trimethyl-	n.d.	0.009 ± 0.003[a]	n.d.	n.d.
	Benzene, 1,2,3,5-tetramethyl-	n.d.	0.005 ± 0.002[a]	n.d.	n.d.
	Phenol, 3-ethyl-	0.073 ± 0.008[a]	0.261 ± 0.451[a]	n.d.	n.d.
	Benzene, 1-ethyl-2,4-dimethyl-	n.d.	0.013 ± 0.023[a]	n.d.	n.d.
	Benzene, 1,2,4,5-tetramethyl-	n.d.	0.001 ± 0.002[a]	n.d.	n.d.
Ketones (3)	Methyl Isobutyl Ketone	0.029 ± 0.011[a]	0.108 ± 0.003[a]	0.056 ± 0.012[a]	0.048 ± 0.017[a]
	Acetoin	2.193 ± 0.578[a]	1.976 ± 0.371[a]	2.521 ± 0.116[a]	2.565 ± 1.506[a]
	Acetophenone	0.022 ± 0.003[a]	0.02 ± 0.001[a]	0.054 ± 0.003[a]	0.006 ± 0.003[a]
Phenol (2)	Phenol, 2-ethyl-	0.066 ± 0.006[a]	0.79 ± 0.075[a]	0.247 ± 0.033[a]	0.009 ± 0.004[a]
	Phenol, 2,4-bis(1,1-dimethylethyl)-	0.017 ± 0.013[b]	4.254 ± 3.686[a]	0.312 ± 0.049[b]	0.455 ± 0.353[b]
Terpenes (3)	Benzene, 1-methyl-3-(1-methylethyl)-	0.001 ± 0.001[a]	0.043 ± 0.003[a]	0.011 ± 0.002[a]	0.001 ± 0.001[a]
	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	n.d.	0.034 ± 0.002[a]	n.d.	n.d.
	Linalool	0.011 ± 0.006[a]	0.227 ± 0.032[a]	0.043 ± 0.013[a]	n.d.
Others (3)	Acetic acid, 1-methylpropyl ester	0.061 ± 0.021[a]	0.001 ± 0.002[a]	0.07 ± 0.015[a]	0.001 ± 0.002[a]
	Pyrazine, trimethyl-	n.d.	n.d.	0.027 ± 0.002[a]	n.d.

Disulfide, dimethyl	n.d.	n.d.	n.d.	0.002 ± 0.001[a]

[a-d] Within the same row, mean values ± standard deviation (n = 3) with different superscript letters indicate significant differences ($p < 0.05$), two-way ANOVA with Tukey's post hoc test.

Quantitative data of VOCs combined with multivariate analysis revealed clear compositional differences among the four vinegar types, as illustrated by the PCA (**Figure 6**). In detail, the first two principal components of the PCA explained about 66% of the total variance. Using those two components, the vinegar types were clearly distinguished. Interestingly, AV and the commercial organic apple vinegar were in the same quadrant of the plain. CV was well-separated from the commercial vinegar, mainly associated with presence of terpenic (e.g., alpha-terpeneol) and phenolic (e.g. 2,4-bis(1,1-dimethylethyl)-phenol) compounds, which are characteristic of citrus-derived matrices. PV was very well distinguished from CV, because of its higher content of benzenoid compounds, namely benzaldehyde and benzyl alcohol, typically associated with stone-fruits.

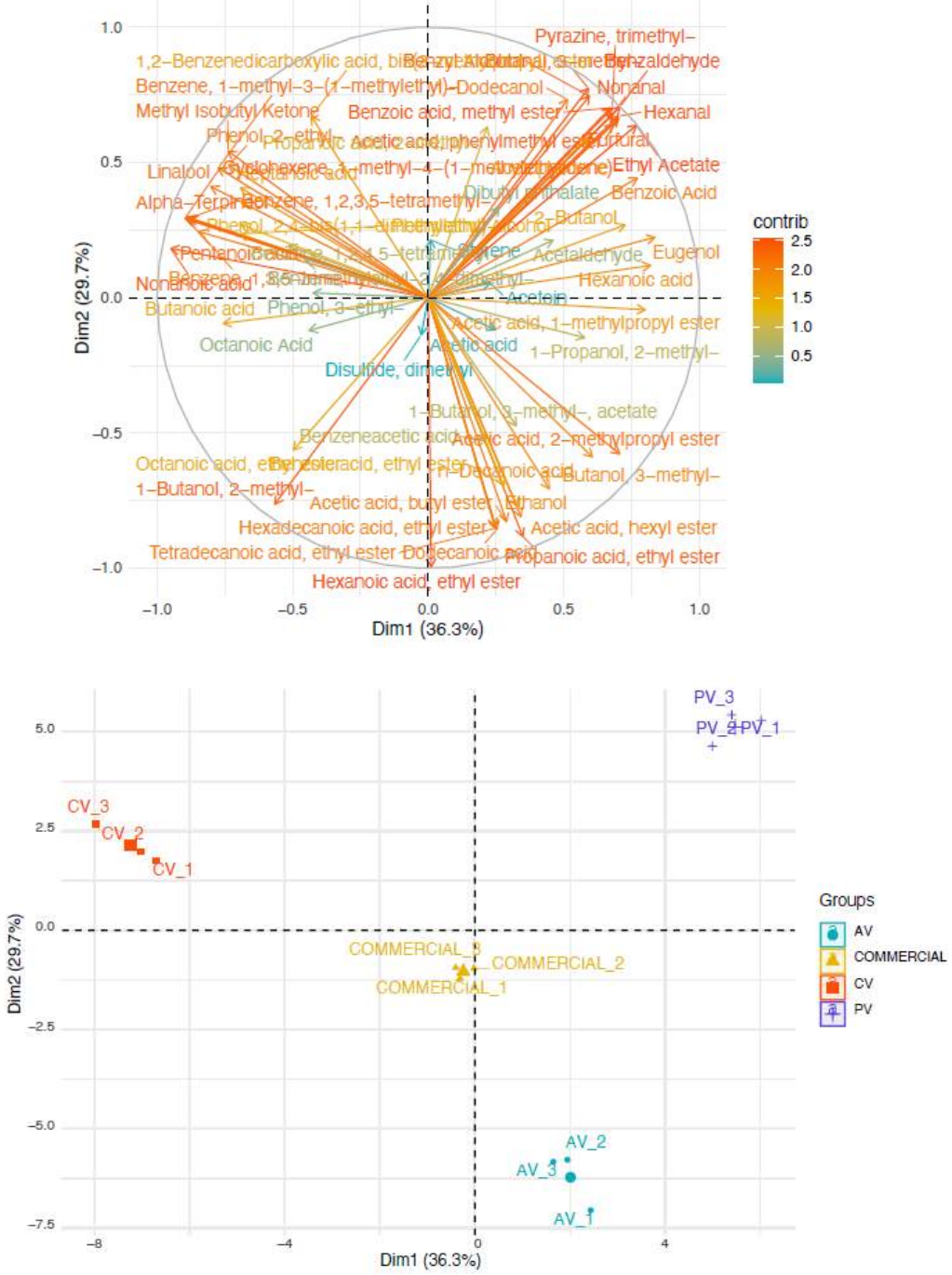


Figure 6. PCA of VOCs of apple vinegar (AV_1, AV_2, AV_3), peach vinegar (PV_1, PV_2, PV_3), clementine vinegar (CV_1, CV_2, CV_3), and commercial apple vinegar. Results are shown as mean of 3 replicates.

To better highlight the differences of VOCs profile among the vinegars, a clustered heatmap was built using just those compounds that showed at least one statistically significant difference (**Figure 7**).

AV samples were clustered because of their relative richness in medium-chain fatty acids, 1-buthanol,2-methyl, 1-buthanol,3-methyl, and acetic acid,2-methylpropyl ester. PV was characterized by high contents of hexanoic and benzoic acids, ethyl acetate and phenylmethyl ester of acetic acid, furfural, benzaldehyde, and benzoic acid. CV samples clustered together because of their relatively high levels of alpha-terpeneol. Overall, commercial vinegar exhibited a balanced but acetic-acid-centered VOCs profile (**Figure 7**).

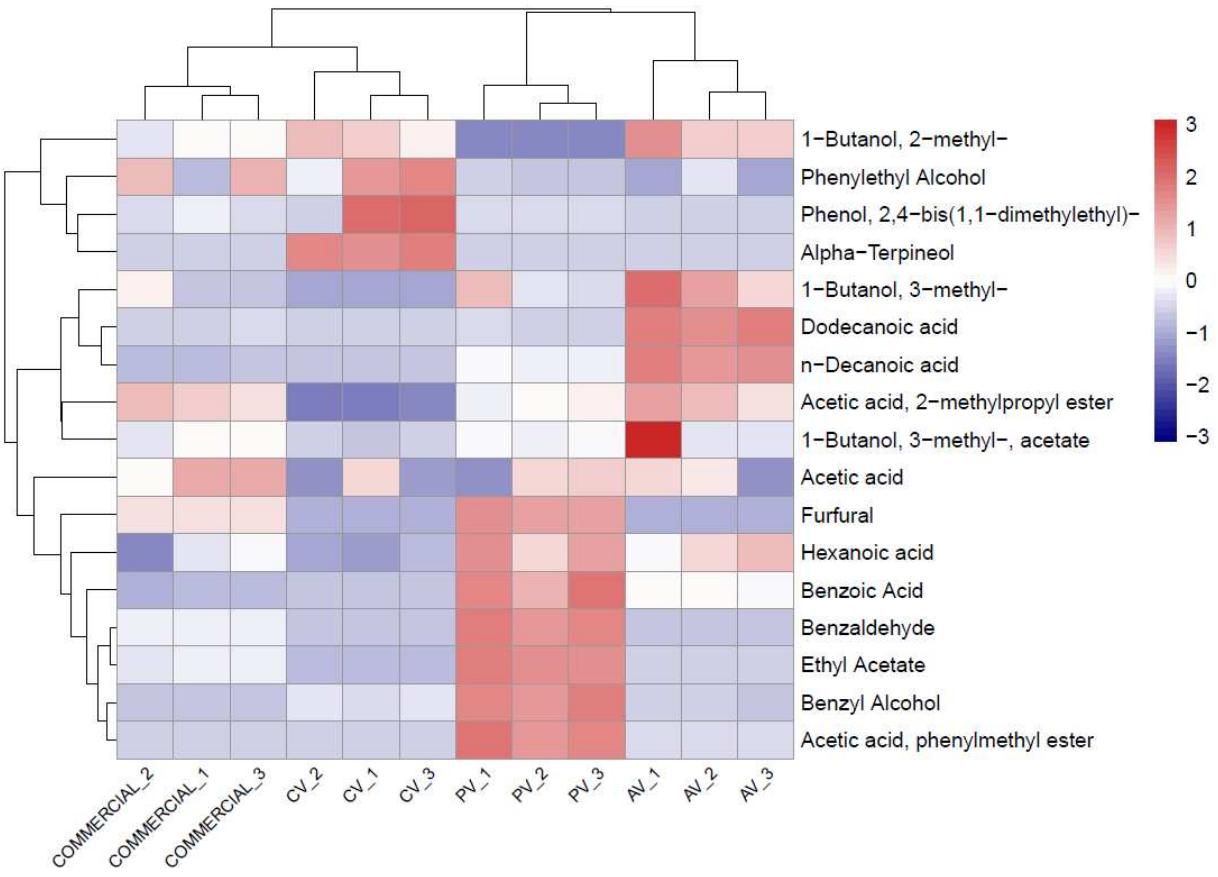


Figure 7. Heatmap of statistically significant VOCs (p-value<0.05) VOCs in apple vinegar (AV_1, AV_2, AV_3), peach vinegar (PV_1, PV_2, PV_3), clementine vinegar (CV_1, CV_2, CV_3), and commercial apple vinegar. Results are shown as mean of 3 replicates.

4.3.6 Descriptive sensorial analysis

The descriptive sensory analysis revealed some differences among the vinegar samples (**Figure 8**), especially in terms of color intensity, aroma characteristics, taste attributes, and overall acceptability. PV recorded the highest color (9.5 ± 0.9) and aroma (10.7 ± 0.9) intensity, together with the strongest floral perception (5.4 ± 1.2). Despite this, PV obtained the lowest acceptability score (5.7 ± 1.4), suggesting that those intense attributes were not uniformly favored by panelists. CV was characterized by a higher sweetness perception (6.3 ± 0.8) and a notable citrus zest note (5.1 ± 0.9). It also achieved the highest acceptability rating (8.0 ± 0.7), indicating a favorable balance of taste and aroma. AV was perceived as the most acidic (7.5 ± 0.5) and its overall acceptability remained moderate (6.5 ± 0.8). Among all the vinegars, the commercial one showed comparatively lower color intensity (2.7 ± 0.4) and aroma intensity (4.9 ± 0.6) but exhibited a balanced sensory profile. It achieved high acceptability (7.2 ± 0.4) and good taste quality (6.8 ± 0.6), reflecting its role as a standard reference with consistent and familiar sensory characteristics.

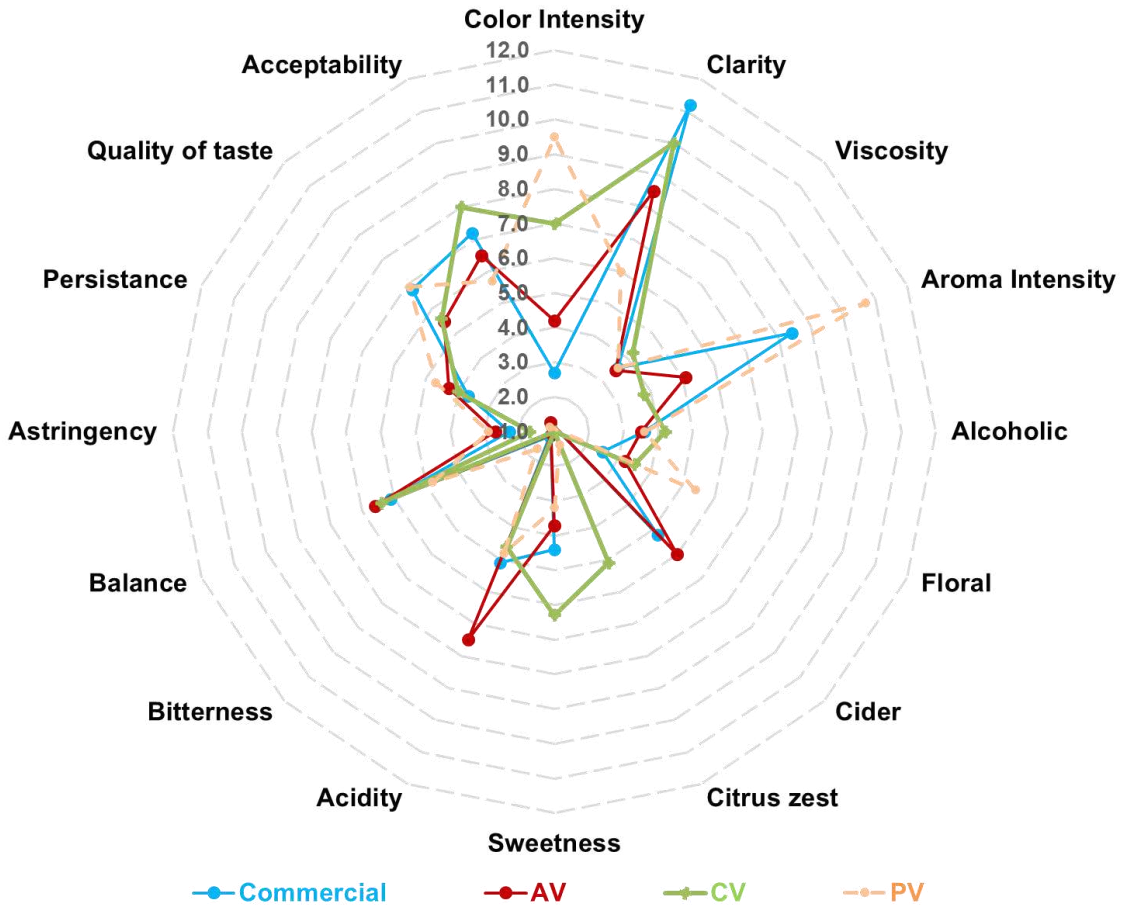
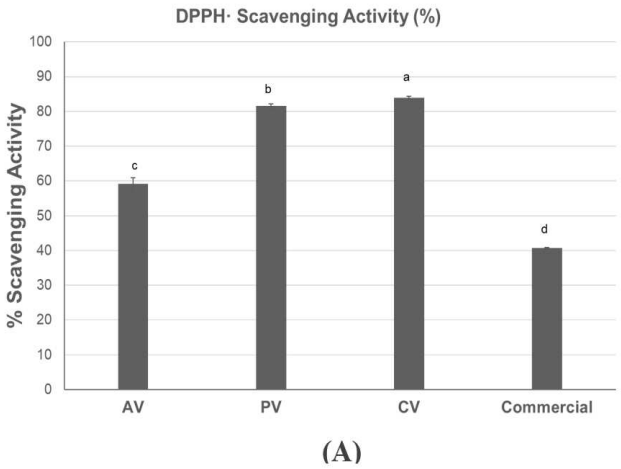


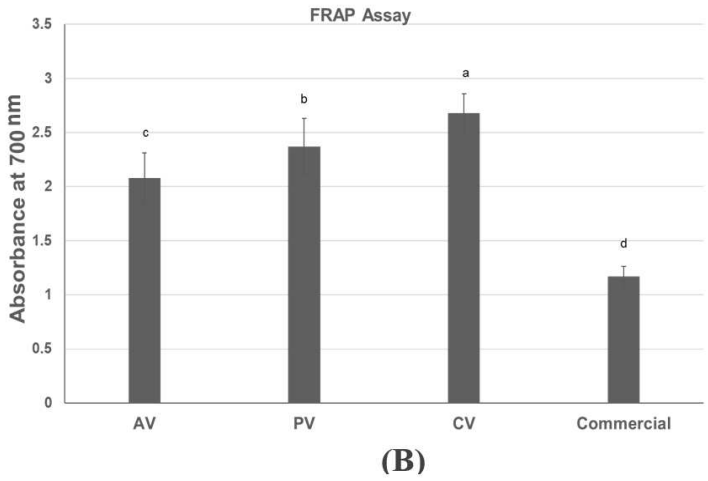
Figure 8. Descriptive sensory analysis of organic vinegars from apple (AV), clementine (CV), and peach (PV) and of commercial organic apple vinegar. Data are the means of scores attributed by nine trained panelists.

4.3.7 Antioxidant activity

The *in vitro* antioxidant capacities of the different vinegar samples were evaluated in terms of radical scavenging activity and capacity to reduce ferric to ferrous ions. CV exhibited the highest radical scavenging activity (≈84%), followed closely by PV with ≈81.5% (**Figure 9A**). AV showed moderate activity (≈59%), while the commercial apple vinegar displayed the lowest scavenging effect (≈41%).



Similarly, the FRAP assay (**Figure 9B**) revealed a consistent trend with the DPPH· results. CV showed the highest ($p < 0.05$) reducing power, with an absorbance of 2.68 U.A.±0.18 followed by PV (2.37 U.A.±0.26) and AV (2.08 U.A.±0.23). The commercial vinegar again showed the lowest ($p < 0.05$) ferric reducing ability (1.19 U.A.±0.09).



Figures 9A & 9B. *In vitro* antioxidant activity of apple (AV), peach (PV), clementine (CV), and commercial vinegars, determined by DPPH· (A) and FRAP (B) assays. Results are shown as mean of 3 replicates, and were expressed as mean ± standard deviation. Different lowercase letters above the bars (a-d) indicate statistically significant ($p < 0.05$) differences.

4.4 Discussion

Vinegar from fruit has attracted attention of researchers, producers, and consumers, because of peculiar sensory traits and possible benefits to human health [3,38]. The characteristics of the raw material, microbial species involved, and fermentation conditions strongly influence process efficiency and final product quality [39]. Among those drivers, fruit characteristics of course play a pivotal role. In this study, we compared the susceptibility of three different fruits to be processed into vinegar, using the same microorganisms (namely *S. cerevisiae* and *G. oxydans*) and fermentation conditions, including initial concentration of sugars. All the fruits were third category fruits from organic farming. Fruits belonging to this category are destined to be wasted with higher probability than fruits of higher quality. We chose apples because they are the most studied for fruit vinegar, whereas clementines and peaches were considered because they are not commonly, or at all, used for vinegar production [2].

Alcoholic fermentation of all the juices proceeded rapidly during the first four days and on day 14 yielded adequate ethanol concentration (4-6%) to start and complete the secondary fermentation. This result was in agreement with previous research about high-sugar fruit substrates [40,41]. Ethanol concentration at the end of the first fermentation is affected by level of fermentable sugars in juice and metabolic characteristics of the yeast used [3]. We found slight differences in ethanol at the end of alcohol fermentation of the juices subjected to study, with PJ reaching the highest level. This likely reflects differences in micronutrient availability across juices, which would modulate *S. cerevisiae* metabolism and ethanol yield.

Acetic acid formation kinetics followed a similar pattern among fermented fruit juices. However, in PJ, acid accumulation began almost immediately and increased steadily, whereas AJ and CJ exhibited a lag phase of roughly four days before acid levels rose sharply. These differences highlight the influence of fruit matrix composition on AAB performance. Peach likely provided a more favorable environment for *G. oxydans* and this would deserve further investigation. Clementine's higher levels of citric-derived components such as limonene and linalool, along with essential oils, may have initially slowed AAB activity [42].

During alcoholic fermentation of all the juices, yeasts increased in the first days and then declined, because of ethanol accumulation and nutrients depletion,

similar to strawberry and persimmon juices patterns of fermentation [43,44]. For all the fruit matrices, we found that dynamics of yeasts and AAB reflected the production of ethanol and its subsequent decrease in favor of acetic acid. The declines in AAB population (probably represented only by *G. oxydans* used as starter) observed after 5-6 days coincided with acetic acid concentrations surpassing 5% and the near-consumption of ethanol, consistent with a previous study that highlighted the dynamics of *Acetobacteraceae* in vinegar production [45]. *G. oxydans* is potentially able to oxidize several substrates, including carbohydrates and alcohols [46]. To the best of our knowledge, no study reported the use of *G. oxydans* as pure culture for driving fermentation of fruit juice to vinegar so far. After acetous fermentation with *G. oxydans*, all the experimental vinegars reached comparable levels of acetic acid and residual ethanol, despite putative compositional differences among fruit substrates. The experimental vinegars showed concentrations of acetic acid and ethanol in compliance with regulatory limits, namely at least 6% and lower than 1%, respectively [47]. Similar results were reported by Maske et al., who obtained apple vinegar using a natural starter containing *Saccharomyces*, *Leuconostoc*, and *Acetobacter*, among others [45]. In our study, all the vinegars did not harbor coliforms at detectable level, confirming microbiological safety, consistent with vinegar's antimicrobial properties [48]. Specific testing for *Salmonella* spp. and *Escherichia coli* was not performed, as coliform counts remained below detection limits, indicating no evidence of potential fecal contamination.

Colorimetric analysis indicated that overall, the fruit vinegars produced at laboratory, particularly those from peach and clementine juices, exhibited darker, warmer, and more saturated tones, associated with natural pigments and non-enzymatic browning. On the other hand, commercial vinegar was brighter but less chromatic, possibly due to industrial clarification and filtration practices. The values observed for L^*, a^*, and b^* fell within the range reported for traditional and industrial vinegars [49]. Those color coordinates may be affected by raw material, processing conditions, and storage [50].

Sensory analysis highlighted large differences among the vinegars, assigning the highest visual intensity score to PV, followed by clementine, apple, and commercial apple vinegar; this confirmed the results of instrumental color metrics. Among other attributes evaluated by the panelists, large differences among the

vinegars were found also for aroma intensity, which was quite high for PV and commercial vinegar, sweetness (highest score attributed to CV), and acidity (highest score attributed to AV). Interestingly, notwithstanding the highest scores of color and aroma intensity, and floral aroma received by the PV, panelists evaluated this vinegar with the lowest acceptability. It may be hypothesized that excessively intense aromatic complexity, combined with low perception of acidity negatively affected overall acceptability. Unexpectedly, the experimental vinegar obtained from fermentation of CJ was appreciated more than commercial vinegar, possibly due to its sweet flavor, citrus zest note, and moderate color intensity and acidity. These results would suggest that clementines are particularly suitable for producing vinegar that could be well accepted by consumers. To our knowledge, no research studies were conducted on vinegar produced from clementine, although previous studies reported the descriptive sensory analysis on experimental vinegar from other citrus fruits, such as lemon [51], mandarin [52], and orange [53]. However, they did not evaluate overall acceptability of vinegars and did not compare experimental vinegars with a commercial one.

Some studies about fruit vinegar analyzed VOCs, organic acids, and free amino acids because all of them may be considered important drivers of odor, taste, and flavor of vinegar [51,54,55]. However, we preferred to focus just on VOCs because of their low odor threshold value to be perceived, compared to most organic acids and free amino acids. Profiles of VOCs for all the vinegars were consistent with the chemical diversity reported in fruit vinegars [56]. VOCs in vinegar may originate from fruits used as raw material, or may be generated by microorganisms driving fermentation of fruit juices [57]. However, since we used the same microorganisms (*S. cerevisiae* and *G. oxydans*) for all the vinegars, we expect that most of differences could be attributable to the different fruits used. Multivariate analysis based on VOCs profile showed that our experimental AV was quite like the commercial apple vinegar, possibly because they were produced from juices of the same plant species. Overall, commercial vinegar showed the simplest VOCs profile, possibly due to industrial processing that may have reduced volatile complexity [58]. Hexanoic acid ethyl ester, with a fruity aroma, characterized our AV, in agreement with literature [59]. Contrarily to AV, PV and CV were well distinguished from the commercial vinegar. In particular, CV was characterized by relatively high levels of alpha-terpineol, and 2,4-bis(1,1-dimethylethyl)-phenol.

These compounds, along with others (e.g., linalool and phenylethyl alcohol), probably contributed to citrus and floral aromatic characteristics of CV, perceived by the panelists as "citrus zest note". In addition, CV was characterized by high levels of phenylethyl alcohol, another VOC that was previously found as typical of citrus vinegar and possibly produced by yeasts or AAB [52]. PV was characterized by VOCs, such as benzaldehyde, benzyl alcohol, benzoic acid, associated with almond-like aroma, and ethyl acetate and furfural, associated with floral and fruity aromas. Budak et al. (2021) [60] showed that ethyl acetate and, especially benzaldehyde and benzyl alcohol, increased when peach juice was processed to vinegar, whereas furfural decreased. This would suggest that yeasts or AAB are mainly involved in the dynamics of those VOCs.

In vitro antioxidant activity, determined through two assays, was highest in CV. Several compounds may confer antioxidant activity to fruit-based beverages. Anyway, previous studies highlighted strong correlations between some VOCs, such as terpenes and phenols, and antioxidant capacity [56,61]. Therefore, we hypothesize that VOCs such as alpha-terpineol and 2,4-bis(1,1-dimethylethyl)-phenol could be among the main contributors to antioxidant activity found for CV. PV had the second most potent *in vitro* antioxidant activity. It had been previously shown that antioxidant activity of peach juice could decrease or increase in the resulting vinegar, depending on the step of fermentation (alcoholic/acetous) and assay used [60]. Overall antioxidant activity of PV could be attributed to compounds such as phenols and carotenoids contained in the raw matter [62,63]. The analysis of profile of phenolic compounds could have been helpful to try correlating antioxidant activity of experimental vinegars with those bioactive compounds.

From a practical standpoint, the findings of this study provide important insights for potential scale-up and commercialization. Scaling up alcoholic and acetous fermentations would require careful control of oxygen supply and microbial stability, since both *S. cerevisiae* and *G. oxydans* exhibit process-sensitive kinetics that may differ in larger bioreactors. For small producers or artisanal processors, the approach tested here appears economically viable, as third-category fruits are low-cost raw materials and fermentation infrastructure can be kept relatively simple. In particular, CV presents notable commercial promise, given its high acceptability score and strong antioxidant activity, suggesting a differentiated

premium product for niche markets such as gourmet, potentially functional, or locally sourced food products. The vinegars produced also showed microbiological stability and acetic acid levels compatible with long shelf life, although long-term storage behavior (clarity, sediment formation, color stability, and VOC retention) should be characterized in future work and storage under cool and dark conditions would be advisable to preserve color and volatile profile. When compared with typical industrial vinegar production, which uses high-efficiency submerged fermentation systems capable of achieving rapid acetic acid accumulation (often within 24–48 h), the batch-type approach used here is slower but preserves more fruit-specific aroma compounds. This suggests a trade-off between production efficiency and sensory distinctiveness, with the latter offering a potential advantage for value-added artisanal products. If compared with industrial vinegar production, the process described here would be less efficient in terms of time and acetic acid yield per unit volume, but it offers added value through enhanced sensory characteristics, unique volatile profiles, and the valorization of otherwise wasted fruit resources.

4.5 Conclusion

This study demonstrated that alcoholic and acetous fermentation processes can be effectively applied to transform third-category organic fruits—specifically apples, peaches, and clementines—into high-quality vinegars with distinct chemical and sensory attributes. The resulting products reached acetic acid concentrations of 5.0–5.6% (w/v), confirming the successful conversion of low-grade fruit biomass into microbiologically stable vinegars. Among the evaluated products, CV showed particular commercial potential, supported by its high consumer acceptance score and superior antioxidant activity, suggesting strong market appeal.

Beyond their technological and sensory value, these findings underscore the potential of low-grade fruits as sustainable raw materials for producing fruit vinegars that can be appreciated by consumers. This approach could support circular bioeconomy principles by transforming underutilized or waste fruit streams into value-added products, thereby reducing environmental burden and enhancing resource efficiency. The valorization of such raw materials shows how

controlled biotechnological processes can contribute to more sustainable and resilient food systems.

The methodology used in this study could be readily adapted to other fruit matrices, while future research should explore the feasibility of scaling up fermentation processes. Additionally, expanded consumer studies and further investigations into the biological activities and functional properties of fruit vinegars are recommended to strengthen the commercial and nutritional relevance of these products.

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Overall conclusions and perspectives

The current trajectory of global food production poses serious concerns for long-term environmental resilience, particularly as the demand for nutritious food continues to rise. Organic systems offer a promising alternative, yet their potential can only be fully realized through processing methods that preserve ecological integrity, minimize resource use, and valorize materials that would otherwise be discarded. In this context, fermentation emerges as a pivotal biotechnological tool capable of reconciling the need for sustainable transformation of raw materials with the regulatory and ethical requirements of organic production.

Against this backdrop, this thesis examined how fermentation—rooted in natural microbial metabolism—can support the development of innovative organic foods while enhancing circularity across value chains. A central component of the research focused on the selection and functionality of starter cultures, comparing the ecological richness of natural starters with the reliability of commercial ones. This work emphasized that the microbiological diversity embedded in natural starters, and their intimate link to local raw materials, can offer distinctive sensory and functional advantages when appropriately managed. At the same time, the analysis highlighted the need for tailored protocols that make these biological systems reproducible and safe for use in organic processing environments.

Building on this conceptual foundation, the experimental chapters demonstrated the practical contributions of microbial fermentation to product innovation. The development of organic sourdough bread enriched with germinated seeds illustrated how fermentative processes can simultaneously improve nutritional value, enhance sensory attributes, and maintain technological performance. The ability to dry and later revitalize sourdough further underscored the feasibility of integrating these methods into small- and medium-scale organic bakeries, where flexibility and minimal processing are key.

Complementing this, the valorization of third-category organic fruits through controlled vinegar fermentation showcased how underutilized resources can be transformed into high-quality, microbiologically stable products. Apples, peaches, and clementines, typically excluded from commercial channels for aesthetic reasons, were successfully converted into differentiated vinegars with distinct volatile and sensory profiles. This not only reduces avoidable waste but also

provides local producers with new avenues for product diversification and economic resilience.

Together, these studies deepen our understanding of how microbial cultures—whether naturally occurring (as in the case of the novel organic sourdough bread) or selected (as in the case of fruit vinegars) —can be leveraged to meet the dual goals of sustainability and product quality in organic food processing. They also highlight the relevance of fermentation as a low-energy, low-additive technology that aligns closely with regulatory expectations for organic foods and with consumer preferences for authenticity, healthfulness, and minimal processing.

Future research should focus on scaling-up these protocols for other different organic fermentation matrices, exploring multi-strain starter consortia that combine stability with ecological complexity, and assessing consumer acceptance at larger scales. Additional emphasis should be placed on shelf-life optimization and on extending these approaches to other underused organic raw materials. The insights gained through this thesis offer a foundation for developing new fermented products that support environmental goals, respond to evolving market demands, and reinforce the transition toward more circular and sustainable organic food systems.