



UNIVERSITY OF BARI ALDO MORO

Department of Soil, Plant and Food Science

PhD course in Soil and Food Sciences

(XXXVII cycle)

Scientific Disciplinary Sector AGRI-o8/A (formerly AGR/16)

INNOVATIVE FERMENTED FOODS: FROM THE CHARACTERIZATION OF STARTER MICROORGANISMS TO THE DEVELOPMENT OF THE PRODUCTION PROTOCOLS

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Introduction

Conventional food production is no longer sustainable and imposes a significant strain on environment. The global food system contributes to greenhouse gas emissions, biodiversity loss, destruction of terrestrial ecosystems, freshwater consumption, and nutrient loading (Rockström *et al.*, 2020). With the world's population expected to approach nearly 10 billion by 2050 (Rockström *et al.*, 2020; Desa, 2022), one of the major challenges we face today is how to feed this growing population while preserving natural ecosystems. To achieve this, drastic changes are necessary, including a shift towards healthy diets, regenerative agricultural practices, and waste valorization. In the search for new or alternative crops to meet the increasing demand for food and feed, resources such as algae, insects, and food by-products present interesting opportunities for sustainable and innovative transformations (Garofalo *et al.*, 2022).

Furthermore, innovations—understood as new products, processes, or services—are recognized as vital tools for companies in the food industry to distinguish themselves from competitors and meet consumer expectations (Menrad, 2004). Recent innovations in the food industry have focused on novel scientific and technical approaches to food processing, as well as the introduction of new food products. In this context, functional foods have gained particular prominence, driven by the rising cost of healthcare, the increasing life expectancy, and the desire of elder people for a better quality of life (Kotilainen *et al.*, 2006; Robertfroid, 2000a, Robertfroid, 2000b).

Innovations in the food industry often aim to develop key replacement products, align with nutritional guidelines, and comply with food additive regulations. These innovations can occur at any stage of the food supply chain and can be classified into several categories: new food ingredients and materials, innovations in fresh foods, new food processing techniques, improvements in food quality, new packaging methods, and new distribution or retailing approaches (Bigliardi & Galati, 2013).

In this thesis, special focus will be placed on innovations involving the use of non-conventional matrices (e.g., food by-products), advancements in production technologies (such as fermentation with unconventional starters or fermentation combined with emerging production technologies), and improvements in food quality and functionality.

1.1 Food waste and by-products

Before addressing food loss throughout the entire agri-food chain, it is important to clarify the differences between "food waste" and "by-products." Food loss is defined as 'the decrease in quantity or quality of food'. Food waste, a subset of food loss, refers to the discarding or alternative (non-food) use of food that is still safe and nutritious for human consumption, occurring at any point along the food supply chain, from primary production to household consumption (FAO 2016). Food by-products, also a component of food loss, are secondary products generated during the agri-food supply chain (FAO 2016). Food waste and by-products are generated in large quantities by the food industry worldwide each year. Approximately 38% of this loss occurs during food processing. These losses originate from a variety of sources, including animal-derived waste and by-products such as carcasses, hides, hooves, heads, feathers, manure, offal, viscera, bones, fat and meat trimmings, and blood. In the seafood industry, animal-derived waste and by-products includes skins, bones, oils, and blood, while dairy processing produces by-products like whey, curd, and milk sludge from separation processes. Plant-based food processing generates by-products such as peelings, stems, seeds, shells, bran, and residues after the extraction of oils, starches, juices, and sugars (Helkar, Sahoo, & Patil, 2016). The disposal of these food industry losses poses significant challenges to ecosystems due to their poor biological stability, substantial nutritional content, high concentration of organic compounds, elevated water activity, poor oxidative stability, and optimal conditions for enzymatic activity (Helkar, Sahoo, & Patil, 2016). The sheer volume of food loss, along with its microbial decomposition, can have negative impacts on the environment and human health, while also imposing considerable costs for waste treatment. For food manufacturers, who already operate on low profit margins, these additional waste and by-products processing costs are detrimental not only to the food industry but also to the agricultural sector and the broader economy (Helkar, Sahoo, & Patil, 2016). There is a growing demand for measures to minimize the environmental and health impacts of organic food losses, as well as to reduce the financial burden of waste treatment. Agricultural waste, co-products and by-products play a crucial role in animal feed production worldwide, while biomass residues are widely used in the production of bioenergy. Efficient use of by-products can greatly influence a country's economy and reduce environmental

pollution. Moreover, food by-products streams also present a promising source of functional compounds that, due to their nutritional and rheological properties, can be repurposed. These valuable components—such as polysaccharides, proteins, fats, minerals, fibers, flavor compounds, phytochemicals, and bioactive compounds—can offer significant health benefits when utilized effectively (Helkar, Sahoo, & Patil, 2016). Indeed, innovative valorization strategies increasingly focus on recovering valuable ingredients from fruit and vegetable by-products, utilizing them as natural sources of bioactive compounds for drug development and functional food formulation (Lu *et al.*, 2019).

Recent innovative and technological solutions have been proposed to selectively extract bioactive compounds while adhering to environmentally friendly standards. Among these, advanced techniques such as high-pressure processes (subcritical and supercritical states), high hydrostatic pressure, pulsed electric fields, membrane filtration, enzyme extraction, ultrasounds, and microwaves offer promising alternatives (Ferrentino, Asaduzzaman, & Scampicchio, 2018). At the same time, fermentation-based valorization strategies are gaining attention, with some examples represented by anaerobic digestion of organic feedstocks (De Groof *et al.*, 2019), fermentation of date palm waste to produce cost-effective alternative to expensive raw materials (Azam & Ahmad, 2019), bioconversion of cocoa by-products to generate enzymes, polysaccharides, and nutraceuticals (Vásquez *et al.*, 2019), and sourdough fermentation to produce baked goods that release bioactive compounds and may help alleviate irritable bowel syndrome symptoms (Gobbetti *et al.*, 2019; Sabater *et al.*, 2020)

These valorization strategies not only contribute to food loss reduction but also enhance the sustainability of food systems by transforming by-products into valuable resources. By applying these approaches, the food industry can reduce its environmental footprint while generating high-value compounds with potential applications in pharmaceuticals, nutraceuticals, and functional foods. As research advances, combining extraction technologies with fermentation processes holds great promise for creating more efficient and sustainable methods to tap into the full potential of food by-products.

1.2 Fermentation process

Fermentation is an ancient natural process that has been utilized for food preservation for thousands of years. For much of this time, the production of fermented products occurred without a comprehensive understanding of the biological, biochemical, and chemical principles involved. The process relied on the indigenous microbiota present in the food substrate, which, influenced by various environmental factors, resulted in products with characteristics closely linked to their geographic origins (Mannaa *et al.*, 2021). While fermentation has been practiced for millennia, the specific role of microorganisms in this process was only elucidated more recently by Louis Pasteur, who identified living cells as the key agents responsible for fermentation (Ross, Morgan, & Hill, 2002).

Upon a strictly biochemical point of view, fermentation is a biological process in which microorganisms such as bacteria, yeast, or fungi convert organic compounds into simpler ones. For example, yeast enzymes can convert sugars and starches into alcohol, while proteins can be converted to peptides/amino acids (Sharma *et al.*, 2020). In contemporary practices, driven fermentation is favored over spontaneous fermentation to improve process monitoring and enhance reproducibility. This approach allows for greater control over the fermentation environment, enabling producers to maintain consistent conditions and optimize outcomes. By using specific starter cultures and carefully managing variables such as temperature, pH, and fermentation time, manufacturers can achieve uniformity in flavor, texture, and quality across batches. This shift towards driven fermentation not only ensures product safety and reliability, but also meets the increasing consumer demand for standardized and high-quality fermented foods (Galimberti *et al.*, 2021; Mannaa *et al.*, 2021).

Fermentation is widely used in food production because it offers several advantages. Firstly, fermented foods have a longer shelf-life compared to their raw matrices, thanks to the lower pH, the production of antimicrobial compounds, and other preservative factors. Additionally, fermentation enhances the organoleptic properties of food, improving taste and aroma. Fermentation also aids in removing harmful or undesirable components from raw materials. A notable example is the reduction of antinutritional factors in fermented legumes. Indeed, the consumption of legume protein is still limited due to issues such as digestive discomfort, reduced mineral bioavailability, and decreased protein

digestibility (Díaz-Batalla *et al.*, 2006). However, lactic acid fermentation appears to be one of the potential solutions to mitigate these drawbacks (Emkani, Oliete, & Saurel, 2022). Additionally, fermented products frequently exhibit enhanced antioxidant activity; for example, fermented milk and yogurt have been shown to possess greater antioxidant properties compared to fresh milk, owing to the release of biopeptides during the breakdown of proteins such as α -casein, α -lactalbumin, and β -lactoglobulin (Sharma *et al.*, 2020). Moreover, as mentioned in the previous paragraph, fermentation plays a vital role in processing food by-products. It helps to revalorize these materials by reducing undesirable traits, such as bitterness and off-flavors, while enhancing their functional and nutritional value. Fermentation can be used either as a standalone biotechnological approach or in combination with other innovative technologies (e.g., ultrasound) (Ran *et al.*, 2019).

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

1.2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are historically defined as an ubiquitous and heterogeneous family of microbes that can ferment a variety of nutrients into, primarily, lactic acid although recent molecular evidence has challenged this definition (Brooijmans *et al.*, 2009). They are found in environments rich in available carbohydrate substrates, such as food and feed, but also in human and animal cavities, and in sewage and plant material. Indeed, strains have been isolated from all these environments (Kandler and Weiss, 1986; Liu *et al.*, 2014).

LAB are a group of Gram-positive bacteria that can be differentiated by various morphological, microscopic, and biochemical tests. These tests analyze factors such as cell structure, glucose fermentation properties, sugar utilization, and optimal growth temperatures. Traditionally, the core LAB group consisted of four genera: *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Lactococcus*. However, advancements in molecular biology techniques have expanded the number of recognized genera, including *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, and *Weissella*, among others. Notably, in 2020, the genus *Lactobacillus* underwent significant reclassification, resulting in its division

into approximately 25 new genera. These include *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lentilactobacillus*, *Levilactobacillus*, *Limosilactobacillus*, and others, reflecting the increasing complexity and diversity within the LAB group (Niamah *et al.*, 2024).

LAB derive their energy through substrate-level phosphorylation, as they lack a functional respiratory system. They rely on two main hexose fermentation pathways: the homofermentative pathway, which primarily produces lactic acid, and the heterofermentative pathway, which generates lactic acid, CO₂, and either ethanol or acetate (Kandler, 1983; Lahtinen *et al.*, 2011, Liu *et al.*, 2014).

As LAB are very important in many food applications, the food industry is always seeking strains with superior characteristics and properties to enhance sensory and product quality. Moreover, during LAB fermentation, various bioactive compounds can be produced. For instance, peptides like valyl-prolyl-proline and isoleucyl-prolyl-proline from fermented milk show potential as anti-hypertensive agents (Nongonierma, & FitzGerald, 2015). LAB also produce exopolysaccharides (EPS), which have demonstrated anti-diabetic, cholesterol-lowering, antioxidant, and immunomodulatory effects. EPS reduce cholesterol by limiting its absorption in the gut and binding to it (Nampoothiri *et al.*, 2017). LAB strains can also produce enzymes like amylase and protease, aiding in nutrient absorption by breaking down complex substrates. Additionally, LAB-fermented foods enhance mineral absorption, help with lactose intolerance, prevent antibiotic-associated diarrhea, and show anti-tumor potential in some studies (Gao *et al.*, 2013). Antimicrobial compounds like bacteriocins are also produced during fermentation (Mathur, Beresford, & Cotter, 2020).

1.2.2 Yeasts: *Saccharomyces* and non-*Saccharomyces*

Yeasts are eukaryotic microorganisms that thrive in various ecological environments, including water, soil, air, and on plant and fruit surfaces. One of their most important habitats is ripe fruit, where they play a key role in decomposition and fermentation. Compared to other microorganisms like LAB, yeasts have simpler nutritional needs, requiring only basic compounds like fermentable sugars, amino acids, vitamins, minerals, and oxygen to grow. These nutrients enable yeasts to thrive in various environments, allowing them to efficiently carry out fermentation and metabolic activities (Walker, & Stewart,

2016). Yeasts are known for their ability to ferment different sugars like glucose, fructose, sucrose, maltose, and maltotriose, commonly found in ripe fruit and cereals. They can also tolerate acidic conditions, with pH levels around 3.5 or lower. Morphologically, yeasts exhibit a wide range of shapes, with round, ellipsoidal, and oval forms being most common. In the identification process, microscopic evaluation is typically followed by microbiological and biochemical testing. Additionally, ethanol, organic acid, and sulfur dioxide (SO₂) tolerance are important tools for distinguishing between yeast species (Maicas, 2020).

From a technological perspective, yeasts are divided into two major groups: *Saccharomyces* and non-*Saccharomyces*.

Saccharomyces, particularly *S. cerevisiae*, is the most extensively studied eukaryote and serves as an invaluable model organism in many areas of basic research on eukaryotic life. Its unicellular nature simplifies experimental work and offers the advantage of sharing almost all essential biological functions present in higher eukaryotes, which are highly conserved in *S. cerevisiae* (Parapouli *et al.*, 2020). Additionally, it is easily manipulated genetically. Unlike many other model organisms, *S. cerevisiae* is also of significant importance in various biotechnological fields, some of which have been utilized for thousands of years. The organism's biotechnological relevance stems from its distinctive biological features, such as its ability to ferment, producing alcohol and CO₂, as well as its resilience to challenging conditions like high osmolarity and low pH (Parapouli *et al.*, 2020). It also exhibits greater tolerance to SO₂ compared to non-*Saccharomyces* yeasts, although this trait varies across different strains due to natural genetic differences (Maicas, 2020). Notably, *S. cerevisiae* plays a central role in industries like food, beverages—particularly wine—and biofuel production.

Non-*Saccharomyces* yeasts, such as *Kloeckera*, *Hanseniaspora*, *Brettanomyces*, *Pichia*, *Lachancea*, and *Kluyveromyces*, are essential in many fermentation processes due to their diverse metabolic capabilities, making them particularly valuable for traditional and non-traditional beverage production (Maicas, 2020). These yeasts dominate the early stages of spontaneous fermentation until ethanol concentrations reach around 4-5% v/v, at which point their growth is inhibited by the increasing alcohol and depletion of oxygen. Despite the sustained presence of certain non-*Saccharomyces* yeasts, the majority do disappear during the early stages of a vigorous fermentation and

Saccharomyces spp., more resistant to ethanol, take over and complete the fermentation process (Jolly *et al.*, 2014). Recent research has highlighted the importance of non-*Saccharomyces* yeasts in positively contributing to the sensory qualities of wine (Borren & Tian, 2020; Padilla, Gil, & Manzanares, 2016; Tufariello *et al.*, 2021). Although these yeasts typically have lower fermentative power and ethanol production compared to *S. cerevisiae*, they excel in producing aromatic compounds such as esters, higher alcohols, and fatty acids, making them valuable in fermentation. In contrast, non-*Saccharomyces* yeasts belonging to the genera *Debaryomyces*, *Hansenula*, *Candida*, *Pichia* and *Hanseniaspora* possess various degrees of β -glucosidase activity and can play a role in releasing volatile compounds from non-volatile precursors. Additionally, their fermentation activity increases in the presence of small amounts of oxygen, leading to increased biomass and reduced ethanol production, a useful approach for lowering alcohol levels in wines when used in co-culture with *S. cerevisiae* (Maicas, 2020).

In conclusion, yeasts, both *Saccharomyces* and non-*Saccharomyces*, exhibit remarkable diversity not only among genera but also within species and strains of the same species. This variability is reflected in their metabolic and fermentative characteristics, significantly impacting fermentation processes and the sensory qualities of the final products. In the following chapters of this thesis, some species will be explored in greater detail.

1.3 Innovative technologies

With increasing demand to obtain processed foods with better attributes than have been available to date, food researchers have pursued the discovery and development of improved preservation processes with minimal impact on the fresh taste, texture, and nutritional value of food products. In food preservation, thermal treatment has proven to be very effective in terms of microbial inactivation but may also cause loss of nutrients and impairment of desirable quality properties (Lado & Yousef, 2002; Ortega-Rivas, & Salmerón-Ochoa, 2014). Combined methods can be used in food treatment to reduce textural and nutrient damage and obtain microbiologically safe products. Preservation of foods by combined methods consists of applying a combination of stress factors, often simultaneously, to minimize processing. By avoiding any single preservation technique, the synergistic effect allows the production of high-quality food

products (Alzamora *et al.*, 1998; Ortega-Rivas, & Salmerón-Ochoa, 2014). There are also a series of individual non-thermal methods investigated recently in food processing that do not involve direct heat and are said to be able to produce safe foods with improved quality attributes. These methods include ultrahigh hydrostatic pressure (HHP) and high-voltage pulsed electric fields (HV PEF) and could have an enormous impact on the food industry (Flickinger, 1999; Ortega-Rivas, & Salmerón-Ochoa, 2014). Some other non-thermal food processing techniques comprise ultraviolet (UV) radiation, gamma irradiation, ultrasound, and non-conventional chemical reagents (Ortega-Rivas, & Salmerón-Ochoa, 2014). Some of the most important innovative technologies and their impact on food composition are listed and discussed below.

Ionizing radiation: The use of ionizing radiation for the preservation of food has been studied for over a century as a method to preserve food, making it one of the most researched techniques for enhancing food safety and extending shelf life (Diehl, 1995). Although food irradiation is considered an authentic nonthermal process, and so it is supposed to keep quality attributes of processed foods, it can sometimes lead to off-flavors, tissue softening, and increased permeability in fresh fruits and vegetables (Stewart, 2009). Radiation affects key food components like carbohydrates, proteins, and lipids, but the changes, even at high sterilization doses, are generally minimal and comparable to those from other methods like pasteurization. As for any other food processing method, irradiation has advantages and disadvantages. Some foods are sensitive to ionizing radiation and too high a dose may cause organoleptic changes, rendering the food unacceptable to the consumer. Therefore, the actual dose employed is a balance between what is needed and what can be tolerated by the product without unwanted changes (Farkas, 2006). The right radiation dose is crucial to avoid unwanted sensory changes while ensuring food safety (Ortega-Rivas, & Salmerón-Ochoa, 2014).

Ultraviolet radiation: UV light in the food industry is used for disinfection, e.g., water or air, but irradiation of food with high-energy ionizing UV radiation is a used physical treatment of food. It can be used to prolong the shelf-life of food products and/or to reduce health hazards associated with certain products due to the presence of pathogenic microorganisms. For instance, Mansourbahmani *et al.* (2017) in their study found that a combination of CaCl_2 and UV-C irradiation can increase the shelf-life of tomatoes. Ultraviolet radiation an efficient, nonchemical

method that eliminates bacteria, viruses, yeasts, molds, and their spores. UV disinfection is environmentally friendly, requiring minimal maintenance and leaving no chemical residues or changes in the food's taste, odor, or pH. As UV has no residual effect, the best position for a treatment system is immediately prior to the point of use. This ensures incoming microbiological contaminants are destroyed and there is a minimal chance of post-treatment contamination (Ortega-Rivas, & Salmerón-Ochoa, 2014).

Ultrasound: Application of sound waves with frequencies above 20 kHz may induce cavitation in liquids that can lead to localized high temperature and pressure bursts, improved mass transport, and induction and acceleration of radical-driven reactions. These physical and chemical effects provide platforms for potential applications of ultrasound in food processing operations ranging from microbial inactivation and modification of texture, color, taste, and odor to enhancement in the functionality of food ingredients. Nowadays, power ultrasound is an emerging and promising technology for industrial food processing. Ultrasound has two main applications: as a diagnostic tool for non-destructive evaluation and as a source of energy in sonochemistry. These applications involve different frequency ranges of ultrasound and the uses of both ranges in the food industry are an active subject for research and development (Ortega-Rivas, & Salmerón-Ochoa, 2014). The potential use of this novel technology to produce permanent changes in food materials in liquid systems is, as previously mentioned, through the generation of intense cavitation. This can lead to the inactivation of microorganisms and enzymes for food preservation or decontamination by ultrasonic irradiation demonstrates the benefits of ultrasound as a food preservation tool. In addition, there are an increasing number of industrial processes that employ power ultrasound as a processing aid, including the mixing materials, foam formation or destruction, agglomeration and precipitation of airborne powders, the improvement in efficiency of filtration, drying, and extraction techniques in solid materials, and the enhanced extraction of valuable compounds from vegetables and food products (Ortega-Rivas, & Salmerón-Ochoa, 2014).

Ultrahigh hydrostatic pressure: HHP, a relatively new technology to the food industry, inactivates microorganisms without causing significant flavor and nutritional changes to foods (Berlin *et al.*, 1999). HHP is a method of food

processing where food is subjected to elevated pressures (up to about 600 MPa), with or without the addition of heat, to achieve microbial inactivation or to alter the food attributes to achieve consumer desired qualities. Pressure inactivates most vegetative bacteria at pressures above 400 MPa. HHP retains food quality, maintains natural freshness, and extends microbiological shelf-life. HHP causes minimal changes in the freshness characteristics of foods by eliminating thermal degradation. As compared with thermal processing, HHP produces foods with better appearance, texture, and nutrition. For instance, Lee *et al.* (2018) sought to replace thermal process with a HHP treatment in red bean powder production to improve the digestibility by protein destruction and to ensure microbiological stability. Due to the use of HHP treatment trypsin inhibitor activity was reduced by 80.5 % and phytic acid levels were decreased to 8.19 %, while organoleptic and nutritional qualities remained unchanged.

Moreover, HHP can be conducted at ambient or refrigerated temperatures, thereby eliminating thermally induced cooked off-flavors. This technology is especially beneficial for heat-sensitive products (Ortega-Rivas, & Salmerón-Ochoa, 2014).

High-voltage pulsed electric fields: PEF processing involves the application of an externally generated electric field across a food product with the intent of inactivating pathogenic microorganisms, modify enzymes, intensifying some processes, or achieving some specific transformation in the product. The technology has long been used for cell hybridization and electrofusion in genetic engineering and biotechnology. It works by disrupting cell membranes under a high electric field, leading to increased permeability and electrical conductivity of the cellular material. This phenomenon, known as dielectric breakdown or electroporation, occurs through two main mechanisms: electroporation, where pores are formed in the membrane due to polarization, and membrane denaturation caused by ohmic heating from the high resistance of the membrane compared to the cell's contents. PEF technologies have been demonstrated to be a viable alternative to high-temperature inactivation of microbial load in liquid foods such as fruit juices and milk (Knorr *et al.*, 1994; Barbosa-Canova *et al.*, 1999; Ortega-Rivas, & Salmerón-Ochoa, 2014). Several studies highlight the impact of PEF treatment on the shelf-life of fruits and vegetables, which face significant losses annually due to issues in harvesting, storage, packing, transport, and market

dynamics. Factors such as respiration, ethylene production, water loss, microbiological infection, mechanical injury, and enzymatic reactions contribute to quality deterioration. To mitigate these, various methods have been explored, with the industrial use of HV PEF showing promise for enhancing product quality while reducing energy consumption. HV PEF, often applied as a pretreatment, helps by destroying ethylene and volatile compounds, removing fungal spores, and releasing ozone, negative ions, and other reactive species into the storage environment (Atungulu *et al.*, 2005; Dalvi-Isfahan *et al.*, 2016; Shiina *et al.*, 2009).

1.4 Innovative plant-based products

The innovative sector of plant-based food has seen significant growth, particularly in the category of fermented products, driven by shifting consumer preferences towards health-conscious and sustainable options. In 2021, the global market for plant-based products produced through fermentation was valued at USD 329.29 million and is projected to reach USD 422.26 million by 2026, growing at a compound annual growth rate (CAGR) of 5.0% (Research and Markets Fermented Plant-Based Alternatives Market, 2021). Europe leads the global market, accounting for 73% of the total market share and generating USD 96.99 million in revenue in 2020, with projections reaching USD 129.47 million by 2026 (BIS Research)(Boukid *et al.*, 2023). This diverse market includes both conventional items, such as bakery products (Gómez & Martinez, 2018), and emerging categories like meat (Ahmad *et al.*, 2022) and dairy alternatives (Harper *et al.*, 2022).

Central to the rise of plant-based innovation is the use of plant-derived proteins as substitutes for animal proteins. These proteins are valued for their nutritional content, functional properties, and ability to meet the growing demand for sustainable food options. They play a key role in producing analogues for meat and dairy products, such as fish, cheese, and yogurt, offering comparable textures, flavors, and sensory experiences (Hur *et al.*, 2014). Sources like mycoproteins, algae, vegetables, cereals and food by-product are being explored for their potential to mimic animal proteins while meeting consumer expectations for health benefits (Alrosan *et al.*, 2022). Moreover, plant-based products are naturally rich in bioactive compounds, including polyphenols and flavonoids, which contribute to their strong antioxidant properties. These antioxidants help combat oxidative stress and reduce the risk of chronic diseases, boosting their appeal to health-

conscious consumers. Fermentation further enhances their nutritional value by improving nutrient bioavailability and introducing probiotics, which support gut health and overall well-being (Farid *et al.*, 2024). In particular, fermented plant-based products stand out due to their enriched nutritional profiles, achieved through fermentation with lactic acid bacteria, yeast, or fungi. These processes increase the presence of flavonoids, polyphenols, prebiotics, probiotics, vitamins, and organic acids. Together, these innovations reflect the synergy of functional nutrition and sustainability, addressing the growing demand from vegan, vegetarian, and flexitarian consumers worldwide (Farid *et al.*, 2024).

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Fermentation of pomegranate matrices with *Hanseniaspora valbyensis* to produce a novel food ingredient

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This chapter represents a modified version of that published in LWT, 2023 (180, 114687)

2.1 Introduction

Pomegranate (*Punica granatum L.*) fruits are consumed all over the world, and the most widespread cultivation is in the Mediterranean basin (e.g., Italy, Spain, Turkey, and Greece), Southern Asia, India, and America (Ferrara *et al.*, 2014). The fruit contains (per 100 g), on average, 48–52 g of a juicy red-pink pulp, called arils, consisting of about 78% of juice and 22% of seeds (Dhumal *et al.*, 2014), variable depending on cultivar and environmental conditions (Venkitasamy *et al.*, 2019). Arils are rich in valuable compounds such as polyphenols, flavonoids, anthocyanin, ascorbic acid, ellagic acid, carotenoids, and tannins (Gül & Şen, 2017; Jing *et al.*, 2012; Tasaki *et al.*, 2008). Due to the potential benefices associated to its consumption (Viuda-Martos, Fernandez-Lopez, & Perez-Alvarez, 2010; Vučić *et al.*, 2019), worldwide interest in pomegranate fruits has highly increased in recent years, causing an increment of demand for fresh fruits or juice. Pomegranate arils, juice and/or their by-products are suitable for the development and optimization of novel functional foods or, as such, as ingredients for novel functional formulations with high nutritional value, because of their richness in bioactive compounds (Ayoubi *et al.*, 2022; Nuzzo *et al.*, 2022; Sahraee, Ghanbarzadeh, & Falcone, 2022). Recently, juice and foods fortified with different part of pomegranate have been proven to have anti-oxidant, anti-inflammatory, anti-diabetic, anti-hypertensive, and anti-tumor effects even in *in vivo* studies (Moga *et al.*, 2021; Seyed Hashemi *et al.*, 2021). In addition, pomegranate juice has been used as suitable substrate in fermentation with various biocatalysts and/or as good probiotic carrier targeting the production of functional beverages with enhanced health benefits (Di Cagno, Filannino, & Gobbetti, 2017; Filannino *et al.*, 2013; Mantzourani *et al.*, 2020; Mousavi *et al.*, 2013; Mustafa *et al.*, 2019; Pontonio *et al.*,

2019; Valero-Cases, Nuncio-Jaiuregui, & Frutos, 2017). As a result of the higher production of juice, pomegranate by-products (seeds and peels) are also available in large amount. Compared to arils, pomegranate seeds contain even higher amounts of nutritionally valuable components, such as proteins, fibre, ellagitannins, polysaccharides and minerals (Gül & Şen, 2017; Venkitasamy *et al.*, 2019). It has been reported that they contain on average 13-14 g of carbohydrates and proteins, 30 g of fats and 35-40 g of fibre on 100 g of seeds (Rowayshed *et al.*, 2013), depending on the cultivar. As regard minerals, seeds contain, on average, 30 mg of calcium, 45.0 – 50.0 mg of potassium, 1.3 – 3.1 mg of iron, 1.0 – 3.4 mg of zinc on 100 g (El-Nemr, Ismail, & Ragab 1990; Venkitasamy *et al.*, 2019). Due to climate change (with atmospheric CO₂ levels playing a pivotal role), a decrease in many minerals (e.g., iron and zinc) was observed in plants (Loladze, 2014). The depletion of minerals contents may contribute to increase the prevalence of health problems in the world population, such as: overweight (caused by the excessive introduction of food to compensate for the deficiency) (Loladze, 2014) and malnutrition (especially in subjects characterized by exclusion diets linked to allergies or intolerances) (Schreiner *et al.*, 2020).

Enhancement of large-scale consumption foods through valuable by-products or zero-kilometer local production, added as additional and non-conventional ingredients, would be perfectly in line with Sustainable Development Goals of the United Nations 2030 Agenda, to preserve environment and combat the climate change. Some studies tried to efficiently re-use pomegranate by-product, including it as an ingredient in preparation of large-scale consumption food. Gül & Şen (2017), for example, included pomegranate seeds flour as ingredient in bread-making for enriching the food with fibre. They found that the breads enriched in highest percentage of pomegranate seeds flour were not appreciated by panellists, especially cause of the loaf volume, color, chewiness, and aroma. The only acceptable fibre -enriched bread was the one containing only 5 g of seeds flour on 100 g of bread (Gül & Şen, 2017). Fermentation using lactic acid bacteria (LAB) and other microorganisms has been proven as a feasible strategy to improve chemical composition and structure of the enriched food (Christ-Ribeiro *et al.*, 2021; Sabater *et al.*, 2020). Although fermentation of pomegranate matrix could seem challenging because of its composition (e.g., presence of many bactericidal compounds, low pH) (Howell & D'Souza, 2013), some researchers focused on

fermentation of pomegranate juice, as mentioned above. However, none of those studies tested the use of autochthonous microorganisms (i.e., isolated from the matrix and used as starters for fermenting the same matrix) as starters of pomegranate fermentation. The selection and use of autochthonous microorganisms as fermentation starters could improve shelf-life, nutritional and sensory traits of food (Di Cagno *et al.*, 2013).

Based on the above considerations, the aim of this study was to lay the basis for a more efficient exploitation of pomegranate seeds and juice, starting from the characterization of the microbiota of this niche, in order to develop, on pilot plant, a large-scale consumption food, added with a novel food ingredient, corresponding to fermented pomegranate matrices, to be able to meet consumer's needs, in terms of nutrition and sustainability.

2.2 Material and methods

2.2.1 Plant material

Fresh pomegranate fruits (cv. *Wonderful*) were purchased from retail and cut into quarters. Arils were manually separated from peels and pith and collected. Aliquots of native arils were stored at -20 °C until their use. The remaining aliquot was stored at 4 °C and subjected to microbiological analysis within 24 h.

2.2.2 Production of pomegranate juice and seeds

Pomegranate juice (PJ) and seeds (PS) were obtained from native or chemically sterilized arils. To obtain the chemically sterilized arils, prior the preparation of PJ, arils were submerged in NaClO solution (10 ml/l in tap water), in an arils:water ratio of ca. 1:2, w/v, at room temperature for five min. To remove residual NaClO, arils were washed under tap water, at ca. 10 °C, for two min. Juice and seeds were extracted from native or chemically sterilized arils by squeezing them through a lab blender mixer (Bag Mixer 400 P, Interscience International, France) in a stomacher bag with lateral filter for 3 cycles of 3 min each (total treatment time: 9 min), with a PJ and PS yields, respectively, of ca. 70 g and 30 g starting from 100 g of arils. After squeezing, PJ and PS were separately collected and stored at -20 °C until analyses and processing/direct use.

2.2.3 Chemical analyses on juice and seeds

S.A.Mer. – Servizio Analisi Chimico-merceologiche (Bari, Italy) analysed pomegranate unprocessed juice and seeds for concentration of ashes (gravimetric method, Reg. CE 1833/2015), saturated fats (Soxhlet method), unsaturated, mono-unsaturated, and poly-unsaturated fats (gas chromatography) (Reg. CE 1833/2015), proteins (Kjeldahl method) (D.M. 2307/94 n. 4), calcium and potassium (ion chromatography) (internal method), iron and zinc (Inductively Coupled Plasma – Optical Emission Spectroscopy, ICP-OES, according to the UNI EN ISO 11885:2009). Total carbohydrates were determined by differences. Crude fibre was determined only for seeds, through the Weende method (Reg. CE 152/2009).

2.2.4 Antioxidant activity

Sample for the determination of antioxidant activity in PJ was prepared by mixing 10 ml of juice and 90 ml of demineralized water (1:10 v/v). In case of solid substrates (e.g., PS), antioxidant activity was measured on ethanolic extract, prepared following the procedure reported in Caponio *et al.* (2020), with modifications, by mixing 1 g of seeds and 10 ml of ethanol:water solution (80:20, v/v). The mixture was stirred (VM4 IDL stirrer) for 10 min, speed 6, at room temperature, then treated using an ultrasonic bath for 15 min and centrifuged at $13552 \times g$, for 10 min, 25 °C. Supernatant was collected, whereas the pellet was subjected to two further extractions, in the same conditions as above. The supernatants from the three extractions were combined and stored at -20 °C until analysis.

Antioxidant activity was estimated in terms of scavenging activity towards the radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH $\cdot$), and determination of the ferric reducing antioxidant power (FRAP). The free radical scavenging activity against DPPH $\cdot$ was measured as described by Caponio *et al.* (2020). In details, 50 μ l of each sample were added to 950 μ l of DPPH $\cdot$ solution (0.0031 g/100 ml in ethanol). Blank and positive control containing 50 μ l of ethanol-water solution or the synthetic antioxidant butylated hydroxytoluene (BHT) as reference (1 g/l in ethanol-water solution), respectively, instead of sample, were included in the analysis. After incubation (in dark, 30 min at 25 °C), the absorbance was measured at 517 nm. The free radical scavenging activity was calculated as follows:

$$\begin{aligned}
 &DPPH \cdot \text{ scavenging activity (\%)} \\
 &= [(blank \text{ absorbance} \\
 &- sample \text{ absorbance})/blank \text{ absorbance}] \times 100.
 \end{aligned}$$

FRAP was evaluated according to Son *et al.* (2018) modified by Minervini *et al.* (2019). In detail, 200 µl of sample (juice or ethanolic extracts) were mixed with 200 µl of 0.2 M sodium phosphate buffer (pH 6.6) and 200 µl of potassium ferricyanide (1 g/100 ml in demineralized water). The reaction mixture was incubated at 50 °C for 20 min, then stopped by adding 200 µl of trichloroacetic acid (10 g/100 ml in demineralized water). After centrifugation (8000 × g, 10 min, 4 °C), 500 µl of the collected supernatant were added of 100 µl ferric chloride (0.1 g/100 ml in demineralized water) and 400 µl of demineralized water. After incubation (10 min, 25 °C), the absorbance of ferrous ion was measured at 700 nm. BHT (0.1 g/l in ethanol-water solution) was used as a positive control.

2.2.5 Microbiological characterization of juice and seeds

Microbiological analyses were carried out on 10 g of PJ and PS obtained from native fresh arils stored at 4 °C, using culture media and supplements purchased from Oxoid (Dublin, Ireland), after serial dilutions in sterile saline solution (9 g of NaCl per litre of demineralized water). Dilutions were plated using the following agar media and incubation conditions: Plate Count (PCA) (30 °C, 48 h, for total mesophilic aerobic microorganisms), de Man Rogosa Sharpe (MRS) with cycloheximide (0.1 g/l) (30 °C, 48 h, for mesophilic rod-shaped LAB), M17 supplemented with glucose solution (10 g/100 ml in demineralized water) and cycloheximide (0.1 g/l) (30 °C, 48 h, for mesophilic coccus-shaped LAB), Violet Red Bile Glucose (VRBGA) (37 °C, 24 h, for *Enterobacteriaceae*), Baird Parker supplemented with egg yolk-tellurite emulsion (37 °C, 48 h, for staphylococci), Slanetz and Bartley (37 °C, 48 h, for enterococci), Sabouraud Dextrose (SDA) (30 °C, 48 h, for moulds and yeasts), Wort agar (WA) (0.1 g/l) (25 °C, 48 h, for moulds and yeasts). For the enumeration of LAB, *Enterobacteriaceae*, moulds and yeasts, pour plate technique was performed; instead, spread plate technique was carried out for the enumeration of staphylococci and enterococci. In detail, the identification of moulds and yeasts was accomplished by visual analysis of colony morphology.

2.2.6 Isolation of pro-technological microorganisms from PJ and PS

PJ and PS from native fresh arils were used as source of isolation of pro-technological microorganisms after spontaneous fermentation. To obtain a substrate for spontaneous fermentation of seeds, a semi-solid matrix was prepared adding tap water to PS (seeds:water ratio of ca. 4:1, w/v). Both juice and water-seeds mixture were incubated at 30 °C for 16 h. Spontaneously fermented juice and seeds were then analysed to isolate presumptive LAB and yeasts, using the above serial dilutions method. After incubation of plates, only yeast colonies were found. Therefore, yeasts were isolated by picking up colonies from the SDA plates inoculated with the highest diluted samples. Twelve yeast pure cultures were obtained after two consecutive streaks on the same agar medium. All the isolates were stored at -20 °C in Sabouraud Dextrose broth with glycerol (0.2 ml of glycerol per ml).

2.2.7 Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and genotypic ideation of yeasts

Yeasts DNA was extracted using the Wizard® Genomic DNA purification kit (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. The concentration of extracted DNA was estimated by spectrophotometric determination, using the Nanodrop 2000c (Thermo Fisher Scientific Inc, Wilmington, U.S.A.). Yeasts were biotyped by RAPD-PCR analysis using two primers: M13m (5'- GAGGGTGGCGGTTC -3'), and RP11 (5'- GAAACTCGCCAAG -3') (Del Bove *et al.*, 2009). The reaction mixture contained 200 µmol/l of each dNTP, 2 µmol/l primer, 4 mmol/l MgCl₂, 4 mmol/l KCl, 0.1 U of GoTaq® G2 Flexi DNA polymerase (Promega, Madison, Wisconsin, USA), 1.0 µl PCR buffer, 20 ng DNA and sterile molecular reagent grade water to 25 µl. PCR was performed in the same conditions reported by Del Bove *et al.* (2009) and PCR products were separated by agarose gel electrophoresis at 110 V for 2 h and detected by transilluminator. The size (in bp) of the PCR products was estimated using the HyperLadder™ 1kb (Bioline, London, United Kingdom) marker. Gel images were acquired through the Gel Doc 2000 Documentation System (Bio-Rad Laboratories) and four isolates (one from PS and three from PJ) were selected because their RAPD profile was representative of all the isolates.

Yeasts identification was obtained upon partial sequencing of 26S rRNA gene, amplified using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). The reaction mixture was prepared on ice by adding the following reagents: 200 µmol/l of each dNTP, 0.2 µmol/l of each primer, 2 mmol/l MgCl₂, 2 mmol/l KCl, 0.04 U of GoTaq® G2 Flexi DNA polymerase (Promega, Madison, Wisconsin, USA), 10.0 µl PCR buffer, ca. 10 ng DNA and sterile molecular reagent grade water to 50 µl. PCR was performed in the same conditions reported by Kurtzman & Robnett (1998). PCR products were separated as indicated above. The purification of the PCR product (ca. 600 bp) was carried out using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

PCR products were sequenced by MacroGen Europe BV (Amsterdam, The Netherlands) and the DNA sequence homology (higher than 99%) was determined through pair-wise sequence alignments, using BLAST within the NCBI nucleotide collection database.

2.2.8 Fermentation of PJ and PS and characterization of the fermented products

Yeast strains isolated from pomegranate matrices were singly used as starters for fermenting PJ and PS (seeds:water ratio of ca. 4:1, w/v), obtained from chemically sterilized arils. When used for fermentation, yeasts were cultivated (48 h, 30 °C) in Sabouraud Dextrose broth, harvested by centrifugation (10000 × g, 10 min, 4 °C), washed twice with 50 mM sterile potassium phosphate buffer (pH 7.0), re-suspended in juices samples or in sterile distilled water and used to inoculate PJ or PS, respectively (initial cell number corresponding to ca. 7.0 log cfu/g). PJ and PS were fermented at 30 °C for 24 h. PJ and PS from native (unsterilized) arils were spontaneously fermented without the addition of starters at 30 °C for 24 h and used as the control. At the end of fermentations, PJ and PS were cooled to 10 °C. Cell density of presumptive yeasts was determined using SDA under the same conditions indicated above. Chemical analyses and determination of antioxidant activity were carried out as described above.

Based on the results obtained from chemical characterization of fermented products, the most suitable yeast strain was selected for fermenting seeds that would be further treated for food application.

2.2.9 Treatment of PS for obtaining food ingredient

PS fermented by the selected yeast strain, as well as unprocessed (unfermented) seeds, were dried in an oven at 60 °C, overnight. Then, dried seeds were ground through a coffee mill and the flours obtained were used as an ingredient for production of *granola* snack. Three theses of *granola* snacks were produced at the pilot plant of the Department of Soil, Plant and Food Sciences of the University of Bari: (i) *granola* snack without seeds flour (G-C), used as a control; (ii) *granola* snack with unprocessed pomegranate seeds flour (G-US); (iii) *granola* snack with fermented pomegranate seeds flour (G-FS). *Granola* snack protocol (percentage of ingredients, time, and temperature of baking) had been preliminarily set-up. Ingredients of *granola* snacks are detailed in Table 1.

Table 1. Recipes of *granola* snacks. *Granola* snack without the addition of the pomegranate seeds flour (control) (G-C); *granola* snack with the addition of unprocessed pomegranate seeds flour (G-US); *granola* snack with the addition of flour obtained by pomegranate seeds fermented by *Hanseniaspora valbyensis* S-L1 (G-FS). Ingredients are expressed as percentage of total weight.

	G-C	G-US	G-FS
Water	18.2	18.2	18.2
Seeds oil	7.3	7.3	7.3
Honey	7.3	7.3	7.3
Brown sugar	1.7	1.7	1.7
Fermented pomegranate seeds flour	- [*]	-	18.2
Unprocessed pomegranate seeds flour	-	18.2	-
Whole oat	18.2	9.1	9.1
Puffed barley	18.2	9.1	9.1
Almonds	10.9	10.9	10.9
Hazelnut	10.9	10.9	10.9
Goji berries	7.3	7.3	7.3

-* Ingredient not added

The *granola* production protocol consisted of four phases: (i) preparation of a syrup by heating water, seeds oil, brown sugar, and honey for 10 min; (ii) addition of pomegranate (fermented or unfermented) seeds flour, whole oats, puffed barley, almonds, and hazelnuts to the syrup; (iii) baking the granola in an oven for 30 min at 160 °C, with open valve; (iv) cooling and breaking the *granola snack with subsequent addition of the goji berries*.

2.2.10 Characterisation of *granola* snacks

After 24 h from baking, *granola* snacks were subjected to the determination of antioxidant activity and to sensory analysis. Antioxidant activity was estimated in terms of scavenging activity towards the radical DPPH· and FRAP on ethanolic extracts of *granola* snacks, using the methods described above for seeds. As regard the sensory analysis, the panel was composed of thirteen volunteers (5 males and 8 females, with a mean age of 27-years-old, a range of 24–30 years old) from laboratory staff, which had been previously trained about the meaning of the sensory attributes and scores. Each *granola* snack was identified by a code number and served, at room temperature, under daylight illumination, in random order. Each panellist evaluated two spoons of *granola* snack per thesis. The attributes were: size, shape regularity, colour, typical granola odour, toasted odour, caramel odour, off-odour, breakage, adhesiveness, hardness, gumminess, persistence of particles difficult to swallow, sweet taste, bitter taste, caramel taste, typical *granola* taste, palatability and overall acceptability. The score for each sensory attribute ranged from 1 (lowest) to 9 (highest).

2.3 Results

2.3.1 Chemical composition and microbial cell densities of fresh unprocessed PJ and PS

As expected, overall PS showed higher concentration of all the nutrients than PJ (Table 2). The only exception was potassium, whose concentration in PJ was ca. two-fold higher than in seeds. Potassium and calcium were the most abundant minerals in both PJ and PS. Total mesophilic aerobic microorganisms were found at higher ($P < 0.05$) cell density in PS than in PJ (Figure 1). No significant ($P > 0.05$) differences in cell density of moulds and yeasts were found between seeds and juice, regardless of the agar medium used. Fungal biota was in the order of 5 log

cfu/g in all the samples (Figure 1). Colonies found on agar media elective for LAB were revealed as yeast colonies, upon microscopic observation. Enterococci, staphylococci, and *Enterobacteriaceae* were below the detection limit (2 and 1 log cfu/g for cocci and *Enterobacteriaceae*, respectively) (data not shown).

Table 2. Chemical analyses on pomegranate juices (PJ) and seeds (PS) unprocessed (UN), fermented by *Hanseniaspora valbyensis* J-L1 (J-L1), *H. valbyensis* J-L5 (J-L5), *H. valbyensis* J-L6 (J-L6) and *H. valbyensis* S-L1 (S-L1) strains, and spontaneously fermented (SF). Values in the same column with common letter (^{a-i}) were not significantly ($p > 0.05$) different.

	Total carbohydrates (g/100 g)	Proteins (g/100 g)	Unsaturated fats (g/100 g)	Mono-unsaturated fats (g/100 g)	Poly-unsaturated fats (g/100 g)	Saturated fats (g/100 g)	Crude Fibre (g/100 g)	Ashes (g/100 g)	Ca (mg/100 g)	K (mg/100 g)	Fe (mg/100 g)	Zn (mg/100 g)
UN-PJ	16.70 ^b	0.68 ^g	0.25 ^d	0.14 ^c	0.11 ^{cd}	0.18 ^d	- ^a	0.73 ^b	7.50 ^{fg}	200.70 ^a	0.90 ^c	0.20 ^{de}
J-L1 PJ	8.20 ^e	0.43 ^h	0.13 ^e	0.07 ^d	0.06 ^d	0.09 ^e	-	0.40 ^f	8.20 ^f	167.70 ^{cd}	0.50 ^d	0.20 ^{de}
J-L5 PJ	7.20 ^{fg}	0.37 ^h	0.11 ^e	0.06 ^d	0.05 ^d	0.08 ^e	-	0.65 ^{cd}	7.40 ^g	152.70 ^f	1.00 ^c	0.20 ^{de}
J-L6 PJ	7.40 ^f	0.44 ^h	0.12 ^e	0.06 ^d	0.05 ^d	0.08 ^e	-	0.62 ^d	9.70 ^e	189.50 ^b	1.00 ^c	0.20 ^{de}
S-L1 PJ	6.80 ^g	0.36 ^h	0.11 ^e	0.06 ^d	0.05 ^d	0.08 ^e	-	0.43 ^f	8.20 ^f	155.90 ^{ef}	0.50 ^d	0.10 ^e
SF-PJ	16.60 ^b	0.74 ^{fg}	0.26 ^d	0.14 ^c	0.12 ^c	0.19 ^d	-	0.56 ^e	5.90 ^h	127.30 ^g	1.10 ^c	0.20 ^{de}
UN-PS	18.10 ^a	1.34 ^a	0.48 ^a	0.26 ^a	0.22 ^a	0.35 ^a	12.40 ^c	0.92 ^a	25.70 ^b	103.80 ^h	1.00 ^c	0.40 ^{bc}
J-L1 PS	10.80 ^d	0.93 ^{de}	0.29 ^{cd}	0.16 ^c	0.13 ^c	0.21 ^{cd}	9.70 ^d	0.74 ^b	15.30 ^c	60.80 ⁱ	3.70 ^a	0.50 ^b
J-L5 PS	7.60 ^{ef}	0.85 ^{ef}	0.29 ^{cd}	0.16 ^c	0.13 ^c	0.21 ^{cd}	14.50 ^a	0.72 ^{bc}	13.10 ^d	64.00 ⁱ	1.10 ^c	0.40 ^{bc}
J-L6 PS	10.10 ^d	1.07 ^c	0.36 ^{bc}	0.20 ^{bc}	0.16 ^{bc}	0.26 ^{bc}	13.80 ^{ab}	0.75 ^b	13.50 ^d	62.30 ⁱ	1.70 ^b	0.30 ^{cd}
S-L1 PS	10.30 ^d	1.04 ^{cd}	0.32 ^{cd}	0.18 ^{bc}	0.14 ^{bc}	0.23 ^{cd}	12.40 ^c	0.72 ^{bc}	35.30 ^a	161.70 ^{de}	1.90 ^b	0.70 ^a
SF-PS	14.70 ^c	1.22 ^b	0.42 ^{ab}	0.23 ^{ab}	0.19 ^{ab}	0.31 ^{ab}	13.30 ^b	0.87 ^a	26.50 ^b	172.90 ^c	0.90 ^c	0.50 ^b

⁻ Crude fibre content was not determined in juices

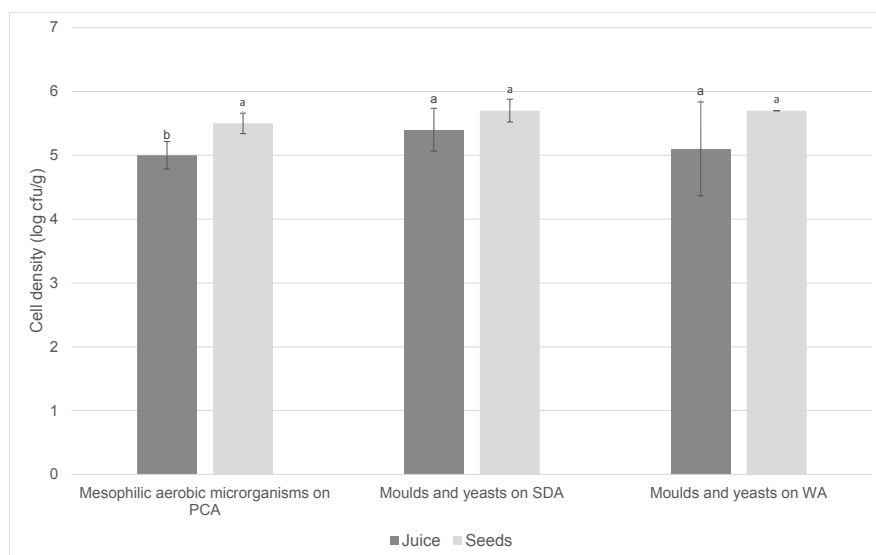


Figure 1. Cell density (expressed as log cfu/g) of total mesophilic aerobic microorganisms (determined on Plate Count agar (PCA)), moulds and yeasts (determined on Sabouraud Dextrose agar (SDA) and Wort agar (WA)) in fresh pomegranate juice and seeds. For each microbial group, bars sharing one letter (^{a-b}) indicate not significantly ($p > 0.05$) different values of cell density.

2.3.2 Identification and selection of yeasts isolated from spontaneously fermented pomegranate

Upon 16 h of spontaneous fermentation, yeast cell density reached the order of 7 ± 0.1 log cfu/g both in PJ and PS (data not shown). Colonies observed on agar media elective for LAB revealed as yeasts. Therefore, we proceeded to isolate yeasts from SDA plates. Six yeasts colonies from spontaneously fermented PJ and six from spontaneously fermented PS were isolated and biotyped through RAPD-PCR analysis. At the similarity level of 80%, the twelve isolates were grouped into four clusters (I-IV) (data not shown). Clusters I, II and III included isolates from PJ. The IV cluster only grouped isolates from PS. Isolates representative for each cluster (namely J-L1, J-L5, J-L6 and S-L1 from juice and seeds, respectively) were subjected to identification by sequencing the D1/D2 domain of the DNA encoding the 26S rRNA. All the isolates were identified as *Hanseniaspora valbyensis* and subsequently used, as single starter, for driven fermentation of PJ and PS (water-seeds mixture). After 24 h of fermentation, cell density of yeasts in PJ and PS fermented by *H. valbyensis* strains increased significantly ($P < 0.05$) in all the

theses (Table 3). All isolates grew up to ca. 7.7 log cfu/g and 7.9 log cfu/g in PJ and PS, respectively (average value). *H. valbyensis* J-L5 and *H. valbyensis* J-L6 were characterized by higher, although not significantly different, values of cell yield both in PJ and PS. *H. valbyensis* S-L1 showed the lowest, although not significantly different, increase of the cell yield both in PJ and PS (ca. 7.6 log cfu/g and 7.8 log cfu/g, respectively). After 24 h of spontaneous fermentation, the cell number of presumptive yeasts showed increases from ca. 2.0 to 4.1 and from 2.3 to 4.8 log cfu/g in PJ and PS, respectively (Table 3).

Table 3. Cell density of yeasts (log cfu/g) in pomegranate juices and seeds fermented by *Hanseniaspora valbyensis* J-L1, *H. valbyensis* J-L5, *H. valbyensis* J-L6 and *H. valbyensis* S-L1 strains, and spontaneously fermented before (To) and after (T24) 24 h of fermentation at 30 °C. Values in the same line with common letter (a–b) were not significantly ($p > 0.05$) different.

	Pomegranate juice		Pomegranate seeds	
	To	T24	To	T24
<i>Hanseniaspora valbyensis</i> J-L1	7.0 ± 0.2 ^b	7.6 ± 0.0 ^a	7.0 ± 0.1 ^b	7.9 ± 0.2 ^a
<i>Hanseniaspora valbyensis</i> J-L5	7.0 ± 0.0 ^b	7.9 ± 0.0 ^a	7.1 ± 0.0 ^b	7.9 ± 0.2 ^a
<i>Hanseniaspora valbyensis</i> J-L6	7.0 ± 0.0 ^b	7.8 ± 0.0 ^a	7.0 ± 0.2 ^b	8.0 ± 0.1 ^a
<i>Hanseniaspora valbyensis</i> S-L1	7.0 ± 0.2 ^b	7.6 ± 0.0 ^a	7.1 ± 0.2 ^b	7.8 ± 0.1 ^a
Spontaneously fermented	2.0 ± 0.0 ^b	4.1 ± 0.2 ^a	2.3 ± 0.0 ^b	4.8 ± 0.0 ^a

PJ and PS fermented by *H. valbyensis* strains were characterized by lower ($P < 0.05$) concentrations of carbohydrates, proteins, and fats than unprocessed (unfermented) matrices (Table 2). Those macro-nutrients were found in spontaneously fermented PJ and PS at concentrations that were not significantly ($P > 0.05$) different from those in unprocessed matrices. Carbohydrates represented the only exception, because they decreased ($P < 0.05$) during seeds fermentation. Overall, ashes decreased ($P < 0.05$) during fermentation (either spontaneous or driven by *H. valbyensis*), with the exception of spontaneously

fermented PS. As regards calcium, potassium, iron, and zinc, they generally decreased in fermented PJ, compared to unprocessed (Table 2). Two exceptions were: calcium, in juices fermented by *H. valbyensis* J-L1, J-L6, S-L1, and iron in spontaneously fermented, and J-L5- and J-L6-fermented juices, which either increased or were found at not significantly ($P > 0.05$) different concentrations. Comparing fermented to unprocessed seeds, calcium and potassium decreased ($P < 0.05$) in fermented PS, except for those spontaneously fermented or fermented by *H. valbyensis* S-L1, wherein both minerals increased ($P < 0.05$). During fermentation of seeds, iron concentration generally increased, except for spontaneously fermented PS. In detail, iron concentration was ca. three and two-fold higher than unprocessed seeds, when *H. valbyensis* J-L1 and S-L1 were used as starters, respectively. Overall zinc concentration was not affected by fermentation, excepted for PS fermented by *H. valbyensis* S-L1, wherein this mineral increased ($P < 0.05$). Mineral content of pomegranate seeds was overall higher than juices. Fibre concentration overall increased during seeds fermentation, except for those fermented by *H. valbyensis* S-L1 (no variation) or by *H. valbyensis* J-L1 (decrease) (Table 2).

Antioxidant activity of pomegranate juices was overall higher than seeds, considering both the results of radical scavenging activity (Figure 2) and those of FRAP (Figure 3). Positive control (BHT) showed a radical scavenging activity of 92.11% (data not shown in the figure). Apart from the positive control, the highest ($P < 0.05$) radical scavenging activity (82.9%) was found for unprocessed PJ. Upon fermentation, the scavenging activity of PJ decreased ($P < 0.05$). Among fermented PJ, the spontaneously fermented one and those fermented by *H. valbyensis* J-L1 or J-L6 showed the highest ($P < 0.05$) activity. Fermented PS were characterized by lower ($P < 0.05$) antioxidant activity than unprocessed seeds (Figure 2). A value of FRAP of 2.7 was found for positive control (data not shown in the figure). The highest ($P < 0.05$) FRAP was found for unprocessed matrices (2.7 for juice and 1.3 for seeds) (Figure 3). Among fermented matrices, those spontaneously fermented showed the highest ($P < 0.05$) antioxidant activity.

Based on the mineral results, *H. valbyensis* S-L1, isolated from PS, was selected as the autochthonous starter and the corresponding fermented seeds was chosen as novel food ingredient for enriching the *granola* snack.

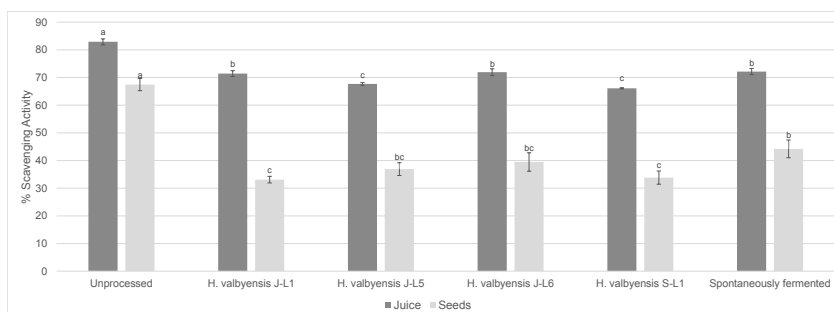


Figure 2. DPPH· Scavenging activity (%) of pomegranate juices (dark grey) and seeds (light grey) unprocessed, fermented by *Hanseniaspora valbyensis* J-L1, *H. valbyensis* J-L5, *H. valbyensis* J-L6 and *H. valbyensis* S-L1 strains, and spontaneously fermented. Antioxidant activity was measured, in case of seeds, on ethanolic extracts and, in case of juices, on juices diluted in demineralized water (1:10). For each matrix (juice and seeds), bars sharing at least one letter (^{a-c}) indicate not significantly ($p > 0.05$) different values of radical scavenging activity.

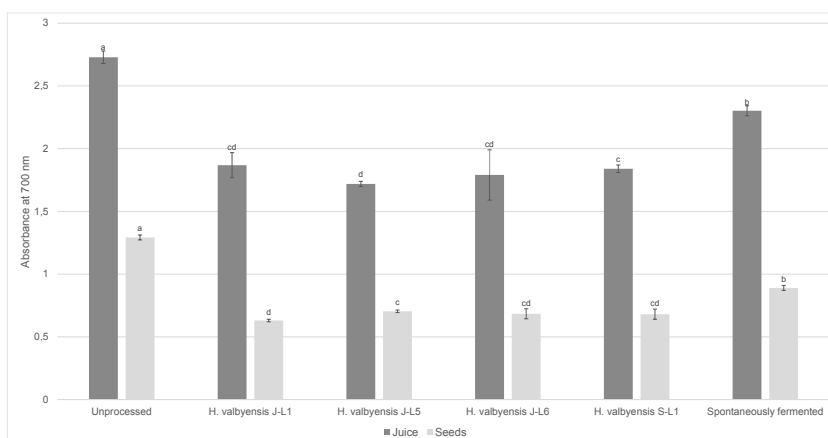


Figure 3. Ferric reducing antioxidant power (FRAP) of pomegranate juices (dark grey) and seeds (light grey) unprocessed, fermented by *Hanseniaspora valbyensis* J-L1, *H. valbyensis* J-L5, *H. valbyensis* J-L6 and *H. valbyensis* S-L1 strains, and spontaneously fermented expressed as absorbance at 700 nm. Antioxidant activity was measured, in case of seeds, on ethanolic extracts and, in case of juices, on juices diluted in demineralized water (1:10). For each matrix (juice and seeds), bars sharing at least one letter (^{a-d}) indicate not significantly ($p > 0.05$) different values of antioxidant activity.

2.3.3 Antioxidant activity and sensory analysis of *granola* snacks

As expected, *granola* snacks containing pomegranate seeds flour were characterized by a higher ($P < 0.05$) antioxidant activity than *granola* snack without pomegranate seeds flour (control, G-C) (Figure 4). No significant differences ($P > 0.05$) were observed in radical scavenging activity between *granola* added with unprocessed seeds flour (G-US) and *granola* added with seeds flour previously fermented with *H. valbyensis* S-L1 (G-FS) (Figure 4a). On the contrary FRAP was higher ($P < 0.05$) in G-US than G-FS (Figure 4b). Table 4 reports the expected nutritional values of the three snacks, calculated based on the amounts of each different ingredient. Visual and olfactory analyses, as well as most tactile descriptors (breakage, adhesiveness, hardness, gumminess) and caramel taste, showed almost no differences between *granola* snacks (Figure 5 and Table 5). Highest presence of particles difficult to swallow and highest bitter taste were perceived in G-US. G-C was perceived as sweeter than G-US ($P < 0.05$), and as sweet as G-FS. In addition, compared to G-US, G-FS was characterised by higher ($P < 0.05$) palatability, although lower ($P < 0.05$) than G-C. Overall acceptability was highest for G-C ($P < 0.05$); nevertheless, considering *granola* snacks enriched with pomegranate seeds flour, the panellists preferred the thesis G-FS, obtained upon fermentation by *H. valbyensis* S-L1.

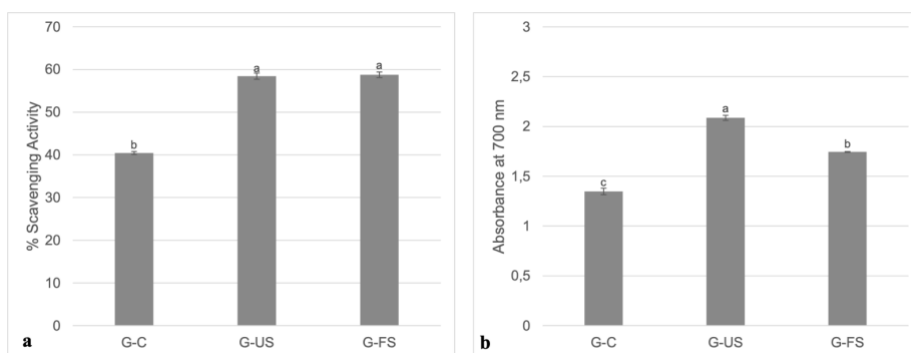


Figure 4. Antioxidant activity on ethanolic extracts of *granola* snacks as assessed through determination of 2,2-DiPhenyl-1-PicrylHydrazyl (DPPH) radical scavenging activity (panel a) and ferric reducing antioxidant power (FRAP) assay expressed as absorbance at 700 nm (panel b). *Granola* snack without the addition of the pomegranate seeds flour (control) (G-C); *Granola* snack with the addition of unprocessed pomegranate seeds flour (G-US); *Granola* snack with the addition of flour obtained by pomegranate seeds fermented by

Hanseniaspora valbyensis S-L1 (G-FS). For each assay, bars sharing at least one letter (^{a-c}) showed no significant differences ($p > 0.05$).

Table 4. Expected nutritional values of the *granola* snacks. *Granola* snack without the addition of the pomegranate seeds flour (control) (G-C); *granola* snack with the addition of unprocessed pomegranate seeds flour (G-US); *granola* snack with the addition of flour obtained by pomegranate seeds fermented by *Hanseniaspora valbyensis* S-L1 (G-FS). The nutritional values were calculated based on the amounts of each different ingredient of the *granola* snacks and on the values reported on the nutrition labels of ingredients and as regards G-US and G-FS, on the chemical analyses carried out on the seeds in this study.

Expected nutritional values (on 100 g of product)	G-C	G-US	G-FS
Energy value (kcal)	456	390	390
Fats (g)	25.68	24.96	24.86
which saturated fats (g)	2.40	2.29	2.26
Carbohydrates (g)	46.36	33.30	33.56
Fiber (g)	7.37	8.20	8.20
Proteins (g)	9.90	8.10	8.03
Calcium (mg)	78.60	76.00	78.10
Iron (mg)	2.90	2.50	2.80
Potassium (mg)	252.80	234.70	247.60
Zinc (mg)	1.42	1.20	1.30



Figure 5. Appearance of *granola* snacks: a) without pomegranate seeds flour (G-C, control); b) with unprocessed pomegranate seeds flour (G-US); c) with *Hanseniaspora valbyensis* S-L1-fermented pomegranate seeds flour (G-FS).

Table 5. Sensory analysis of *granola* snacks. *Granola* snack without the addition of the pomegranate seeds flour (control) (G-C); *granola* snack with the addition of unprocessed pomegranate seeds flour (G-US); *granola* snack with the addition of flour obtained by pomegranate seeds fermented by *Hanseniaspora valbyensis* S-L1 (G-FS). Values in the same line sharing at least one letter (^{a-c}) showed not significantly ($p > 0.05$) different score, ranging from 1 (lowest) to 9 (highest).

	Descriptors	G-C	G-US	G-FS
Visual analysis	Size	4.2 ± 0.9 ^a	4.4 ± 1.1 ^a	4.1 ± 1.0 ^a
	Shape regularity	3.5 ± 2.7 ^a	4.1 ± 2.7 ^a	3.9 ± 2.4 ^a
	Colour	7.0 ± 1.5 ^a	7.8 ± 1.6 ^a	7.8 ± 0.8 ^a
	Typical granola			
Olfactory analysis	odour	4.8 ± 2.0 ^a	3.5 ± 1.9 ^a	3.9 ± 2.4 ^a
	Toasted odour	5.9 ± 2.3 ^a	5.1 ± 2.6 ^a	5.3 ± 2.9 ^a
	Caramel odour	5.2 ± 2.6 ^a	3.4 ± 2.4 ^a	4.1 ± 2.4 ^a
	Off-odour	0.3 ± 0.9 ^a	1.6 ± 1.9 ^a	1.0 ± 1.5 ^a
Tactile analysis	Breakage	4.1 ± 2.9 ^a	4.3 ± 2.2 ^a	3.7 ± 2.8 ^a
	Adhesiveness	2.8 ± 2.2 ^a	2.5 ± 2.2 ^a	1.4 ± 1.8 ^a
	Hardness	5.5 ± 2.0 ^a	6.0 ± 2.1 ^a	6.0 ± 1.8 ^a
	Gumminess	1.4 ± 1.3 ^a	0.7 ± 0.9 ^a	1.2 ± 1.9 ^a
Taste analysis	Persistence of particles difficult to swallow	0.5 ± 1.0 ^c	3.4 ± 1.1 ^a	1.8 ± 1.7 ^b
	Sweet taste	5.1 ± 1.7 ^a	3.1 ± 1.5 ^b	4.0 ± 2.1 ^{ab}
	Bitter taste	1.9 ± 1.9 ^c	4.7 ± 0.7 ^a	3.6 ± 1.9 ^b
	Caramel taste	3.8 ± 1.7 ^a	2.8 ± 1.3 ^a	2.5 ± 2.0 ^a
Overall acceptability	Typical granola taste	6.1 ± 1.6 ^a	3.2 ± 1.5 ^b	3.9 ± 2.5 ^b
	Palatability	7.9 ± 0.7 ^a	4.7 ± 1.3 ^c	6.5 ± 1.3 ^b
		7.5 ± 0.8 ^a	4.9 ± 1.4 ^c	6.3 ± 1.7 ^b

2.4 Discussion

To fight against nutritional deficiency and climate change are among the most important challenges for food researchers and industries. A sustainable strategy to face with such challenges is represented by the valorization of agri-food by-products or zero-kilometer local productions (Santos, Pintado, & da Silva, 2022). In this study we focused on juices and seeds obtained by squeezing pomegranate arils, rich in phenolic compounds, tannins, flavonoids, sterols, fatty acids, dietary fibre, vitamins, and minerals (Venkitasamy *et al.*, 2019), and on autochthonous yeasts capable to grow in pomegranate matrices and potentially improve their nutritional value. Overall, as regard chemical composition, higher level of minerals was found in PS compared to PJ. This can be explained considering that concentrations of all the nutrients were not expressed on a dry weight base, and PJ mainly consists of water. Compared to a previous study (Rowayshed *et al.*, 2013), PS seemed to be quite poor in proteins, but the data cannot be straightforwardly compared because Rowayshed *et al.* (2013) showed the concentrations of nutrients as percentage on dry weight base.

From the literature available to us, we could not find any study reporting the composition of pomegranate microbiota. We found that pomegranate microbiota was dominated by yeasts. This microbial group, probably environment-borne, typically contaminates fruit peels (Kalia & Gupta, 2006). Notwithstanding the use of culture media selective (e.g., VRBGA) or elective (e.g., MRS) for specific groups of bacteria, we did not find bacteria at detectable cell density, even after spontaneous fermentation. This could be due to the low pH and the presence of many bactericidal compounds (e.g., hydrolysable tannins, ellagitannins, gallotannins, anthocyanins and flavonols) in the pomegranate matrices (Howell & D'Souza, 2013; Reddy, Gupta, Jacob, Khan, & Ferreira, 2007). The only yeasts species identified in both PJ and Ps was *Hanseniaspora valbyensis*. This non-conventional yeast is often found as one of the microbial drivers of balsamic vinegar and cider fermentations (Bellut *et al.*, 2018), although it does not fall within the list of microorganisms with Qualified Presumption of Safety (QPS) status. *H. valbyensis* is known for its outstanding pectinolytic (Panon, Massiot, & Drilleau, 1995) and endo-glucanase (Abd-El-Al & Phaff, 1969) activities, and for its tolerance to high levels of selenium, osmotolerance and acid tolerance (Golubev & Golubev, 2002). It does not produce high levels of ethanol, but it forms significant amounts

of ethyl and phenethyl acetate, positively impacting odour and taste (Xu, Zhao, & Wang, 2006). Indeed, Bellut *et al.* (2018) tried to use “non-*Saccharomyces* yeasts” (including *H. valbyensis*) to produce alcohol-free beer. *H. valbyensis* showed excellent performance during propagation and fermentation, and the experimental alcohol-free beer received positive feedback from the panellists (Bellut *et al.*, 2018). Its osmotolerance and acid tolerance justify the occurrence of this yeast species in the pomegranate-derived matrices subject of the current study and make it of biotechnological interest.

The four representative autochthonous strains of *H. valbyensis* were able to drive the fermentation of pomegranate matrices, causing modifications of the chemical composition. Overall, concentrations of carbohydrates, proteins, fats, and ashes were lower in matrices fermented with *H. valbyensis* than in unfermented juice and seeds, because microorganisms reasonably used these nutrients for their growth. However, we found neither a unique trend for all the nutrients nor a perfect correspondence between increase of cell density and modification in nutritional composition of matrices during fermentation. For instance, the highest increase in minerals (calcium, potassium, iron, and zinc) concentration was observed in seeds fermented by *H. valbyensis* S-L1, isolated from PS, which was the strain showing the lowest (although not significantly different) increase of cell density after 24 h. Parallel to the increase of those minerals, and in apparent contrast with that, we found that ashes decreased during fermentation. This agreed with a previous study on biscuits (Adebiyi, Obadina, Adebo, & Kayitesi, 2017), wherein it was hypothesized that decrease of ashes during fermentation was due to leaching of soluble inorganic salts. At the same time, fermentation of seeds seemed, in some cases, to increase some minerals. Indeed, it may occur that during fermentation, some microbial enzymes (e.g., phytases) could act on mineral-chelating compounds (e.g., phytic acid), causing an increased availability and/or extractability of some minerals (Sade, 2009). On the other hand, a significant decrease in Ca and K was observed in pomegranate seeds fermented by *H. valbyensis* J-L1, J-L5, and J-L6, isolated from PJ. The loss of those minerals could be due to their utilization by the microorganisms involved in the fermentation process, in agreement with a previous study (Torres, Frías, Granito, & Vidal-Valverde, 2006). We could hypothesize that *H. valbyensis* J-L1, J-L5, and J-L6 would require higher quantity of Ca and K for satisfying their growth requirements,

compared to S-L1 (Dallal, Zamaniahari, Davoodabadi, Hosseini, & Rajabi, 2017; Kiczorowski, Kiczorowska, Samolińska, Szmigielski, & Winiarska-Mieczan, 2022). In the current research, during pomegranate fermentation with *H. valbyensis*, the antioxidant activity decreased, compared to the unprocessed matrices. This result disagreed with those found in several studies reporting an increase of antioxidant activity upon fermentation (Cheng, Choi, Yang, & Suh, 2016; Victoria, Rodica, & Georgeta, 2015; Xu *et al.*, 2021). We may hypothesize that this result could be attributed to the ability of *Hanseniaspora* spp. to form acetates, which cause a reduction of antioxidant activity (Andlauer, Stumpf, & Fürst, 2000; Coelho, Genisheva, Oliveira, Teixeira, & Domingues, 2017; Su & Chien, 2007).

Among the four autochthonous yeasts, we selected *H. valbyensis* S-L1, because besides being able to grow in the pomegranate matrices, it caused the best increase in mineral content in seeds. Based on the chemical composition of PS fermented by S-L1, we tried to design a fortified food that, being supplemented with this fermented by-product, could be of nutritional interest. *Granola* is a food generally eaten for breakfast or as a snack during the day. This “breakfast cereal” generally includes cereals, nuts, and fruits as main ingredients. Unlike other large-scale consumption foods (e.g., bread), granola crunchy texture eases the use of additional and non-conventional ingredients (e.g., alternative flours) without severely affecting sensory and structural aspects (Pathare, Baş, & Byrne, 2012). Numerous studies reported the use of by-products, other than pomegranate seeds flour, as additional ingredient to produce breakfast cereals (Cecchi *et al.*, 2019; Dos Santos *et al.*, 2019; Oliveira, Marques, Kwiatkowski, Monteiro, & Clemente, 2013). In this study, we compared three theses of *granola* snack, aiming to highlight the added value of using pomegranate seeds flour, previously fermented (G-FS) or not (G-US), with respect to a conventional *granola* (G-C), serving as a control.

Compared to G-C, the use of pomegranate seeds flour, as an additional ingredient of *granola*, seemed to increase *in vitro* antioxidant activity of the snack. In agreement with antioxidant activity found for pomegranate seeds, FRAP of G-US was higher than G-FS. Based on the expected nutritional values of each ingredient of *granola* snacks, G-FS and G-US could contain less proteins and, especially, carbohydrates than G-C (control *granola*); in addition, the two fortified snacks could be characterized by higher content of fibre. However, all the *granola* snacks could be labeled as “high fibre”, because they theoretically contain more than 6 g

for 100 g of product (EU Regulation No 1924/2006 and subsequent modifications). Regarding minerals, the expected contents of calcium, iron, potassium, and zinc were higher in G-FS and G-C, compared to G-US. This was in accordance with the results of chemical analyses carried out on (unprocessed) seeds and seeds fermented with *H. valbyensis* S-L1. Finally, we expect that the two *granola* snacks fortified with pomegranate seeds flour would have lower (ca. 15%) energetic values than control *granola*. Overall, these results would mean that, thanks to the use of a novel food ingredient, namely pomegranate fermented seeds flour, G-FS could be daily consumed in a higher dose than conventional *granola*, with no change of carbohydrates and proteins intake, but with an overall higher intake of minerals and fibre. Fermentation of seeds also contributed to significantly improve the overall acceptability of *granola* snacks with seeds flour, making it more similar to the control and more appreciated than *granola* containing unprocessed seeds, for the lower presence of particles difficult to swallow, and the higher sweetness and palatability. Numerous studies underlined how by-products fermentation positively affects the final quality of novel foods. Katina *et al.* (2012), and Pontonio, Dingo, Gobetti, & Rizzello (2019) fortified wheat bread with fermented milling by-products, showing how fermentation positively impacted textural properties and sensory profile of the breads, compared to bread added with unfermented by-products. Christ-Ribeiro *et al.*, (2021) used fermented rice bran as an ingredient in the preparation of gluten-free cookies, showing that fermentation positively affects the nutritional quality, texture, and consumer acceptability.

2.5 Conclusions

The present study demonstrates the potential of fermentation by yeasts isolated from the matrix to convert pomegranate seeds, commonly considered as food by-products, into nutritive improvers. The use of fermented pomegranate seeds flour as additional novel food ingredient of *granola* snacks allows the increase in antioxidant activity, crude fibre, and minerals (calcium, potassium, iron, and zinc) and the decrease of energetic values compared to a conventional *granola* snack. Moreover, *H. valbyensis* S-L1 fermentation positively affected sensory properties of the enriched *granola*. The results of this study could be of interest for food industries searching for novel foods, with added value in terms of nutritional quality and sustainability.

2.6 References

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Characterization of *Hanseniaspora valbyensis* and identification of potential fructose transporters

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3.1 Introduction

For thousands of years, fermented products have been produced without understanding the biological, biochemical and chemical basis responsible for this type of production. Fermentation was based on autochthonous microbiota of the food substrate that were affected by the surrounding conditions and environment, leading to fermented products with characteristics strongly related to their geographical location (Mannaa *et al.*, 2021). A complex microbial consortium is generally involved in spontaneous fermentation of foods (Roudil *et al.*, 2020). Due to the increasing demand for fermented products that should be safe and standardized, the use of starter cultures and the control of fermentation protocols spread on industrial scale (Galimberti *et al.*, 2021; Mannaa *et al.*, 2021). Alcoholic fermentation is probably the most ancient biotechnological transformation in human history (Kraus *et al.*, 1983; Gonzalez & Morales, 2022). Among the yeasts that perform alcoholic fermentation, *Saccharomyces* spp. are those most frequently used as starter to produce alcoholic beverages (e.g., wine, beer, cider), primarily thanks to their high fermentation efficiency and strong alcohol resistance (Budroni *et al.*, 2017; Corich *et al.*, 2022; Wu *et al.*, 2023). Besides good fermentation efficiency, selection of yeasts as starter for alcoholic beverages is also based on the characteristic aroma and flavours that yeasts can give to the final product (Budroni *et al.*, 2017). Apart from *Saccharomyces* spp., non-*Saccharomyces* yeasts, commonly indicated as “non-conventional” yeasts (e.g., *Hanseniaspora* (*Kloeckera*) *valbyensis*, *Hanseniaspora* *guilliermondi*, *Kloeckera* *apiculata*, *Metschnikowia* *pulcherrima*, *Starmerella* *bobicola* (formerly *Candida* *stellata*) have attracted much attention as pure or mixed-culture starters for fermented alcoholic beverages production. Non-*Saccharomyces* yeasts are generally present on the fruit surfaces; in some traditional productions non-*Saccharomyces* yeasts represent potential sources of deterioration; however, numerous recent studies underline the potential of non-*Saccharomyces* yeast as a

starter due to their abilities to ferment challenging matrices and provide peculiar flavour characteristics to the final product (Rojas *et al.*, 2003; Romano *et al.*, 2003; Xu, Zhao, & Wang, 2006; Zohre, & Erten, 2002). Among non-*Saccharomyces* yeasts, the genus *Hanseniaspora* is known for its high capacity to produce aroma compounds (Bilbao *et al.*, 1997, Rojas *et al.*, 2003; Xu, Zhao, & Wang, 2006). *Hanseniaspora* is dominant in the early stages of wine (spontaneous) fermentation, but after 48–72 hours give way to *Saccharomyces cerevisiae* (Zott *et al.*, 2008). This shift occurs because *Hanseniaspora* is sensitive to ethanol and to antimicrobial peptides eventually synthesized by *S. cerevisiae* during fermentation (Seixas *et al.*, 2019; Valera *et al.*, 2020). Therefore, reputation of *Hanseniaspora* as poor fermenter might be due to the presence of inhibitors rather than to its reduced ability to ferment sugars (Valera *et al.*, 2020). Additionally, *Hanseniaspora* species produce different secondary metabolites and exhibit various fermentative behaviours, with differences even among strains of the same species (Martin *et al.*, 2018; Moreira *et al.*, 2011). Although several studies have been focused on the phenotypic traits of *Hanseniaspora* species throughout fermentation (such as, *H. uvarum*, *H. vineae*, *H. guilliermondii*) this genus, and particularly *Hanseniaspora valbyensis*, is still little known. Recent studies tried to use *H. valbyensis* as starter to produce alcoholic beverages (e.g., beer, wine, cider) (Bellut *et al.*, 2018; Wang *et al.*, 2020; Xu, Zhao, & Wang, 2006) or to produce nutritionally improved ingredients (Limongelli, Minervini, & Calasso, 2023); however, none of these focused on *H. valbyensis* behaviour on different fermentation conditions. Several studies reported the capability of *Hanseniaspora* species to grow on fructose (e.g. *H. uvarum*, *H. vineae*, *H. guilliermondii*, *H. valbyensis*), although no fructose-specific transporters were identified. However, Seixas *et al.* (2019) identified the protein UTAD222 in the *H. guilliermondii* proteome as a potential fructose transporter, because phylogenetically close to fructose transporter *Zygosaccharomyces bailii* Ffz1. Ffz transporters are phylogenetically distinct from all the other previously characterized hexose transporters that belong to the Sugar Porter family. The Ffz proteins belong to a newly described family of sugar transporters that is phylogenetically more similar to drug transporters (Leandro *et al.*, 2011).

Based on the above considerations, the aim of this study was to investigate the behaviour of four strains of *H. valbyensis* under various growth conditions (both

optimal and suboptimal), with the goal of determining the ability of the strains to utilize specific sugars, assessing their growth rate, and evaluating the main metabolites produced under specific conditions. In addition, capability of *H. valbyensis* to grow on fructose was explored, both through phenotypic characterization and detailed *in silico* genomic analysis.

3.2 Materials and methods

3.2.1 Yeast strains

The *Hanseniaspora valbyensis* strains used in this study were obtained from the Culture Collection of the Department of Soil, Plant and Food Science of the University of Bari Aldo Moro (Italy). *H. valbyensis* strains J-L1, J-L5, J-L6 were previously isolated from pomegranate juice, whereas *H. valbyensis* S-L1 was from pomegranate seeds (Limongelli, Minervini, & Calasso, 2023). Two yeast strains, namely the wild-type *Saccharomyces cerevisiae* CEN PK and *Kluyveromyces marxianus* CBS 397, obtained from the Culture Collection of the School of Microbiology of the University college of Cork (Ireland) were used as control. Yeast strains were routinely cultured at 30 °C in YP medium (1% yeast extract; 2% bactopectone) with 2% d-glucose.

3.2.2 Drop test

Serial dilutions were used to determine the capacity of the strains to use specific sugars. For these experiments, a single colony was inoculated into 5 ml YP medium with 2% d-glucose (YPD) in a 15 ml sterile tube, and this was incubated overnight at 30 °C. The day after, cell suspensions were diluted to the absorbance at 600 nm (A_{600}) of 0.5 U.A. in 5 ml of fresh medium and incubated at 30 °C until A_{600} from 1 to 2 U.A. was reached (around 3 h). Cells were then harvested by centrifugation (MICRO STAR 17R, VWR™) at 5000 rpm for 5 min and washed with sterile distilled water. Cells were resuspended in sterile distilled water, and the A_{600} of all the samples was normalized to that of the sample with the lowest A_{600} value. Serial dilutions were prepared in sterile distilled water in Falcon™ 96-Well Cell Culture Microplates, using a different row for each strain. About 5 µl of each dilution were spotted onto agar plates containing Synthetic minimal media (MM) with one of the following sugars at a final concentration of 20 g/l: d-glucose, d-fructose, α-lactose, d-xylose, d-maltose or sucrose. MM agar plates were prepared as follows: 5 g/l ammonium sulphate, 3 g/l anhydrous potassium dihydrogen phosphate, 0.5

g/l magnesium sulphate heptahydrate; pH 5.5; after autoclaving: 100 ml/l of 20% appropriate sugar solution, 1 ml/l 1000x vitamin solution, and 1 ml/l 1000x trace elements solution were added (Donzella *et al.*, 2021). After inoculation, plates were incubated for 24 h at 30 °C and the growth was assessed using a scanner (Epson perfection 4180 photo).

3.2.3 Growth kinetics on flasks

Growth kinetics of *H. valbyensis* J-L1, J-L5, J-L6, S-L1 and of *S. cerevisiae* CEN PK and *K. marxianus* CBS 397 on MM with glucose 2%, fructose 2% or glucose-fructose 2% were performed in triplicate in 250 ml flasks as follows. Overnight inoculum in YPD was prepared as for drop test experiments and 50 ml of MMs were inoculated at a starting A_{600} of 0.1 U.A.. The flasks were incubated at 30 °C on an orbital shaker (Incu-Shaker™ 10l, Benchmark Scientific, Sayreville, NJ, USA) at 200 rpm and A_{600} was measured every 3 h for 36 h. At each sampling time, 1 ml of culture were harvested by centrifugation at 13300 rpm for 5 min and supernatant was stored at -20 °C. Metabolites such as glucose, fructose, ethanol, glycerol and organic acids were determined through a High-Performance Liquid Chromatography (HPLC) system, equipped with a Rezex ROA-Organic Acid H+ (8%), LC Column (300 x 7.8 mm) and a refractive index detector (Agilent Technologies, Santa Clara, CA, USA). The mobile phase was 0.6 mL/min of HPLC grade water (Merck, Darmstadt, Germany) supplemented with 0.01 N Sulfuric acid (Merck, Darmstadt, Germany) and the oven temperature was 65 °C. Before analysis, supernatant was diluted 1:5, the injected volume was of 20 µl, and the run time was approximately of 25 min. The outputs were quantified through OpenLab Chromatography Data Systems (CDS) (Agilent Technologies, Santa Clara, CA, USA).

3.2.4 Growth kinetics on CLARIOstar plus

Growth kinetics of *H. valbyensis* J-L1, J-L5, J-L6, S-L1 and of *S. cerevisiae* CEN PK and *K. marxianus* CBS 397 on different sugars and under different environmental conditions (different % of glucose, NaCl, and pH) were performed in triplicate by CLARIOstar plus plate reader (BMG Labtech, Ortenberg, Germany). Overnight inoculum in YPD was prepared as for the drop test experiments and 10 ml of MM medium was inoculated at a starting A_{600} of 0.1 U.A. Several MMs were prepared according to the conditions to be tested: MM with 2% of sugar (d-glucose or d-fructose or α -lactose, d-xylose, d-maltose or sucrose), pH 5.5; MM with 1% of

glucose, pH 5.5; MM with 0.5% of glucose, pH 5.5; MM with 2% of glucose and 0.5% of NaCl, pH 5.5; MM with 2% of glucose and 1% of NaCl, pH 5.5; MM with 2% of glucose and 2% of NaCl, pH 5.5; MM with 2% of glucose, pH 4.5; MM with 2% of glucose, pH 3.5. 200 µl of each culture were inoculated, in triplicate, into a sterile Falcon™ 96-Well Cell Culture Microplates. The 96-Well plates were incubated at 30 °C under orbital shaking at 200 rpm. A₆₀₀ was measured every 15 min for 36 h. Data were analysed using the MARS Data Analysis Software.

3.2.5 *In silico* analysis on sugar transporters

Potential sugar porters were identified by the combination of different bioinformatic approaches, in accordance with Donzella *et al.*, 2021. The predicted genome of *H. valbyensis* NRRL Y-1626 was retrieved from the NCBI Assembly platform (<https://www.ncbi.nlm.nih.gov/>) and then compared against the TransportDB 2.0 database to identify possible sugar transporters (Elbourne *et al.*, 2017; Elbourne *et al.*, 2023). All proteins predicted to contain 11 or more transmembrane domains were considered as potential sugar transporters. Sequences belonging to the MFS superfamily were extracted from this list of putative sugar transporters and compared with known sugars transporters. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones, Taylor & Thornton, 1992). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. This analysis involved 309 amino acid sequences. There were a total of 1533 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Stecher, Tamura & Kumar, 2020; Tamura, Stecher & Kumar, 2021).

3.3 Results

3.3.1 Preliminary growth tests on different carbon sources

The growth of *H. valbyensis* strains and the two controls (*S. cerevisiae* CEN PK and *K. marxianus* CBS 397) on MM with different carbon sources was assessed by the drop test and subsequently verified by growth kinetics on CLARIOstar Plus. As shown by the drop test, after 24 h of incubation all the strains of *H. valbyensis* were able to grow on MM glucose 2% and fructose 2%; however, a halo was observed also in MM sucrose 2% and maltose 2% (Figure 1). Both control strains (*S.*

cerevisiae CEN PK and *K. marxianus* CBS 397) were able to grow on MM glucose 2%, fructose 2%, sucrose 2% and maltose 2%. No growth was found on MM xylose 2% and lactose 2%, except for *K. marxianus*. Overall, *S. cerevisiae* and *K. marxianus* growth on MM agar plates seemed faster than *H. valbyensis* strains, regardless of the sugar tasted (Figure 1).

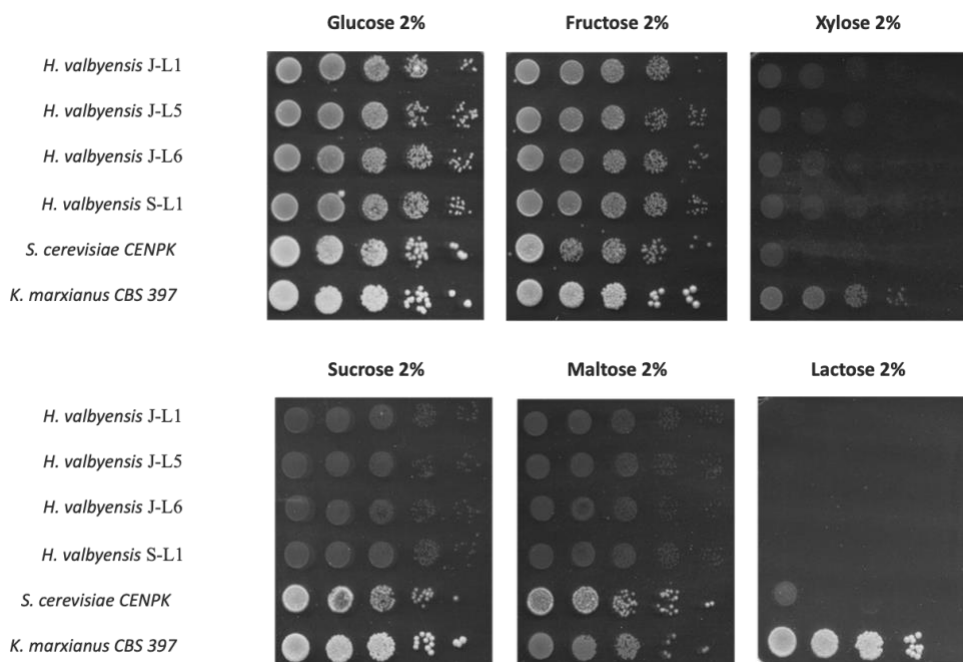


Figure 1. Drop test on MM agar plates with 2% of different Carbon sources (D-glucose or D-fructose or α -Lactose, D-xylose, D-maltose or Sucrose) of *Hanseinaspora valbyensis* J-L1, J-L5, J-L5, S-L1, *Saccharomyces cerevisiae* CEN PK and *Kluyveromices marxianus* CBS 397. Plates were incubated for 24 hours at 30 °C and the growth was assessed using a scanner (Epson perfection 4180 photo).

Growth kinetics on CLARIOstar showed only few differences between the strains tasted, when cultured in broth at pH 5.5 containing glucose 2% or fructose 2% (Figure 2). *H. valbyensis* strains were able to grow slightly better on MM glucose (Figure 2a) than on MM fructose (Figure 2b). Moreover, the stationary phase was reached after ca. 18 hours by all the strains (including the two control strains) tested in both MM media, reaching slightly higher values of A_{600} in *H. valbyensis* strains.

No growth was observed on MM maltose except for the two controls (Figure 2c). *S. cerevisiae* reached the highest value of cell density and reached the stationary phase after ca. 18 h, whereas *K. marxianus* seemed to be still in exponential growth phase after 36 hours.

The two control yeast strains were able to grow on MM sucrose 2%, whereas slight growth was observed for *H. valbyensis* strains (Figure 2d). In addition, on CLARIOstar was preliminary tested also the grow on MM with binary sugar mixture (glucose-fructose 2%) (Figure 2e). No differences were observed between the strain tested and the growth rate was comparable to the growth on MM glucose and fructose.

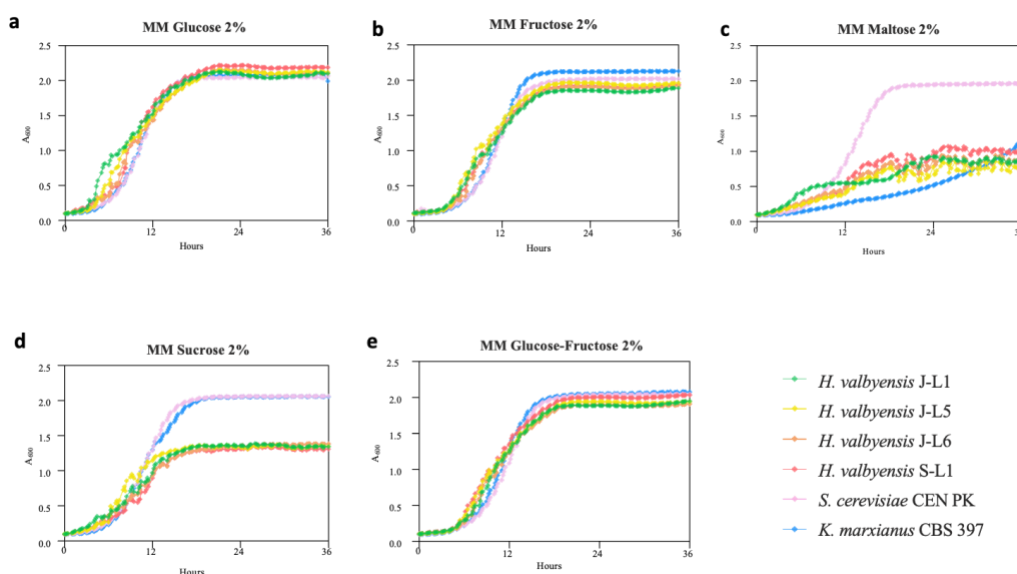


Figure 2. Growth kinetics on CLARIOstar plus of *Hanseniaspora valbyensis* J-L1, J-L5, J-L6, S-L1, *Saccharomyces cerevisiae* CEN PK and *Kluyveromyces marxianus* CBS 397 on MM glucose 2% (a), fructose 2% (b), maltose 2% (c), sucrose 2% (d) and on glucose-fructose 2% (e). Cell density was monitored for 36 hours and expressed as absorbance at 600 nm (A_{600}).

3.3.2 Physiology of *H. valbyensis* on individual and binary sugar mixture

Growth kinetics of all the yeast strains were obtained during cultivation in flask containing MM, with glucose or fructose as single carbon sources or in mixture at 30 °C. All the strains were able to grow on glucose 2%; in particular, *K. marxianus* reached the highest values of absorbance (A_{600} of 23 U.A.) and the stationary phase

after 15 h (Figure 3a). *S. cerevisiae* too reached absorbance values (ca. 15 U.A.) higher than *H. valbyensis* strains and the stationary phase after 15 h. No significant differences were observed between the growth kinetics of *H. valbyensis* strains (highest value of A_{600} of ca. 12 U.A.) and the stationary phase was reached after 21 h. As no differences were observed among the four *H. valbyensis* strains, growth on fructose 2% and glucose-fructose 2% was evaluated only for the strains J-L6 and S-L1. Growth of all the strains on fructose 2% was comparable to that found on glucose 2% (Figure 3b). The two control yeasts were able to grow faster than *H. valbyensis*, reaching the stationary phase after ca. 15 (*K. marxianus*) and 18 (*S. cerevisiae*) h. The two *H. valbyensis* strains (J-L6 and S-L1) reached absorbance of ca. 13 U.A. and the stationary phase after ca. 21-24 h.

Overall, the stationary phase during growth on fructose was reached a few hours later compared to growth in glucose. Overall, kinetics on glucose-fructose showed some differences with respect to those observed with single sugars (Figure 3c). The stationary phase was reached after 15 h by the two control yeasts and after 21 h by *H. valbyensis* J-L6 and S-L1, similarly to kinetics on glucose.

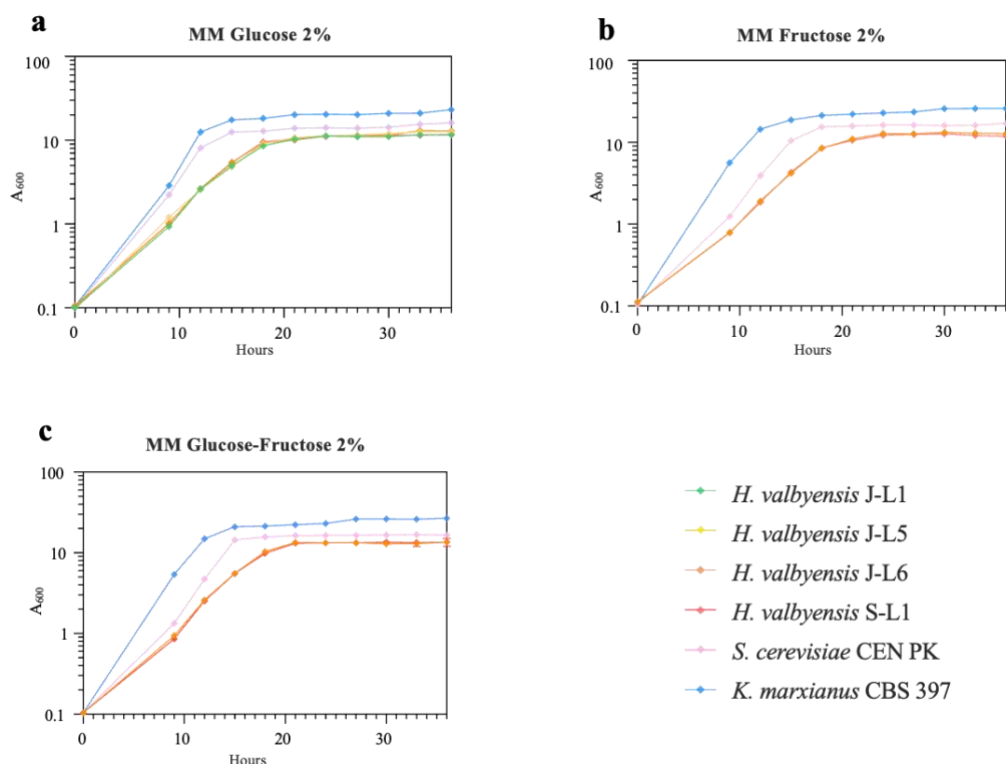


Figure 3. Growth kinetics on flasks of *Hanseniaspora valbyensis* J-L1, J-L5, J-L6, S-L1, *Saccharomyces cerevisiae* CEN PK and *Kluyveromyces marxianus* CBS 397 on MM glucose 2% (a), fructose 2% (b) and glucose-fructose 2% (c). Cell density was monitored for 36 hours expressed as absorbance at 600 nm (A_{600}).

3.3.3 Metabolite production and carbon balances

Growth, sugar consumption, and metabolite production were investigated for *H. valbyensis* strains, and the two controls cultured on glucose, fructose and glucose-fructose. *H. valbyensis* strains were able to completely consume sugar after 21 h of cultivation on MM with glucose (Figure 4a, b, c, d) or fructose (Figure 5a, b). When glucose and fructose were used as binary sugar mixture in MM, *H. valbyensis* seemed to prefer glucose instead of fructose during early fermentation. However, after 18 h of fermentation both sugars were completely consumed (Figure 6a, b). Similarly, also was able to completely consume glucose (Figure 4e) and fructose (Figure 5c) singly used in MM after 21 h of fermentation. When glucose and fructose were used as binary sugar mixture in MM, *S. cerevisiae* preferred to consume glucose before fructose. Indeed, glucose was completely consumed after 18 h, whereas fructose after 21 h (Figure 6c). *K. marxianus* CBS 397 did not completely consumed glucose and fructose or a binary mixture thereof even after 36 h. In detail, in MM glucose (Figure 4f) and MM fructose (Figure 5d), at the end of the fermentation, the sugar concentration was ca. 2 g/l. Instead, in MM glucose-fructose, glucose was completely consumed after 33-36 h of fermentation, and fructose represented the residual sugar (3 g/l) after 36 h (Figure 6d).

All the yeast strain tested were able to produce ethanol. When cultured on glucose 2%, *H. valbyensis* J-L1, J-L5, and J-L6 reached the highest concentration of ethanol (ca. 10 g/l) after 21 h (Figure 4a, b, c). *H. valbyensis* S-L1 continued to produce ethanol that reached the highest concentration (ca. 11 g/l) after 24-27 hours (Figure 4d). All the *H. valbyensis* strains started to produce acetic acid after 21 h of cultivation, reaching maximum levels of about 1 g/l. Ethanol concentration did not remain constant within the system, but with the increase of acetic acid a decrease in ethanol of about 12-18 % was observed (Figure 4a, b, c, d).

When cultured on fructose 2%, the highest concentration of ethanol produced by *H. valbyensis* J-L6 ed S-L1 was slightly lower (9.2 and 9.5 g/l, respectively) compared to glucose (Figure 5a, b). Similarly to what was observed on glucose, an increase of acetic acid (up to 0.8 g/l) was associated to a decrease of ethanol of ca

15-30 % (Figure 5a, b). The production of ethanol and acetic acid in MM glucose-fructose fermented by J-L6 and S-L1 was between what was observed with single sugar (Figure 6a, b). The highest concentration of ethanol reached was about 8.3 and 9.5 g/l for J-L6 and S-L1, respectively, and a decrease (ca. 21-27 %) was found after 27 and 21 h, respectively. Production of acetic acid was found after 21 h and it reached the highest concentration (ca. 1.1 g/l) after 36 h (Figure 6a, b).

S. cerevisiae was able to produce higher level of ethanol when grown on glucose (Figure 4e), than on fructose (Figure 5c). In detail, at the beginning of the stationary phase (18 and 21 h on glucose and fructose, respectively), ethanol concentration reached 10.13 g/l in MM glucose (Figure 4e) and 8.3 g/l in MM fructose (Figure 5c). When cultured in presence of both sugars, *S. cerevisiae* produced ethanol between what was observed in MM with individual sugar, reaching 8.3 g/l of ethanol after 21 h (Figure 6c). A decrease in ethanol was found for *S. cerevisiae* after 24 h, especially when cultured on glucose (ca. 30% decrease) (Figure 4e), but acetic acid was not produced.

K. marxianus was characterized by the lowest production of ethanol; after the exponential phase (12 h), the concentration was about 3 g/l and did not vary until the end of fermentation (Figures 4f, 5d, 6d). Acetic acid was not produced during fermentation.

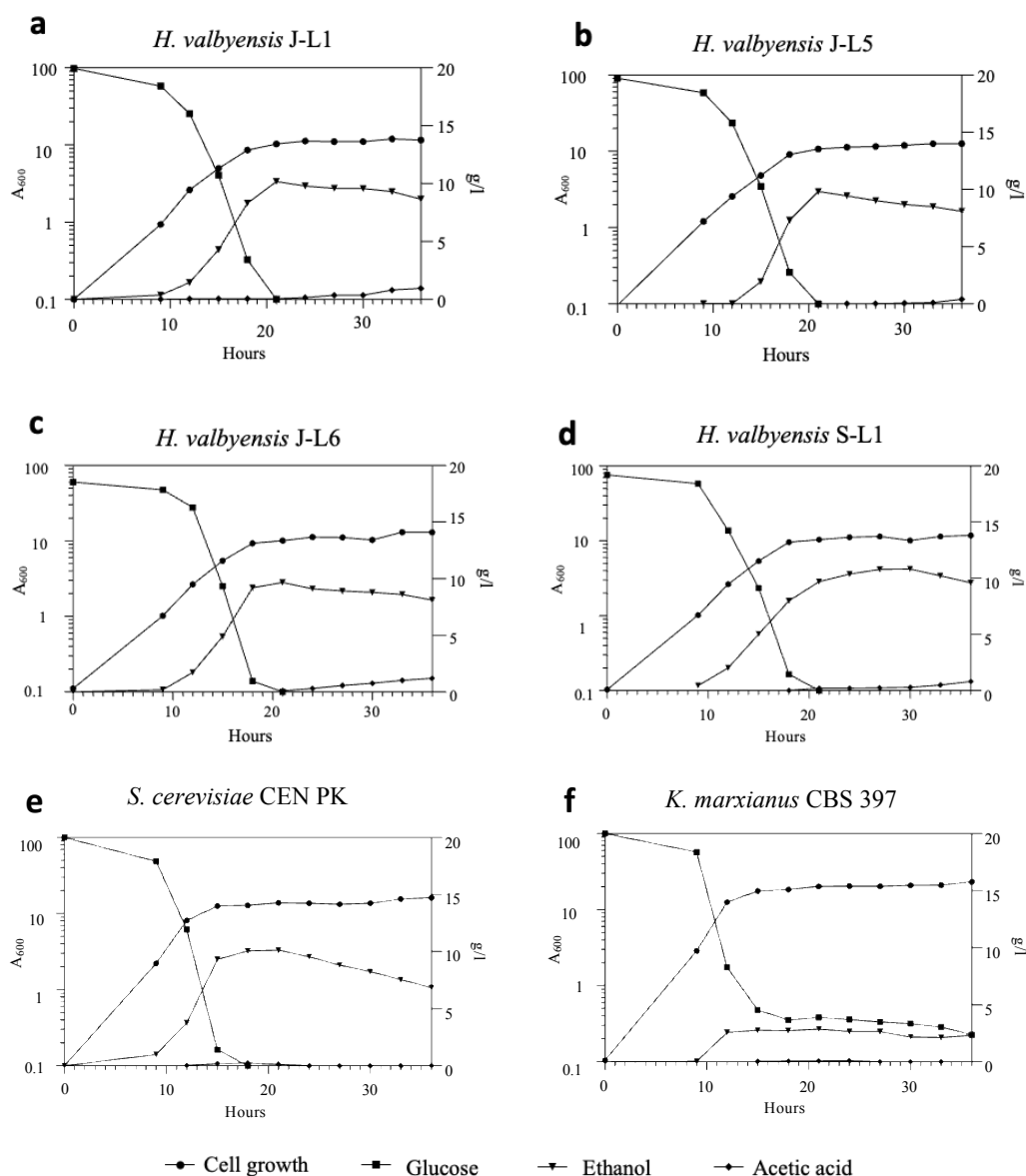


Figure 4. Growth kinetics on flasks of *Hanseniaspora valbyensis* J-L1 (a), J-L5 (b), J-L6 (c), S-L1 (d), *Saccharomyces cerevisiae* CEN PK (e) and *Kluyveromyces marxianus* CBS 397 (f) on MM glucose 2%. Cell density was monitored for 36 hours and expressed as absorbance at 600 nm (A_{600}). Metabolite production and the consumption of glucose were monitored by High-Performance Liquid Chromatography analysis and expressed as g/l.

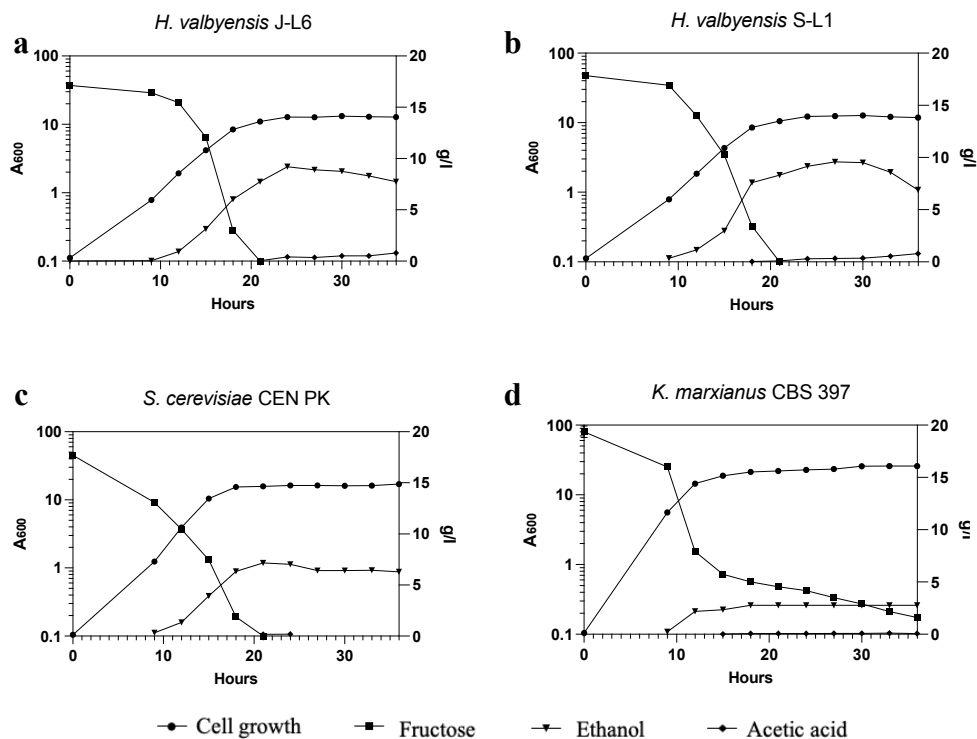


Figure 5. Growth kinetics on flasks of *Hanseniaspora valbyensis* J-L6 (a), S-L1 (b), *Saccharomyces cerevisiae* CEN PK (c) and *Kluyveromyces marxianus* CBS 397 (d) on MM fructose 2%. Cell density was monitored for 36 hours and expressed as absorbance at 600 nm (A_{600}). Metabolite production and the consumption of fructose were monitored by High-Performance Liquid Chromatography analysis and expressed as g/l.

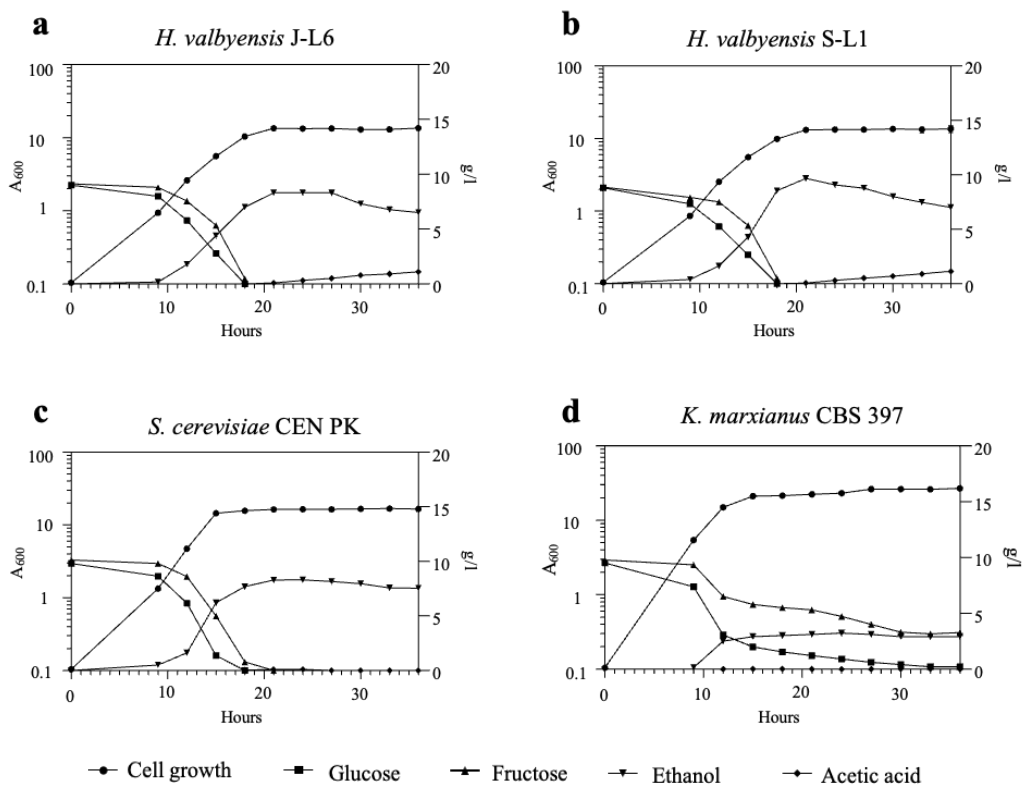


Figure 6. Growth kinetics on flasks of *Hanseniaspora valbyensis* J-L6 (a), S-L1 (b), *Saccharomyces cerevisiae* CEN PK (c) and *Kluyveromyces marxianus* CBS 397 (d) on MM glucose-fructose 2%. Cell density was monitored for 36 hours and expressed as absorbance at 600 nm (A_{600}). Metabolite production and the consumption of glucose and fructose were monitored by High-Performance Liquid Chromatography analysis and expressed as g/l.

3.3.4 Influence of the initial sugar concentration and other conditions on growth

The ability of *H. valbyensis* J-L1, J-L5, J-L6 and S-L1 and the two control yeasts to grow on relatively stressful conditions (lower concentration of sugar, lower pH and higher concentration of NaCl than the optimum) was tested by growth kinetics performed on CLARIOstar plus. *H. valbyensis* J-L6, S-L1, *S. cerevisiae* CEN PK and *K. marxianus* CBS 397 growth rate was highest when the concentration of glucose in MM was 2%. However, J-L6 e S-L1 were less affected by the decrease of glucose concentration. On MM glucose 2%, J-L6 e S-L1 were able to reach A_{600} of 2.0 U.A. after 20 h. Only a slight decrease of the absorbance (1.9 U.A.) was observed on glucose 1%, whereas a higher decrease was observed when glucose concentration was 0.5%, with a value of absorbance of 1.6 U.A. (Figure 7). *S. cerevisiae* CEN PK was the most affected by relatively low concentrations of glucose reaching A_{600} of 1.9, 1.6 and 1.1, respectively on glucose 2%, 1% and 0.5% after 15 h (Figure 7). Similarly, *K. marxianus* was able to grow on MM glucose 2% reaching absorbance values (2.0 U.A.) comparable to those found for J-L6 e S-L1; however, reducing sugar concentration, a decrease of absorbance was observed. The highest absorbance value achieved was about 1.7 and 1.5 U.A., on MM glucose 1% and 0.5%, respectively, after 27 h (Figure 7).

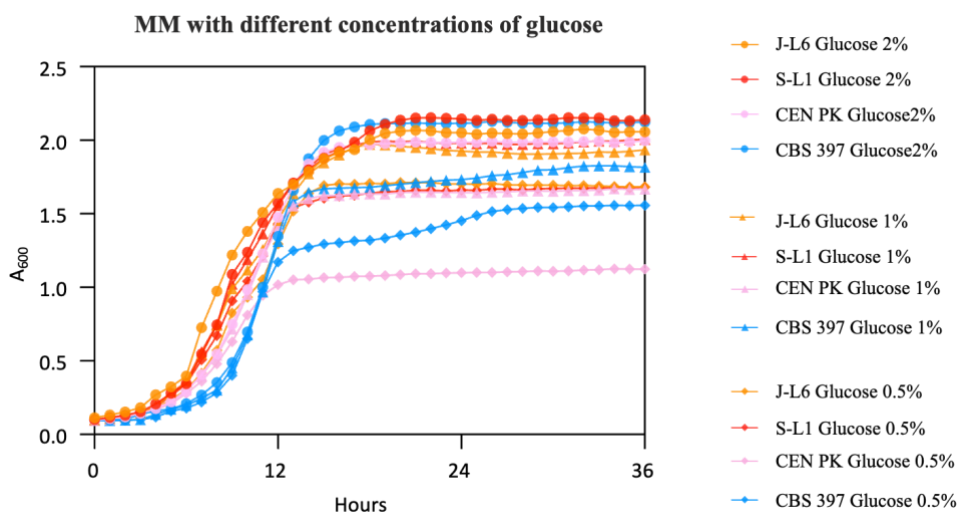


Figure 7. Growth kinetics on CLARIOstar plus of *Hanseniaspora valbyensis* J-L1, J-L5, J-L6, S-L1, *Saccharomyces cerevisiae* CEN PK and *Kluyveromyces marxianus* CBS 397 on MM with different concentration of glucose (MM glucose 2%, 1% and 0.5%). Cell density was monitored for 36 hours and expressed as absorbance at 600 nm (A_{600}).

MM glucose 2% with 0.5%, 1% or 2% of NaCl were used to test the behaviour of strains under osmotic stress on CLARIOstar. No differences were observed between all the strains on MM glucose 2% with 0.5% NaCl (A_{600} of 2 U.A.). J-L6 and S-L1 seemed to better tolerate higher concentration of NaCl, given that no differences in growth were found between the conditions tested. On the contrary, *S. cerevisiae* and *K. marxianus* grew at lower levels (1.8 U.A.) in presence of NaCl at 2 %, compared to the optimal condition (NaCl 0.5%) (Figure 8). Moreover, the same test was repeated on 15ml falcon tubes with also more stringent condition (0.5%, 1%, 2%, 10% and 15% NaCl). *H. valbyensis* strains were able to grow in presence of 10% NaCl, although at lower level than in MM containing 0.5 – 2% of NaCl; however, no growth was found in MM with 15% NaCl (data not shown).

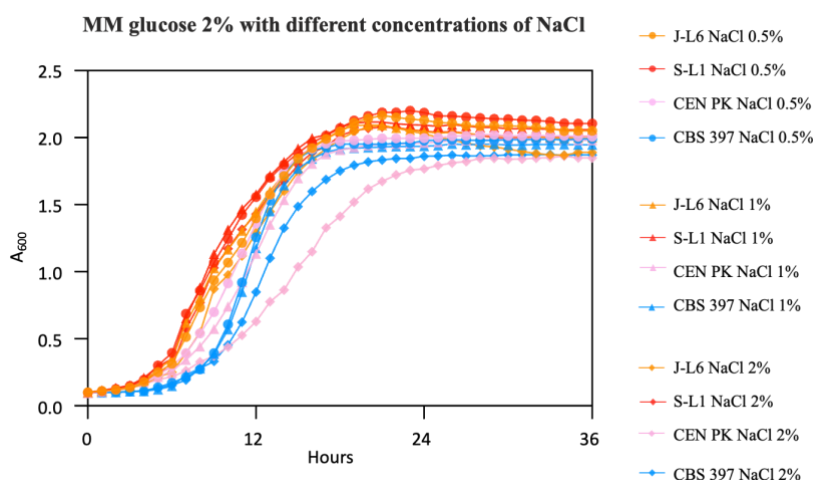


Figure 8. Growth kinetics on CLARIOstar plus of *Hanseniaspora valbyensis* J-L1, J-L5, J-L6, S-L1, *Saccharomyces cerevisiae* CEN PK and *Kluyveromyces marxianus* CBS 397 on MM glucose 2% with different concentration of NaCl (0.5%, 1% and 2%). Cell density was monitored for 36 hours and expressed as absorbance at 600 nm (A_{600}).

MM glucose 2% pH 5.5, 4.5 and 3.5 were used to test the influence of different acid stress condition on strain growth (Figure 9) on CLARIOstar. No differences were observed (A_{600} of ca. 2 U.A.) for all the strains under different pH conditions. The same test was repeated on 15ml falcon tubes with even more stringent condition (pH 5.5, 4.5, 3.5, 3.0). *H. valbyensis* was able to grow at the same levels both at pH 5.5 and pH 3.

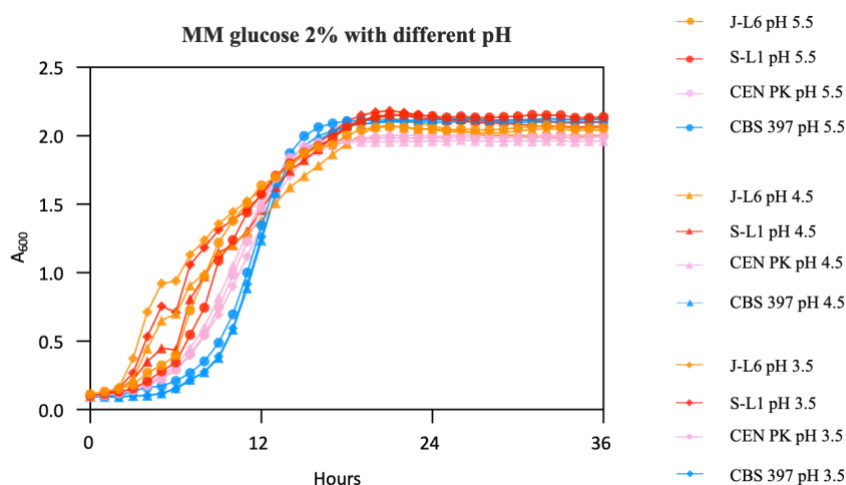


Figure 9. Growth kinetics on CLARIOstar plus of *Hanseniaspora valbyensis* J-L1, J-L5, J-L6, S-L1, *Saccharomyces cerevisiae* CEN PK and *Kluyveromyces marxianus* CBS 397 on MM glucose 2% with different pH (pH 5.5, 4.5, 3.5). Cell density was monitored for 36 hours and expressed as absorbance at 600 nm (A_{600}).

3.3.5 *In silico* identification of potential sugar transporters

A bioinformatic approach was applied to identify all potential sugar transporters in *H. valbyensis*. To do that, the predicted genome of *H. valbyensis* NRRL Y-1626 was compared to the TransportDB 2.0 database to identify candidate MFS transporters. Then, these were screened to select proteins with at least 11 transmembrane domains. This analysis yielded 35 predicted proteins identified as MFS transporters. Subsequently, these 35 proteins were compared to 274 putative sugar transporters belonging to different yeast species previously selected by Donzella, Sousa & Morrissey (2023). The resulting phylogenetic tree showed that the majority of proteins were distributed in un-clustered areas (Figure 10). However, focusing on potential hexose transporters, same transport protein (OBA27516.1 Hv, OBA28435.1 Hv, OBA26974.1 Hv, OBA26972.1 Hv, 26973.1 Hv) fall into the category of HXT-like, phylogenetically close to *S. cerevisiae* HXT-like (Figure 10). No proteins of *H. valbyensis* fall within the fructose transporter cluster (Fsy and Frt); however, some transport proteins are phylogenetically closer to *Zygosaccharomyces bailii* Ffzl (Q7oWR7 ZYGBA), in particular: OBA26907.1 Hv, OBA25439.1 Hv, OBA25375.1 Hv, OBA27466.1 Hv, OBA27465.1 Hv, OBA25998.1 Hv, OBA27030.1 Hv. Moreover, OBA27142.1 Hv fall within the cluster of xylose transporters (Figure 10).

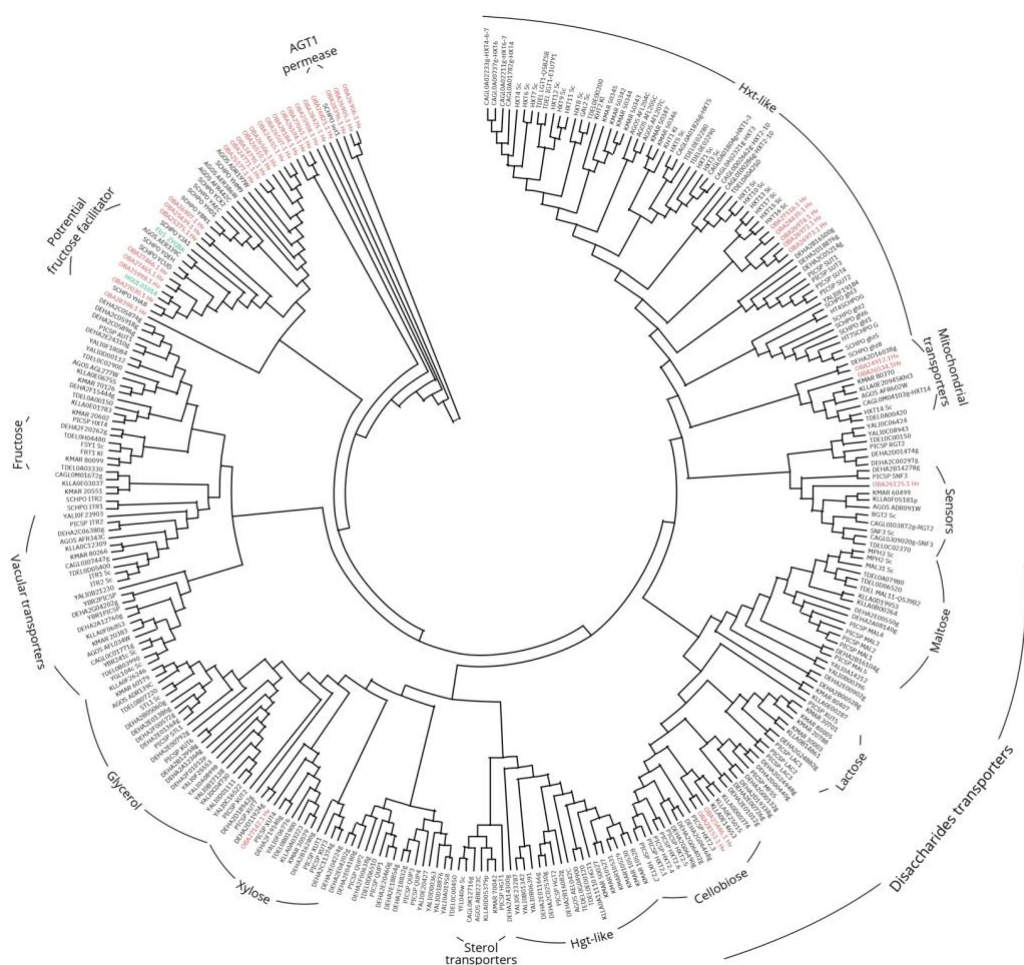


Figure 10. Phylogenetic tree of 35 putative sugar transporters belonging to *Hanseniaspora valbyensis* NRRL Y-1626 (red) compared to 274 putative sugar transporters belonging to different yeast species (black). In green were reported two fructose transporters of interest.

3.4 Discussion

Hanseniaspora valbyensis is an apiculate non-conventional yeast often found in the early stages of wine/cider spontaneous fermentation (Zott *et al.*, 2008). In the last years, non-conventional yeasts, in particular *Hanseniaspora* spp. (e.g., *H. guilliermondii*, *H. opuntiae*, *H. valbyensis*; *H. vineae*), have attracted much attention as pure or mixed-culture starters for fermented alcoholic beverage production for their ability to ferment challenging matrices and for their ability to provide flavour characteristic to the final products (Bourbon-Melo *et al.*, 2021; Medina *et al.*, 2013; Xu, Zhao, & Wang, 2006). However, *H. valbyensis* requires further investigation before it can be used as a starter for industrial fermentation. In the current study the behaviour of four strains of *H. valbyensis*, previously isolated by Limongelli, Minervini Calasso (2023) from spontaneously fermented pomegranate juice (J-L1, J-L5, J-L6) and seeds (S-L1) was investigated, in comparison with two controls *Saccharomyces cerevisiae* CEN PK and *Kluyveromyces marxianus* CBS 397, under various growth conditions (both optimal and suboptimal), focusing on their growth ability, sugar consumption, and the metabolites produced. Moreover, the capability of *H. valbyensis* to grow on fructose was further examined through both phenotypic characterization and *in silico* genomic analysis. *H. valbyensis* strains were able to grow on Minimal Media (MM) with different carbon sources, however the best growth was observed in MM with glucose or fructose. Although many studies reported that *Hanseniaspora* spp. is not able to grow on sucrose and maltose, this ability can vary depending on the strain tested (Čadež *et al.*, 2019). In the present work, considering the kinetics made by CLARIOstar, a slight growth was observed on MM sucrose 2%, but probably due to thermal degradation of sucrose to fructose and glucose, during the autoclave treatment of the media. Therefore, considering the sugars on which *H. valbyensis* was able to grow best and preliminary results, only the behaviour of two selected strains (J-L6 and S-L1, isolated from different part of pomegranate) was analysed more in depth. The consumption of glucose and fructose by apiculate yeasts has been the subject of extensive research over the years, revealing significant variability in the sugar preferences of *Hanseniaspora* species. Most species, such as strains of *H. osmophila*, tend to be glucophilic, preferring glucose as their primary carbon source, while others, such as *Hanseniaspora guilliermondii*, show a preference for fructose (Cabral *et al.*, 2015). Interestingly, certain species,

like *H. uvarum*, can utilize both glucose and fructose at comparable rates (Díaz-Montaña & de Jesús Ramírez Córdova, 2009; Wang *et al.*, 2015). To the best of our knowledge, no studies have yet explored the sugar metabolism of *H. valbyensis* in this specific context, leaving its behaviour with respect to glucose and fructose consumption unexamined. According to the findings of this study, *H. valbyensis* strains fully consumed sugars after 21 hours of cultivation on 2% MM glucose or fructose, at the same rate. When both sugars were present in a binary mixture at the same concentration, J-L6 and S-L1 seemed to prefer glucose over fructose during the early stages of fermentation. However, after 18 hours, both sugars were completely consumed, indicating that *H. valbyensis* can metabolize glucose and fructose at an equal rate. A specific study could be carried out to better understand the behaviour (e.g., glucophilic, fructophilic), of *H. valbyensis* also at lower or higher sugar concentrations on media with binary sugar mixture.

Under the conditions tested (sugar concentration, temperature, stirring, etc.), *H. valbyensis* strains were able to produce ethanol at levels generally comparable to those of *S. cerevisiae*. The growth of *H. valbyensis* was not negatively affected by the ethanol produced. In fact, some studies reported that ethanol level must exceed 6% (v/v) before apiculate yeasts are unable to survive (Nogueira *et al.*, 2008). It is noteworthy that after 21 hours, the ethanol concentration decreased concomitantly with an increase in acetic acid. Acetic acid is a normal co-product of alcoholic fermentation and its production by apiculate yeasts is straightly connected to environmental conditions, such as sugar concentration, nitrogen source and pH (Romano *et al.*, 1992). Under physiological conditions, apiculate yeasts can use acetate as a carbon source by integrating it into respiratory metabolism (Guaragnella & Bettiga, 2021). From the above consideration, we may hypothesize that the increase in acetic acid production could be a physiologic response to the more stressful condition present in the media. In fact, the production of acetic acid began when the sugars were completely consumed, and ethanol reached its maximum concentration.

Based on the growth tests conducted under stressful conditions using CLARIOstar system, *H. valbyensis* appeared to be less affected by reduced glucose concentration, compared to *S. cerevisiae* and *K. marxianus*. However, when the same test was repeated on flasks, the two control yeasts exhibited higher growth than *H. valbyensis*. This suggests that the control yeasts were more sensitive to the

smaller fermentation volumes in the 96-well plates used in CLARIOstar system, and consequently, to the lower surface of contact of broth with the oxygen. *H. valbyensis* growth remained unaffected by NaCl concentrations of 0.5%, 1%, and 2%, but was inhibited at levels above 10%, similarly to *H. gamundiae* (Čadež *et al.*, 2019). As expected, all the strains tested were able to grow at low pH, showing a growth rate comparable to the growth in standard condition (MM glucose 2%, pH 6.5), in agreement with literature (Hakkaart *et al.*, 2020; Solieri & Giudici, 2008; Vivier, *et al.*, 1993).

Focusing on potential hexose transporters, some *H. valbyensis* NRRL Y-1626 proteins fall into the category of HXT-like, showing a higher similarity with HXT 13, HXT 15, HXT 16 and HXT 17 of *S. cerevisiae*. This group of protein transporters is known to play a central role in monosaccharide uptake in yeasts, with specific HXT proteins enabling the efficient import of hexoses (Leandro, Fonseca, & Gonçalves, 2009; Donzella *et al.*, 2021). Some studies reported that this group can contribute to the transport of fructose; in particular, Greatrix & van Vuuren (2006) reported that HXT₁₃ was mainly activated in response to low (<2%) fructose concentration, in agar media with either glucose or fructose from 0.2 to 40%. Moreover, no orthologue of the fructose specific transporters Fsy and Frt were found in *H. valbyensis* proteome. Interestingly, similarly to *H. guilliermondii* UTAD222 (Seixas *et al.*, 2019) some *H. valbyensis* NRRL Y-1626 proteins, predicted to be MFS multi-drug effluxes, showed a higher similarity with *Z. bailii* Ffz1. The Ffz proteins belong to a newly described family of sugar transporters that is phylogenetically more similar to drug transporters of the DHA1 family (Leandro *et al.*, 2011). Ffz transporters are phylogenetically distinct from all the other previously characterized hexose transporters that belong to the sugar porter family, and they are typically found in fructophilic yeasts (Cabral *et al.*, 2015). Further studies are required to better understand the possible function of predicted hexose transporters in *H. valbyensis* and, specifically, whether they are somehow able to contribute to fructose transport.

3.5 Conclusions

In conclusion, the present study has allowed a deeper understanding of the behaviour of *H. valbyensis*, with a focus on its ability to utilize specific sugars, assessing their growth rate, and evaluating the main metabolites produced under specific conditions. In addition, capability of *H. valbyensis* to grow on fructose was explored more in depth, both through phenotypic characterization and *in silico* genomic analysis. Some results obtained during this research, such as the consumption of glucose and fructose at comparable rates and the identification of potential fructose transporters, would need further investigation to be understood more in depth. Nonetheless, this study has contributed to the advancement of knowledge about this promising species, which is still relatively unexplored.

3.6 References

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Impact of autochthonous non-conventional *Hanseniaspora valbyensis* on fermentation and Quality of pomegranate cider

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This chapter represents an extended version of that submitted on Applied Food Research Journal, December 2024

4.1 Introduction

Pomegranate (*Punica granatum L.*) fruits are consumed all over the world, and the most widespread cultivation is in the Mediterranean basin (e.g., Italy, Spain, Turkey, and Greece), Southern Asia, India, and America (Ferrara *et al.*, 2014; Limongelli, Minervini, & Calasso, 2023). Pomegranate is a well-known source of valuable nutrients (e.g., vitamins, fatty acids, and minerals) and compounds with potential antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic activity (Kandylis & Kokkinomagoulos, 2020). However, the consumption of fresh fruits is somewhat inconvenient due to the difficulty of extracting the edible arils (Filannino *et al.*, 2013). Processing fruits into pomegranate juice represents an easy and fast alternative to fresh fruit consumption, able to provide nutritional benefits to humans (Gumienna, Szwengiel, & Górna, 2016). In addition, the consumption of pomegranate is not limited only to fresh fruit or juice; products such as jellies, grenadine syrup (Filannino *et al.*, 2013) and pomegranate alcoholic beverages (Kokkinomagoulos *et al.*, 2020) are also relatively known. Due to the increasing consumers' awareness of potential benefices associated to pomegranate consumption (Limongelli, Minervini, & Calasso, 2023; Viuda-Martos *et al.*, 2010; Vučić *et al.*, 2019), the worldwide demand for this fruit or pomegranate-based foods and beverages has highly increased in recent years. It is estimated that the global size of the pomegranate market was about 248.17 million USD in 2022 and will reach approximately 367.52 million USD by 2030, growing at a Compound Annual Growth Rate (CAGR) of approximately 5% between 2023 and 2030 (Market Research Report 2023, Zion Market Research).

The fermentation process might provide new potential applications for pomegranate (Rios-Corripio & Guerrero-Beltrán, 2019), although it could seem challenging because of fruit's intrinsic parameters (e.g., presence of many antimicrobial compounds, low pH) (Howell & D'Souza, 2013). Indeed, several studies demonstrated that pomegranate juice fermentation (by lactic acid bacteria, yeasts or filamentous fungi) is a promising tool for further improving nutritional, functional and aromatic profile (Cano-Lamadrid *et al.*, 2017; Gumienna, Szwengiel, & Górna, 2016; Kokkinomagoulos *et al.*, 2020; Pontonio *et al.*, 2019, Trigueros, Wojdyło, & Sendra, 2014). Alcoholic fermentation is highly acknowledged in the beverage industry, generating less perishable value-added products, such as wine, beer and cider (Caplice & Fitzgerald, 1999).

Cider is a low alcoholic fermented beverage typically made from blends of several apple cultivars (Karl *et al.*, 2022; Lea & Drilleau, 2003). The global cider market is expected to be valued at 17.9 billion USD in 2023 and reach a valuation of 27.3 billion USD by 2033. Cider is becoming more and more popular among people who drink alcoholic beverages, because it is perceived as much sweeter and more refreshing than beer. Its demand is estimated to record a steady 5.1% CAGR, with UK, USA, China, and India emerging as key producers (Cider market report, Future market insight). Moreover, the growing interest in new and alternative fermented beverages has led to the development of ciders made from fermentable matrices different from apples, such as pears, plums, raspberry, mango, and many others.

The fermentation process of cider is carried out by strains of *Saccharomyces* spp., especially *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, and *Saccharomyces uvarum* which, added as starters, drive the process, reducing the risk of spoilage and unpredictable changes in cider flavour (Javis, 1996; Xu *et al.*, 2006). Nevertheless, apart from conventional starters, non-*Saccharomyces* species, commonly indicated as “non-conventional” species (e.g., *Hanseniaspora* (*Kloeckera*) *valbyensis*, *Kloeckera* *apiculata*, *Metschnikowia* *pulcherrima*, *Starmerella* *bombicola* (formerly *Candida* *stellata*)), have attracted much attention in cider production, because of their ability to ferment challenging matrices (e.g., pomegranate) and provide peculiar flavour characteristics to the final product (Rojas *et al.*, 2003; Romano *et al.*, 2003; Xu *et al.*, 2006; Zohre & Erten, 2002). Among non-*Saccharomyces* yeasts, the genus *Hanseniaspora* showed high capacity to form aroma compounds (Bilbao *et al.*, 1997, Rojas *et al.*, 2003; Xu *et al.*,

2006). *H. valbyensis* strains were recently isolated from pomegranate juice and seeds, showing high ability to ferment the same matrix (Limongelli, Minervini, & Calasso, 2023). To the best of our knowledge, no studies have focused on the use of non-conventional yeasts to drive the fermentation of pomegranate juice to cider. Based on the above considerations, the aim of the present study was to assess the differences, in terms of physico-chemical characteristics, antioxidant properties and flavour profile, among four pomegranate ciders, produced starting from two pomegranate juices with slightly different chemical composition, fermented by a commercial strain of *S. cerevisiae* or a non-conventional, autochthonous (i.e., isolated from the matrix and used as starter for fermenting the same matrix) strain of *H. valbyensis*.

4.2 Material and methods

4.2.1 Pomegranate juices and yeast strains

Two different pasteurized 100% natural pomegranate juices (Lome SuperFruit, Masseria Fruttirossi srl, Castellaneta, Taranto, Italy) were purchased from the retail based on the different sugar content (a juice with a higher (HS) and another with a lower (LS) sugar content). Before use, the juices were analyzed by S.A.Mer. - Service Chemical-Product Analysis (Bari, Italy) for the determination of chemical composition (LS (g/100 g): total fats 0.3; total carbohydrates 15.5, of which sugars 15.5; proteins 0.3, and ash 4.17; HS (g/100 g): total fats 0.3; total carbohydrates 16.8, of which sugars 16.8; proteins 0.3, and ash 4.20).

Hanseniaspora valbyensis J-L6 (Limongelli, Minervini, & Calasso, 2023), belonging to the Culture Collection of the Department of Soil, Plant and Food Science (University of Bari Aldo Moro, Italy) and the commercial yeast (CY) *Saccharomyces cerevisiae* Moz-Cider (Mangrove Jack's, Albany, Auckland, New Zealand) were used as starters for cider making. J-L6 was routinely propagated in Sabouraud Dextrose broth (Oxoid, Basingstoke, Hampshire, UK), whereas CY was a dried preparation that was stored at -20 °C.

4.2.2 Production of pomegranate ciders

H. valbyensis J-L6 was pre-cultured on Sabouraud Dextrose broth at 30 °C for 24 h (late exponential phase of growth). Then, cells were harvested by centrifugation (10,000 × g, 10 min, 4 °C), washed twice with sterile potassium phosphate buffer

(50 mM, pH 7.0) and re-suspended in pomegranate juice samples. CY was activated prior to inoculation through rehydration in pomegranate juice, according to the manufacturers' instruction.

The initial cell density of the starter yeasts was ca. 5.5 log CFU/ml. Juices were fermented at 15 °C, under gentle stirring (160 rpm), for seven days. Four pomegranate ciders were obtained: LS J-L6, HS J-L6, LS CY, and HS CY. LS J-L6 and LS CY were obtained from the same pomegranate juice (LS, "low" sugar) inoculated with either *H. valbyensis* J-L6 or *S. cerevisiae* Moz (CY), respectively; pairwise, HS J-L6 and HS CY were obtained from the same pomegranate juice (HS, "high" sugar) inoculated with either *H. valbyensis* J-L6 or *S. cerevisiae* Moz. After fermentation, pomegranate ciders were matured at 4 °C, for seven days, as-is (untreated) or clarified through centrifugation (20,000 × g, for 20 min) or filtration (0.2 µm).

Microbiological, colorimetric, elemental and gas chromatographic-mass spectrometry analyses were carried out after inoculation (T₀), three (T₃) or seven (T₇) days of fermentation, and after maturation (T₁₄).

4.2.3 Microbiological characterization of pomegranate ciders

Microbiological analyses were carried out using the following agar media and supplements purchased from Oxoid, after serial dilutions of samples in sterile saline (0.9% NaCl) solution: Plate Count Agar (30 °C, 48 h, for total mesophilic aerobic microorganisms), Violet Red Bile Glucose Agar (VRBGA) (37 °C, 24 h, for *Enterobacteriaceae*), Baird Parker supplemented with egg yolk-tellurite emulsion (37 °C, 48 h, for staphylococci), Slanetz and Bartley (37 °C, 48 h, for enterococci), Sabouraud Dextrose Agar (SDA) (30 °C, 48 h, for moulds and yeasts), Wort Agar (WA) (25 °C, 48 h, for moulds and yeasts). For the detection of acetic acid bacteria, Glucose Yeast Calcium Carbonate agar (GYC; 50 g/l of glucose, 10 g/l of yeast extract, 5 g/l of calcium carbonate, 20 g/l agar technical) with cycloheximide (0.1 g/l) was used (30 °C, 96 h).

The pour plate technique was used to inoculate the agar media for the enumeration of total microorganisms, *Enterobacteriaceae*, moulds and yeasts; instead, the spread plate technique was carried out to inoculate the agar media for the enumeration of acetic acid bacteria, staphylococci and enterococci.

4.2.4 Elemental analysis

Before elemental characterization, the pH of all samples was determined by an ultrabasic ub-10 pH meter (Denver Instrument Company; Arvada, CO, USA), equipped with a glass electrode.

The mineral elements composition of all samples was investigated using a S2-Picofox Total reflection X-Ray Fluorescence (TXRF) spectrometer (Bruker Nano GmbH, Berlin, Germany) equipped with a Mo microfocus tube (30 W, 50 kV, 600 μ A), a multilayer monochromator and an XFlash SDD detector with a 30 mm² active area. This multielemental technique has been already used for the analysis of fermented beverages, such as wine (Gruber *et al.*, 2006) and beer (Moreira *et al.*, 2006). In the case of pomegranate juice and cider, samples were preliminarily centrifuged (20,000 $\times$ g, 20 min, at 4 °C) because of their turbidity. Then, supernatants (0.5 ml) were diluted with deionized water (1:1, v/v) in Eppendorf tubes, and added with 10 μ l of a 1000 mg/l Gallium (Ga) standard solution (Sigma-Aldrich) to obtain a final internal standard concentration of 10 mg/l. Thereafter, tubes were vortexed for 30 s and, finally, 10 μ l of sample was pipetted on previously siliconized TXRF quartz reflectors and left drying at 50 °C on a heating plate, under a laminar flow hood (Klockenkämper & Von Bohlen, 2015). All samples were analyzed in triplicate for 1000 s (live time).

4.2.5 Determination of mono-, di-saccharides, acetic acid, and ethanol

Sucrose, fructose, glucose, acetic acid, and ethanol were determined on juice and cider samples (To, T₃, T₇, T₁₄), using the enzyme-based kits K-SUFRG, K-ACET and K-ETOH (Megazyme Ltd., Bray, Ireland) respectively, following the manufacturer's instructions. Before analysis, juice and cider samples were opportunely treated. In detail, for the determination of sucrose, fructose, glucose, and acetic acid, ciders were clarified with Carrez treatment. Specifically, 10 ml of sample were pipetted into a 50 ml volumetric flask containing 30 ml of distilled water. Then, 2.5 ml Carrez I aqueous solution (36 g/l potassium hexacyanoferrate (II)), 2.5 ml Carrez II aqueous solution (72 g/l zinc sulphate) and 5 ml NaOH solution (100 mM) were carefully added, mixing thoroughly after each addition. The suspension was filtered onto a Whatman apparatus (Polycarp 75 SPF; Whatman International, Maidstone, England) equipped with cellulose membrane (pore size of 0.8 μ m, and then 0.45 μ m). Clarified ciders were appropriately diluted before being used in the enzymatic assay. For the determination of ethanol, after

the removal of carbon dioxide by increasing the pH of the solution to approximately 9 with 2 M NaOH and by gently stirring, samples were appropriately diluted and used in the enzymatic assay. For all the enzymatic assays, the absorbance was measured at 340 nm by using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Sweden). The content of sucrose, fructose and glucose, acetic acid, and ethanol were then calculated using the Megazyme Mega-Calc™, accessed from the Megazyme web site (www.megazyme.com), and expressed as g/l.

4.2.6 Volatile organic compound (VOCs) profiling of pomegranate juices and ciders

For the analysis of volatile organic compounds (VOCs), samples were prepared according to the method described by He *et al.* (2021). In detail, 2 ml of pomegranate juice or cider, 0.2 g NaCl and 10 µl of internal standard (4-methyl-2-pentanol with a final concentration of 33 mg/l) were inserted into 20 ml glass vials, which were sealed with a magnetic screw cap with polytetrafluoroethylene/silicone septa and stored at 4 °C until analysis. Extraction of VOCs was performed by Head Space-Solid Phase Micro Extraction (HS-SPME) using a CombiPAL injector sample system (CTC Analytics, Zwingen, Switzerland) and a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Bellefonte, PA). After having equilibrated the samples (45 °C, 15 min), the fiber was exposed in the HS (45 °C, 45 min, under stirring). The extracted VOCs were desorbed into a Clarus 680 gas chromatograph (Perkin Elmer, Beaconsfield, UK) through insertion of the fiber in the injection port (splitless mode, at 220 °C for 3 min). Helium, at a flow rate of 1 ml/min, was used as the carrier gas. The separation of VOCs was carried out using a Rtx-WAX (Restek, Bellefonte, PA, USA) column (30 m x 0.25 mm x 0.25 µm), under the following conditions: the oven temperature was initially set at 35 °C for 8 min, followed by an increase to 60 °C at a rate of 4 °C/min, then followed by an increase to 160 °C at a rate of 6 °C/min, and finally an increase to 200 °C at a rate of 20 °C/min, and held at 200 °C for 15 min. The separated VOCs were detected through a Clarus SQ8MS (Perkin Elmer) quadrupole mass spectrometer, with the ionization source set at 250 °C and the MS transfer line set at 230 °C. Electron ionization masses were recorded at 70 eV in the mass-to-charge ratio range from 34 to 350 m/z. Each generated chromatogram was analyzed for peak identification using the National Institute of

Standard and Technology 2008 library. A peak area threshold of >1,000,000 and a probability of match of 85% or greater were used for VOCs identification, followed by the manual visual inspection of the fragment patterns when required. The relative concentration of each VOC (expressed as $\mu\text{g/g}$ of 4-methyl-2-pentanol) was estimated via interpolation of the relative areas *versus* the internal standard area.

4.2.7 Colour and sensory analysis

Instrumental colour measurements of lightness of pomegranate juices and ciders at T₀, T₃, T₇ and T₁₄ were taken using a Konica Minolta CR-10 reader (Chiyoda, Tokyo, Japan). The calibration of the instrument was performed before measurement by placing the tip of the colour reader against a white ceramic plate. Moreover, centrifugated ciders (LS J-L6, HS J-L6, LS CY and HS CY) were subjected to sensorial analysis. The panel was composed of thirteen volunteers (4 males and 9 females, with a mean age of 30-years) from laboratory staff, which had been previously trained about the meaning of the sensory attributes and scores. Each cider was identified by a code number and served at 4 °C in transparent plastic cups under daylight illumination, in random order (Supplementary Figure 1). Each panelist evaluated 10 ml of each cider thesis. The attributes were: transparency, clarity, and chromatic intensity for the visual evaluation; scent of pomegranate, herbaceous, alcohol, vinegar, and complexity for the olfactory evaluation; sweet, bitter, sour, fruity, alcoholic, tannic taste, and fullness/body for the tasting evaluation, and overall pleasantness. The score for each sensory attribute ranged from 0 (lowest) to 10 (highest).

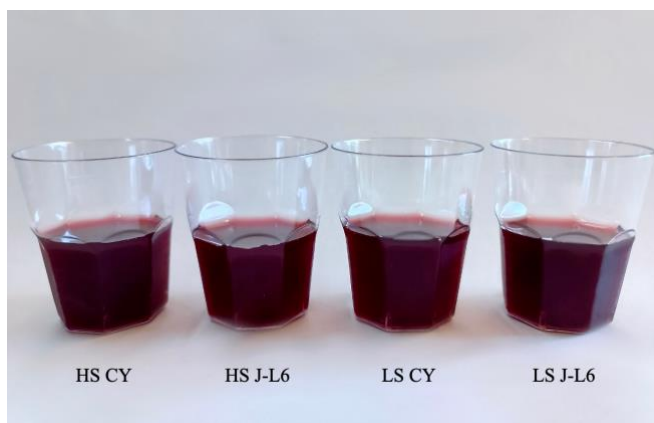


Figure 1. Appearance of centrifugated pomegranate ciders HS-CY, HS J-L6, LS J-L6, LS-CY.

4.2.8 Determination of antioxidant activity

In vitro antioxidant activity was estimated in terms of scavenging activity towards the radical 2,2-DiPhenyl-1-PicrylHydrazyl (DPPH $\cdot$), and determination of the ferric reducing antioxidant power (FRAP) on juice and cider samples (diluted in deionized water, 1:10 and 1:20 respectively for DPPH $\cdot$ and FRAP assay). All samples were analyzed in triplicate. The free radical scavenging activity against DPPH $\cdot$ was measured as reported by Caponio *et al.* (2020), with modifications reported by Limongelli, Minervini, & Calasso (2023). Specifically, the DPPH $\cdot$ assay was performed by preparing a 0.08 mM DPPH $\cdot$ solution in ethanol. Fifty μ l of each sample were added to 950 μ l of DPPH $\cdot$ solution in spectrophotometry cuvettes. After incubation of the reaction mixture (in dark, 30 min at 25 °C), the absorbance was measured at 517 nm by using an Ultrospec 3000 spectrophotometer. A reaction mixture consisting just of 950 μ l of DPPH $\cdot$ and 50 μ l of ethanol (80%) was always inserted as the blank, while another reaction mixture consisting of 950 μ l of DPPH $\cdot$ and 50 μ l of butylated hydroxytoluene (BHT) solution (0.45 g/l in ethanol 80%) was used as a positive control. The free radical scavenging activity was calculated as follows:

$$\text{DPPH} \cdot \text{scavenging activity (\%)} = [(blank \text{ absorbance} - sample \text{ absorbance}) / blank \text{ absorbance}] \times 100.$$

FRAP was evaluated according to the method by Son *et al.* (2018), with modifications reported by Minervini *et al.* (2019). In detail, 200 μ l of sample were mixed with 200 μ l of 0.2 M sodium phosphate buffer (pH 6.6) and 200 μ l of potassium ferricyanide (1%, w/v). The reaction mixture was incubated at 50 °C for 20 min. The reaction was stopped by adding 200 μ l of trichloroacetic acid (10%, w/v). After centrifugation (8000 $\times$ g, 10 min, 4 °C), 500 μ l of the collected supernatant were added with 100 μ l of ferric chloride solution (0.1%, w/v) and 400 μ l of demineralized water. After incubation (10 min, 25 °C), the absorbance of the ferrous ions was measured at 700 nm. BHT solution (0.45 g/l in ethanol 80%) was used as a positive control.

4.2.9 Statistical analyses

Results from microbiological, chemical (including concentrations of mono-, di-saccharides, acetic acid, and ethanol), sensory, color, and antioxidant activity analyses were statistically analyzed using the Student's T-test at a significance level of $P = 0.05$. VOCs were evaluated through unsupervised principal component

analysis (PCA) to assess VOCs profiles in HS and LS pomegranate juice fermented by the commercial yeast or *H. valbyensis* J-L6 after 0, 3 and 7 days of fermentation. Confidence interval ellipses (95%) for both commercial yeast and *H. valbyensis* groups were plotted using the “factoextra” package in R (Kassambara & Mundt, 2017). Pairwise comparisons between *H. valbyensis* and commercial yeast cider were conducted with a Wilcoxon rank-sum test ($P < 0.05$) and fold change analysis ($\log_2FC > 2$) in MetaboAnalyst 3.0. A heatmap of VOCs in matured ciders was created with the “pheatmap” package in R (Kolde, 2018).

4.3 Results

4.3.1 Microbiological characterization of pomegranate ciders

After three days of fermentation (T_3), the cell density of yeasts, determined on SDA and WA, significantly increased ($P < 0.05$) in all the theses, reaching at least $7 \log CFU/ml$ (Figure 2; Table 1). The highest increase, of about three logarithmic cycles, was observed in LS and HS juices fermented by *H. valbyensis* J-L6 (8.6 ± 0.4 and $8.4 \pm 0.4 \log CFU/ml$, respectively). In these juices, after 7 days (T_7), the cell density of yeast was not significantly different ($P > 0.05$), with respect to T_3 . On the contrary, the cell density significantly ($P < 0.05$) increased in the juices fermented by commercial *S. cerevisiae* Mo2 (LS CY, HS CY), reaching at that time values not significantly different ($P > 0.05$) from the juices LS J-L6 and HS J-L6, fermented with the autochthonous starter (Figure 2). The statistical analysis of the cell density of yeasts is shown in Supplementary Table 1. Plate counts on WA gave the same results of cell density as those on SDA (data not shown).

Total mesophilic aerobic microorganisms showed a similar trend to that of yeasts: the initial cell density was ca. 4.5 and 5.7 $\log CFU/ml$ for juices inoculated with *S. cerevisiae* and *H. valbyensis*, respectively. After three days, a significant ($P < 0.05$) increase was observed in all the fermented juices, particularly in LS J-L6 and HS J-L6 (ca. $8.8 \pm 0.1 \log CFU/ml$), where an increase of three logarithmic cycles was registered. No significant differences ($P > 0.05$) in the cell density of total mesophilic aerobic microorganisms were observed after seven days with respect to three days, except for HS juice fermented by commercial yeast, wherein cell density significantly ($P < 0.05$) increased (ca. $7.7 \pm 0.1 \log CFU/ml$). Acetic acid

bacteria, enterococci, staphylococci and *Enterobacteriaceae* were below the detection limit (data not shown).

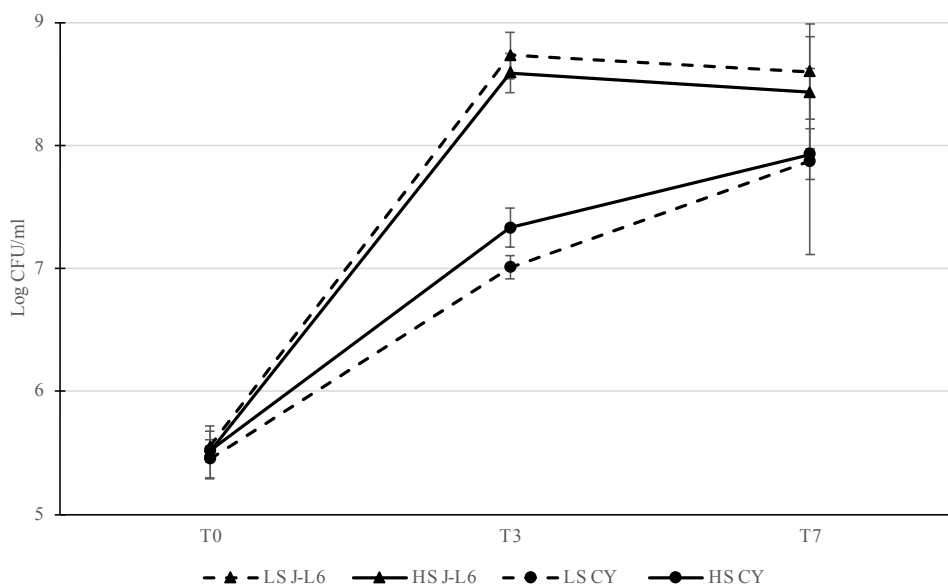


Figure 2. Cell density (expressed as log CFU/ml) of yeasts (determined on Sabouraud Dextrose agar) in “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C.

Table 1. Cell density (expressed as log CFU/ml) of yeasts (determined on Sabouraud Dextrose agar) in “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C. For each thesis, bars sharing one capital letter (A-C) and for each time of fermentation, columns sharing one lower-case letter (a-c) indicate not significantly ($p > 0.05$) different values of cell density.

	To	T3	T7
LS-CY	5.45±0.16 ^{Cb}	7.01±0.09 ^{Bc}	7.87±0.76 ^{Abc}
HS-CY	5.52±0.04 ^{Cb}	7.33±0.16 ^{Bb}	7.93±0.21 ^{Ab}
LS J-L6	5.75±0.12 ^{Ba}	8.93±0.19 ^{Aa}	8.80±0.39 ^{Aa}
HS J-L6	5.71±0.21 ^{Bab}	8.79±0.16 ^{Aa}	8.63±0.45 ^{Aac}

After maturation (i.e., seven days of storage at 4 °C following fermentation), yeast cell density, determined on SDA (Table 2) and WA (data not shown) of all the untreated (U, meaning that they were not subjected to clarification) ciders reached the order of 8 log CFU/ml. Yeast cell density significantly ($P < 0.05$) decreased of at least 3 log cycles in all the ciders clarified by centrifugation (C). Yeasts were found at lower ($P < 0.05$) numbers in the centrifugated ciders obtained by the commercial yeast (LS CY, HS CY) than in the centrifugated ciders obtained with the autochthonous yeast (LS J-L6, HS J-L6). In the ciders clarified by filtration (F), the yeast cell density after maturation was below the detection limit (Table 2).

Table 2. Cell density (expressed as Log CFU/ml) of yeasts (determined on Sabouraud Dextrose agar) in “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented for seven days (15 °C, 160 rpm) by cider commercial yeast (CY) *Saccharomyces cerevisiae* Moz or by *Hanseniaspora valbyensis* J-L6 (J-L6), subsequently treated by centrifugation (C) or filtration (F) or matured “as-is” (untreated, U) at 4 °C, for seven days. For each thesis, bars sharing one capital letter (A-B) and for each treatment (untreated, U; centrifugation, C; and filtration, F), columns sharing one lower-case letter (a-b) indicate not significantly ($p > 0.05$) different values of cell density.

	U	C	F
LS J-L6	8.71±0.6 ^{Aa}	5.28±0.4 ^{Ba}	< 1
HS J-L6	8.44±0.5 ^{Aa}	5.09±0.1 ^{Ba}	< 1
LS CY	8.17±0.5 ^{Aa}	3.88±0.2 ^{Bb}	< 1
HS CY	8.37±0.3 ^{Aa}	3.57±0.3 ^{Bb}	< 1

4.3.2 pH and concentrations of mineral elements in pomegranate juices and ciders
The pH before fermentation ranged from 3.1 to 3.2; during fermentation, it slightly decreased, but not significantly ($P > 0.05$), then remained quite stable until maturation, both in clarified and in untreated ciders (data not shown). Comparing the elemental composition of ciders after seven days of fermentation (T7) with that of juices (To), K and Ca concentrations significantly ($P < 0.05$) increased in all the theses, except for LS-CY, wherein Ca content remained unchanged (Table 2). On the contrary, Fe concentration significantly ($P < 0.05$) decreased in the ciders already after three days, although in HS J-LS it subsequently increased reaching,

after seven days (T7), a similar value compared to that measured at To. Copper decreased in the ciders obtained from HS pomegranate juice, whereas it did not change in the others. The variations of K, Ca, Fe, and Cu concentrations were in some cases progressive during the fermentation, in other cases occurred only in the first three days or only in the last four days of fermentation. No significant differences were found for all the other mineral elements (Table 3). After maturation, K concentration decreased in the ciders that had been clarified through centrifugation (C), except for LS-CY (no significant variation) (Table 4). K concentration significantly ($P < 0.05$) decreased also in J-L6 ciders clarified through filtration (F), with values even lower ($P < 0.05$) than those quantified in J-L6 cider samples clarified through centrifugation (C). Both Ca and Mn content was not affected by the clarification; on the contrary, Cu and Zn content decreased in the clarified ciders. A decrease in Fe concentration was found in the mature HS-ciders clarified by centrifugation or filtration, whereas no variation was revealed in clarified LS-ciders compared to untreated mature cider. Lower Ni concentrations were found in the mature HS-ciders previously clarified by filtration or centrifugation, as well as in LS J-L6 clarified by centrifugation (Table 4). In addition, we found that, among the centrifugated ciders, LS J-L6 contained the highest ($P < 0.05$) K concentration (ca. 1700 mg/l). Among the filtrated ciders, the highest ($P < 0.05$) concentration of K (ca. 1600 mg/l) was found in LS-CY.

Table 3. Mineral content (mg/l) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C. For each thesis, values sharing one letter (A-C) are not significantly ($p > 0.05$) different.

LS J-L6	To	T3	T7
K	813.11 ^C ± 101.06	1091.39 ^B ± 82.01	1536.43 ^A ± 176.34
Ca	29.54 ^B ± 1.57	34.70 ^A ± 2.47	38.35 ^A ± 2.00
Mn	0.40 ^A ± 0.02	0.35 ^B ± 0.03	0.37 ^{AB} ± 0.03
Fe	0.08 ^A ± 0.01	0.03 ^B ± 0.00	0.03 ^B ± 0.01
Cu	0.35 ^A ± 0.22	0.18 ^A ± 0.12	0.24 ^A ± 0.20
Zn	1.20 ^A ± 0.29	1.14 ^A ± 0.24	1.23 ^A ± 0.35
Ni	0.04 ^A ± 0.01	0.04 ^A ± 0.01	0.05 ^A ± 0.01

LS CY	To	T ₃	T ₇
K	965.52 ^B ± 112.59	1027.85 ^B ± 97.35	1593.96 ^A ± 389.59
Ca	35.78 ^A ± 8.79	34.66 ^A ± 8.30	42.17 ^A ± 14.20
Mn	0.43 ^A ± 0.07	0.37 ^A ± 0.11	0.37 ^A ± 0.11
Fe	0.09 ^A ± 0.00	0.04 ^B ± 0.01	0.03 ^B ± 0.01
Cu	0.31 ^A ± 0.20	0.24 ^A ± 0.15	0.13 ^A ± 0.10
Zn	1.28 ^A ± 0.19	1.22 ^A ± 0.22	1.25 ^A ± 0.27
Ni	0.04 ^A ± 0.02	0.04 ^A ± 0.02	0.04 ^A ± 0.00

HS J-L6	To	T ₃	T ₇
K	788.26 ^C ± 71.89	998.72 ^B ± 64.64	1768.29 ^A ± 88.28
Ca	35.63 ^B ± 4.57	38.54 ^B ± 3.26	53.97 ^A ± 3.23
Mn	0.42 ^A ± 0.03	0.33 ^A ± 0.08	0.42 ^A ± 0.07
Fe	0.09 ^A ± 0.04	0.04 ^B ± 0.00	0.06 ^A ± 0.00
Cu	0.35 ^A ± 0.01	0.17 ^B ± 0.03	0.18 ^B ± 0.03
Zn	1.12 ^A ± 0.26	1.05 ^A ± 0.40	1.05 ^A ± 0.24
Ni	0.05 ^A ± 0.01	0.05 ^A ± 0.02	0.06 ^A ± 0.01

HS CY	To	T ₃	T ₇
K	927.04 ^B ± 253.70	969.34 ^B ± 514.17	1689.29 ^A ± 285.10
Ca	44.02 ^B ± 9.37	42.16 ^{AB} ± 17.61	59.02 ^A ± 7.34
Mn	0.44 ^A ± 0.06	0.37 ^A ± 0.12	0.42 ^A ± 0.07
Fe	0.10 ^A ± 0.02	0.06 ^B ± 0.00	0.05 ^C ± 0.00
Cu	0.36 ^A ± 0.02	0.20 ^B ± 0.00	0.18 ^C ± 0.01
Zn	1.06 ^A ± 0.22	0.99 ^A ± 0.25	1.05 ^A ± 0.19
Ni	0.05 ^{AB} ± 0.01	0.04 ^B ± 0.01	0.06 ^A ± 0.01

Table 4. Mineral content (mg/l) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), subsequently clarified through centrifugation (C) or filtration (F) or untreated (U) and matured (4 °C, seven days). For each thesis, values sharing one letter (A-C) are not significantly (p > 0.05) different.

LS J-L6	U	C	F
K	$1849.00^A \pm 0.92$	$1726.39^B \pm 67.0$	$1292.07^C \pm 183.31$
Ca	$45.05^A \pm 5.54$	$47.71^A \pm 8.54$	$42.12^A \pm 2.48$
Mn	$0.41^A \pm 0.03$	$0.44^A \pm 0.04$	$0.38^A \pm 0.04$
Fe	$0.05^A \pm 0.00$	$0.04^A \pm 0.01$	$0.05^A \pm 0.01$
Cu	$0.23^A \pm 0.13$	$0.03^B \pm 0.02$	$0.02^B \pm 0.00$
Zn	$1.28^A \pm 0.25$	$0.18^C \pm 0.08$	$0.36^B \pm 0.02$
Ni	$0.05^A \pm 0.01$	$0.03^B \pm 0.01$	$0.04^{AB} \pm 0.01$

LS CY	U	C	F
K	$1413.26^{AB} \pm 304.51$	$1366.53^B \pm 87.55$	$1657.06^A \pm 111.28$
Ca	$38.86^A \pm 11.81$	$41.36^A \pm 5.34$	$49.90^A \pm 6.54$
Mn	$0.33^A \pm 0.11$	$0.41^A \pm 0.05$	$0.44^A \pm 0.05$
Fe	$0.04^A \pm 0.01$	$0.04^A \pm 0.02$	$0.04^A \pm 0.00$
Cu	$0.14^A \pm 0.09$	$0.03^B \pm 0.01$	$0.02^B \pm 0.01$
Zn	$1.20^A \pm 0.21$	$0.10^C \pm 0.01$	$0.15^B \pm 0.01$
Ni	$0.04^A \pm 0.00$	$0.03^A \pm 0.01$	$0.03^A \pm 0.01$

HS J-L6	U	C	F
K	$1838.29^A \pm 93.73$	$1564.67^B \pm 66.92$	$1164.16^C \pm 91.23$
Ca	$56.46^A \pm 2.36$	$53.86^A \pm 1.10$	$49.44^A \pm 7.50$
Mn	$0.41^A \pm 0.05$	$0.43^A \pm 0.10$	$0.41^A \pm 0.07$
Fe	$0.07^A \pm 0.01$	$0.05^B \pm 0.00$	$0.05^B \pm 0.01$
Cu	$0.18^A \pm 0.02$	$0.05^B \pm 0.01$	$0.03^B \pm 0.01$
Zn	$1.11^A \pm 0.32$	$0.23^B \pm 0.22$	$0.34^B \pm 0.31$
Ni	$0.07^A \pm 0.01$	$0.04^B \pm 0.00$	$0.04^B \pm 0.00$

HS CY	U	C	F
K	$1826.87^A \pm 48.36$	$1650.20^B \pm 127.68$	$1625.12^{AB} \pm 238.75$
Ca	$63.85^A \pm 0.90$	$63.21^A \pm 1.81$	$65.52^A \pm 4.23$
Mn	$0.45^A \pm 0.02$	$0.47^A \pm 0.04$	$0.47^A \pm 0.07$
Fe	$0.09^A \pm 0.03$	$0.05^B \pm 0.00$	$0.05^B \pm 0.00$
Cu	$0.16^A \pm 0.03$	$0.03^B \pm 0.01$	$0.02^B \pm 0.00$
Zn	$1.06^A \pm 0.14$	$0.07^B \pm 0.01$	$0.21^B \pm 0.12$
Ni	$0.06^A \pm 0.00$	$0.04^B \pm 0.01$	$0.04^B \pm 0.01$

4.3.3 Concentrations of monosaccharides, ethanol, and acetic acid in pomegranate cider

The content of glucose and fructose significantly ($P < 0.05$) decreased during fermentation of pomegranate juice to cider (Table 5), whereas sucrose was never detected. The highest decrease in glucose and fructose content was observed after seven days (T7); in detail, pomegranate ciders fermented by the autochthonous yeast *H. valbyensis* (LS J-L6, HS J-L6) were characterized by a significantly lower content of both monosaccharides than ciders fermented by the commercial yeast *S. cerevisiae* (LS CY, HS CY, Table 5). After maturation of ciders, the concentrations of both monosaccharides were below the limit of detection (data not shown).

Table 5. Glucose and fructose content (g/l) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C. Within each thesis, values sharing one capital letter (A-C) are not significantly ($p > 0.05$) different; for each time of fermentation, values sharing one lower-case letter (a-c) are not significantly ($p > 0.05$) different.

	Glucose (g/l)		
	To	T3	T7
LS J-L6	61.78±0.00 ^{Aa}	21.09±0.29 ^{Bc}	1.57±0.10 ^{Cc}
HS J-L6	60.65±0.10 ^{Ac}	26.87±0.44 ^{Bb}	2.16±0.15 ^{Cb}
LS-CY	58.79±0.10 ^{Ad}	42.85±2.59 ^{Ba}	3.13±0.05 ^{Ca}
HS-CY	61.45±0.05 ^{Ab}	42.61±0.59 ^{Ba}	3.24±0.49 ^{Ca}
	Fructose (g/l)		
	To	T3	T7
LS J-L6	49.30±1.87 ^{Aa}	33.81±0.39 ^{Bc}	3.24±0.10 ^{Cb}
HS J-L6	47.94±0.84 ^{Aa}	35.69±0.59 ^{Bb}	3.28±0.15 ^{Cb}
LS-CY	47.94±0.35 ^{Aa}	43.12±1.83 ^{Ba}	4.19±0.05 ^{Ca}
HS-CY	49.13±1.63 ^{Aa}	44.00±0.10 ^{Ba}	4.29±0.79 ^{Ca}

In parallel with the decrease in monosaccharides content, a significant ($P < 0.05$) increase in acetic acid (Table 6a) and ethanol (Table 7a) content after three (T_3) and seven (T_7) days of fermentation was observed. Overall, pomegranate ciders fermented by *H. valbyensis* were characterized by a significantly ($P < 0.05$) higher content in acetic acid and a significantly ($P < 0.05$) lower content in ethanol at the end of fermentation, compared to ciders fermented by *S. cerevisiae*. After maturation, ciders that had been clarified (through centrifugation, C, or filtration, F) were overall characterized by significant ($P < 0.05$) lower concentrations of acetic acid (Table 6b) and ethanol (Table 7b), compared to untreated ciders. In addition, we found a significant ($P < 0.05$) decrease in acetic acid and ethanol content in the ciders fermented by *H. valbyensis* and clarified through filtration, compared to those clarified through centrifugation, except for LS J-L6 ciders, whose ethanol concentration did not differ ($P > 0.05$) regardless of the method of clarification. A similar trend was observed for ethanol in the clarified and mature ciders obtained with the use of the commercial yeast (Table 7b). Acetic acid was found at higher concentration in the filtered ciders obtained with the commercial yeast as compared to the centrifuged ciders obtained with the same starter (Table 6b).

Table 6. Acetic acid content (g/l) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (T_0), and after three (T_3) and seven (T_7) days of fermentation at 15 °C (a) and on ciders subsequently clarified through centrifugation (C) or filtration (F) or matured “as-is” (untreated, U) at 4 °C, for seven days (b). Within each thesis, values sharing one capital letter (A-C) are not significantly ($p > 0.05$) different; for each time of fermentation (a) or treatment (b), values sharing one lower-case letter (a-c) are not significantly ($p > 0.05$) different.

a	T_0	T_3	T_7
LS J-L6	0.06 ± 0.01^{Cc}	0.12 ± 0.01^{Bc}	0.31 ± 0.08^{Aabc}
HS J-L6	0.13 ± 0.00^{Ba}	0.15 ± 0.01^{Bb}	0.41 ± 0.01^{Aa}
LS-CY	0.10 ± 0.01^{Cb}	0.19 ± 0.03^{Bab}	0.24 ± 0.01^{Ac}
HS-CY	0.11 ± 0.05^{Cabbc}	0.23 ± 0.01^{Ba}	0.28 ± 0.01^{Ab}

b	U	C	F
LS J-L6	0.42±0.04 ^{Aa}	0.32±0.01 ^{Bb}	0.21±0.06 ^{Cb}
HS J-L6	0.37±0.02 ^{Aa}	0.35±0.00 ^{Aa}	0.34±0.00 ^{Ba}
LS-CY	0.21±0.01 ^{Ab}	0.07±0.03 ^{Bc}	0.14±0.05 ^{Bbc}
HS-CY	0.11±0.00 ^{Ac}	BLD [*]	0.08±0.02 ^{Bc}

*below the limit of detection

Table 7. Ethanol content (g/l) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C (a) and on ciders subsequently clarified through centrifugation (C) or filtration (F) or matured “as-is” (untreated, U) at 4 °C, for seven days (b). Within each thesis, values sharing one capital letter (A-C) are not significantly ($p > 0.05$) different; for each time of fermentation (a) or treatment (b), values sharing one lower-case letter (a-d) are not significantly ($p > 0.05$) different.

a	To	T3	T7
LS J-L6	0.23±0.01 ^{Cd}	32.75±0.49 ^{Ba}	48.98±0.38 ^{Ad}
HS J-L6	0.36±0.00 ^{Cc}	22.65±0.02 ^{Bc}	50.41±0.68 ^{Ac}
LS-CY	0.64±0.02 ^{Cb}	26.01±0.19 ^{Bb}	58.14±0.61 ^{Ab}
HS-CY	0.80±0.06 ^{Ca}	30.00±0.38 ^{Bb}	59.39±0.31 ^{Aa}

b	U	C	F
LS J-L6	46.33±1.29 ^{Ac}	38.81±1.10 ^{Bd}	37.19±0.52 ^{Bb}
HS J-L6	49.05±1.76 ^{Abc}	48.00±0.09 ^{Ac}	37.02±0.16 ^{Bb}
LS-CY	49.38±0.31 ^{Ab}	48.23±0.00 ^{Bb}	41.04±0.80 ^{Ca}
HS-CY	52.37±0.19 ^{Aa}	51.64±0.21 ^{Ba}	42.65±1.76 ^{Ca}

4.3.4 VOCs profiles of pomegranate juices during fermentation to cider and after maturation

HS-SPME-GC-MS was used to profile VOCs of the juices during fermentation (To, T3, T7) and after maturation (T14). Overall, 97 VOCs were detected and clustered into six chemical classes (alcohols, carboxylic acids, carboxylic esters, hydrocarbons, phenols, terpenes), with 29 compounds, mainly consisting of aldehydes and ketones (“Others”) outside of the above-mentioned classes. The

most dominant compound classes were alcohols and carboxylic acids. Concentrations of VOCs were elaborated through Principal Component Analysis (PCA), with the first two components explaining only 41% of the total variance (Figure 3). Among the variables with the highest loads on the first principal component, we found 3-methyl-1-butanol, nonanoic acid ethyl ester, and 1-hexanol. Ethyl acetate, butanoic acid, and ethyl ester were among the variables with the highest loads on the second principal component. All the juices at the start of fermentation (To) and the juices fermented for three days (T3) by the commercial yeast (CY) *S. cerevisiae* clustered together, mainly because they were characterized by VOCs at low concentrations. During fermentation, some VOCs that were not detected at the start of fermentation, such as ethyl acetate and ethyl ester of decanoic acid, appeared. Pomegranate cider samples obtained after three (T3) and seven (T7) days of fermentation with the autochthonous yeast *H. valbyensis* (J-L6) were grouped in the same quadrant of the plane, well separated from the cider samples fermented for seven days by the commercial yeast (Figure 3).

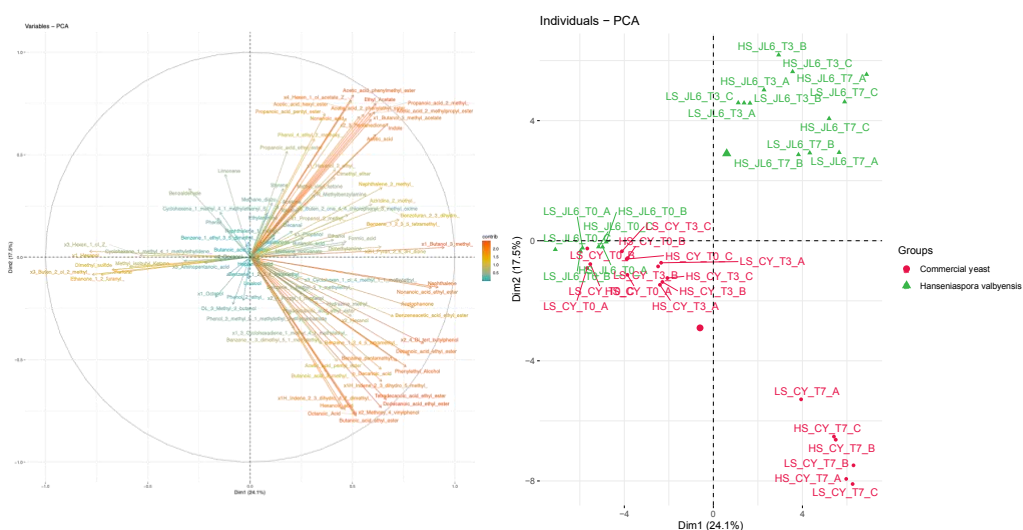


Figure 3. Principal component analysis of VOCs in “low” (LS) and “high” (HS) sugar pomegranate juice fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C. Ellipses were plotted based on confidence interval (95%) of commercial yeast and *Hanseniaspora valbyensis* group marked in red and green respectively.

In order to highlight better the differences in terms of VOCs between the two starter yeasts, we computed a pairwise comparison combining results from fold change analysis and Wilcoxon rank-sum test. The resulting volcano plot suggested that the use of *H. valbyensis* J-L6 as starter differentiated the ciders samples from those obtained using the commercial yeast, in terms of increased amount of: ethyl acetate; acetic acid; acetic acid, 2 phenylethyl ester; acetic acid, phenylmethyl ester; acetic acid, hexyl ester; acetic acid, 2-methylpropyl ester; 1-butanol, 3-methylacetate; 4-hexen-1-ol-acetate (Figure 4). Other compounds (i.e., propanoic acid, 2-methyl; 2-3-pentanedione; nonanoic acid; propanoic acid, pentyl ester; 4-ethyl-2-methoxyphenol) characterized the cider samples obtained using *H. valbyensis* as starter. On the contrary, cider samples fermented by the commercial yeast *S. cerevisiae* were characterized by some medium chain fatty acids (hexanoic acid, octanoic acid) and derivative esters of fatty acids (i.e., dodecanoic acid, ethyl ester; butanoic acid, ethyl ester) (Figure 4).

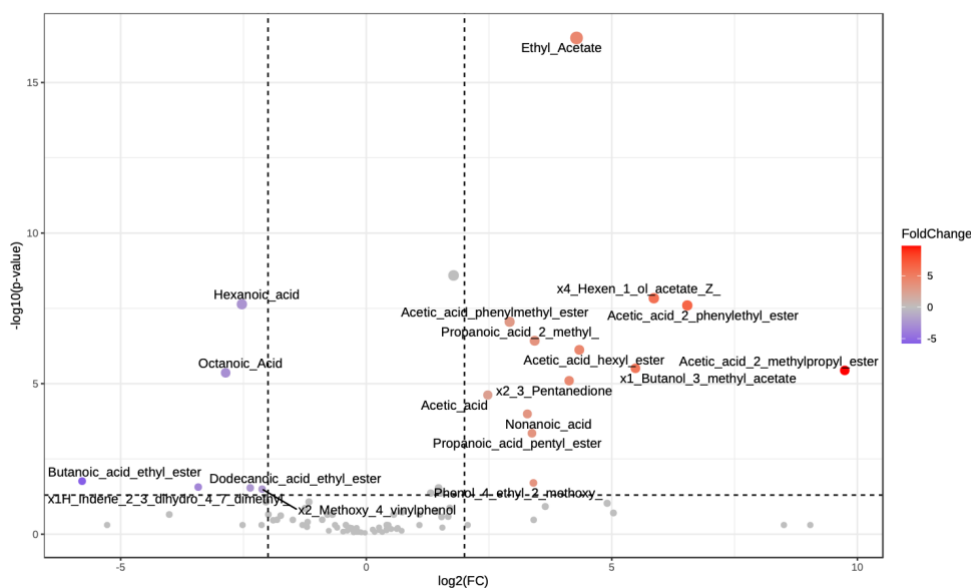


Figure 4. Volcano plot of statistically significant VOCs in fermented pomegranate (*Hanseniaspora valbyensis* vs Commercial yeast). Due to the group comparison direction, increased (red) and decreased (blue) VOC concentrations are relative to different starter implied for juice fermentation *Hanseniaspora valbyensis* (J-L6) and commercial yeast (CY). Pairwise comparison of VOC in samples after inoculation, and after three and seven days of fermentation by meaning of a non-parametric Wilcoxon rank-sum test ($p < 0.05$) combined with a fold change analysis ($\log_2FC > 4$).

After maturation, concentrations of some VOCs (e.g., heptanoic acid, nonanoic acid, 2-methyl-1-propanol) varied depending on the treatment applied (filtration, centrifugation, or no treatment) at the end of fermentation. Three main clusters of cider samples were found (Figure 5): cluster I consisted of mature cider samples obtained with commercial yeast and that were either centrifuged (HS-CY_C, LS-CY_C) or untreated (HS-CY_TQ, LS-CY_TQ), relatively rich in hexanoic, octanoic, and decanoic acids, and their ethyl esters; cluster II grouped cider samples subjected to filtration, especially rich in aldehydes (benzaldehyde, furfural, decanal); cluster III consisted of cider samples fermented by the autochthonous yeast and that were either centrifuged (LS J-L6_C, HS J-L6_C) or untreated (LS J-L6_TQ), and characterized by relatively high concentrations of acetic acid, ethyl acetate and other esters (Figure 5). Samples of mature HS cider not subjected to any treatment after fermentation with the autochthonous yeast did not fall in any of the afore mentioned clusters (Figure 5).

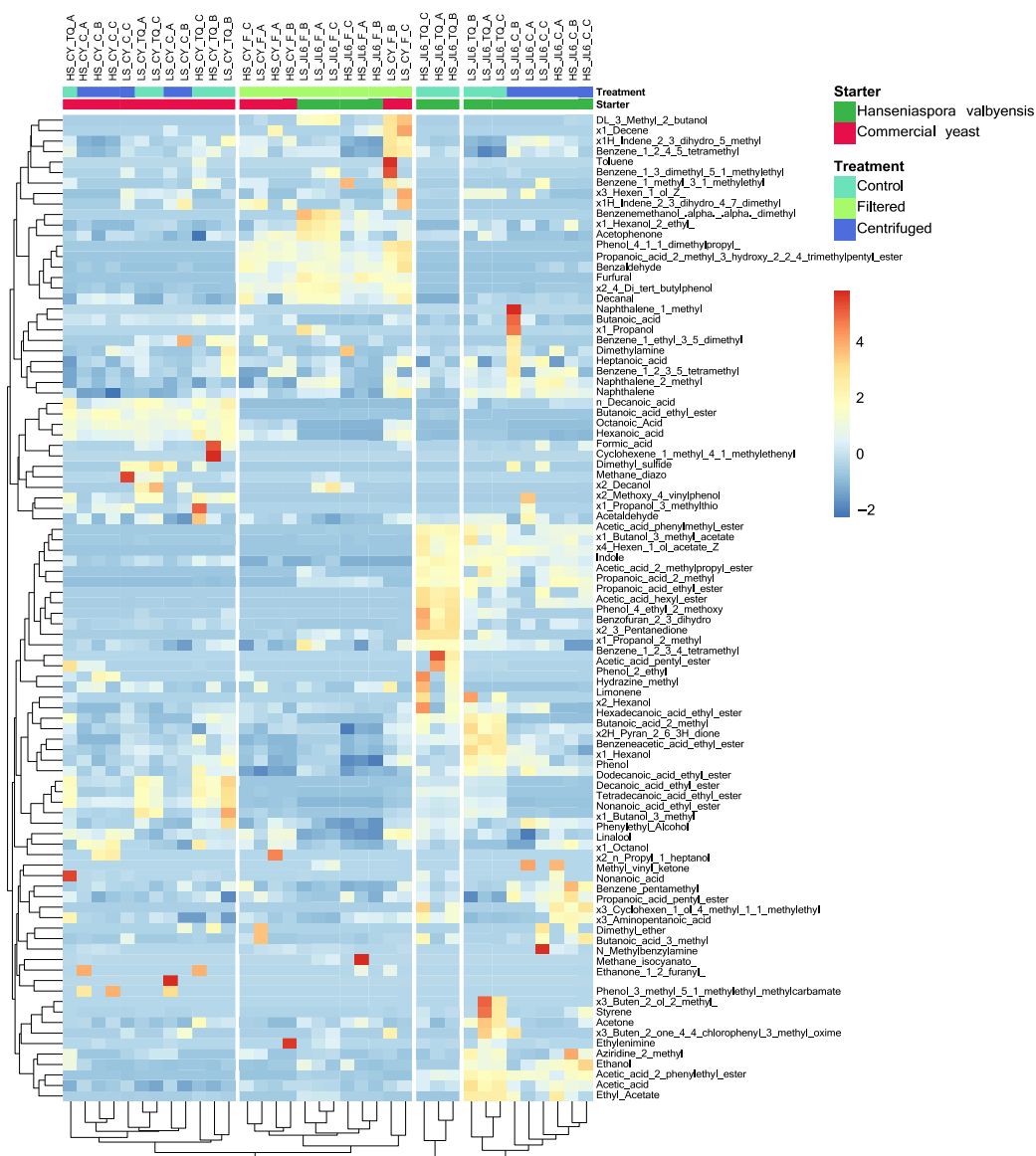


Figure 5. Heatmap of VOCs in mature pomegranate ciders fermented with commercial yeast (CY, red) or *Hanseniaspora valbyensis* (J-L6, green) and that had been centrifuged (C, blue), filtered (F, light green) or untreated (water-green, TQ) prior to maturation.

4.3.5 Colour and sensory analysis of pomegranate ciders

Lightness (expressed as L index) significantly ($P < 0.05$) increased after three and seven days of fermentation of pomegranate juices, compared to the juices at the time of inoculation (To). In detail, the lightness of juices fermented by the autochthonous *H. valbyensis* (J-L6) was significantly ($P < 0.05$) higher than that of the juices fermented by the commercial yeast *S. cerevisiae* (CY) (Table 8a). After seven days of maturation, L index significantly ($P < 0.05$) decreased in clarified (through centrifugation, C, or filtration, F) ciders compared to the theses that had not been clarified (untreated, U); within the latter, HS-CY cider showed the lowest ($P < 0.05$) value (Table 8b). L index varied from ca. 20 (LS-CY cider matured after centrifugation) to ca. 22 (HS J-L6 cider after centrifugation) and from ca. 22 (HS J-L6 and LS-CY, matured after filtration) to ca. 25 (HS-CY after filtration) (Table 8b).

Table 8. Determination of lightness (expressed as L index) in “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C (a) and on ciders subsequently clarified through centrifugation (C) or filtration (F) or matured “as-is” (untreated, U) at 4 °C, for seven days (b). Values sharing one capital letter (A-C) are not significantly ($p > 0.05$) different within the same juice/cider; values sharing one lower-case letter (a-c) are not significantly ($p > 0.05$) different within the same time or clarification treatment.

a	To	T3	T7
LS J-L6	22.47 ± 0.66 ^{Ba}	30.00 ± 0.79 ^{Aa}	29.93 ± 0.93 ^{Aa}
HS J-L6	20.91 ± 0.14 ^{Bb}	28.69 ± 0.81 ^{Aa}	29.21 ± 0.29 ^{Aa}
LS-CY	22.59 ± 0.13 ^{Ca}	23.93 ± 0.56 ^{Bc}	26.79 ± 0.27 ^{Ac}
HS-CY	23.03 ± 0.97 ^{Ca}	25.66 ± 0.74 ^{Bb}	27.36 ± 0.07 ^{Ab}
b	U	C	F
LS J-L6	28.41 ± 0.34 ^{Aa}	21.00 ± 0.03 ^{Cb}	23.34 ± 0.18 ^{Bb}
HS J-L6	28.23 ± 0.28 ^{Aa}	22.70 ± 0.14 ^{Ba}	22.63 ± 0.70 ^{Bb}
LS-CY	27.64 ± 0.51 ^{Aa}	20.58 ± 0.08 ^{Cc}	22.63 ± 0.70 ^{Bb}
HS-CY	25.46 ± 0.56 ^{Ab}	21.09 ± 0.79 ^{Bbc}	25.71 ± 0.19 ^{Aa}

Panel test was carried out just on centrifugated ciders (LS J-L6, HS J-L6, LS CY, HS CY). The results for visual analyses, as well as for most of the olfactory descriptors (scent of pomegranate and herbaceous), showed almost no differences among the four ciders. The lowest hint of ethanol and highest hint of acetic acid, as well as olfactory complexity, were perceived in LS J-L6 and HS J-L6 (data not shown). No significant differences were recognized for sweetness, fruity, and tannic taste; however, ciders obtained upon fermentation by *H. valbyensis* were perceived as more bitter and sour (ca. 5.9 and 7.2, respectively) than those fermented by *S. cerevisiae* (ca. 4.9 and 6.5 for bitterness and sourness, respectively). The alcoholic flavor was higher in ciders fermented by *S. cerevisiae*, compared to those fermented by *H. valbyensis*, with “high” sugar juice fermented by commercial yeast (HS-CY) rated with the highest value (6.5). Ciders obtained upon fermentation with the autochthonous yeast were perceived with more fullness/body than those fermented by the commercial yeast (data not shown). The HS-CY received the highest score for acceptability. Within ciders fermented by the autochthonous *H. valbyensis*, HS J-L6 was rated as the most acceptable (data not shown).

4.3.6 Antioxidant activity of pomegranate juice during fermentation and after maturation to cider

The antioxidant activity of diluted pomegranate juices and ciders was measured during fermentation and after maturation, in terms of radical scavenging activity (%) towards the radical 2,2-DiPhenyl-1-PicrylHydrazyl (DPPH·) and by the absorbance of ferrous ions at 700 nm (FRAP assay). The radical scavenging activity of juices and ciders ranged from 82 to 88%, thus resulting ever higher than BHT, used as positive control (Figures 6 and 7). Before fermentation, the radical scavenging activity ranged from 85% (HS J-L6, HS-CY, and LS-CY) to 88% (LS J-L6); no significant variations were observed for LS J-L6 and HS J-L6 at T₃ and HS J-L6 at T₇, compared to T₀; LS J-L6 decreased significantly ($P < 0.05$) compared to T₀, but not significantly ($P > 0.05$) compared to T₃. “Low” sugar pomegranate juice fermented with *S. cerevisiae* (LS-CY) showed a slight but significant ($P < 0.05$) decrease of radical scavenging activity during fermentation (Supplementary Figure 2). The radical scavenging activity slightly increased ($P < 0.05$), or did not vary, in clarified ciders compared to untreated ones (Figure 7).

The antioxidant activity measured through FRAP assay showed values of absorbance of ferrous ions of about 2.95 in all the pomegranate juices and no

significant variation ($P > 0.05$) was observed during fermentation (Figure 8). After maturation, the antioxidant activity of pomegranate ciders decreased, although being higher than 2.5, thus being comparable to the positive control (Figure 9). No significant differences were observed, within the same cider, regardless of the clarification treatment. Within the same treatment, HS cider obtained upon fermentation with the commercial yeast showed the highest value of FRAP, whereas LSJ-L6 cider (obtained upon fermentation with the autochthonous yeast) overall showed the lowest value (Figure 9).

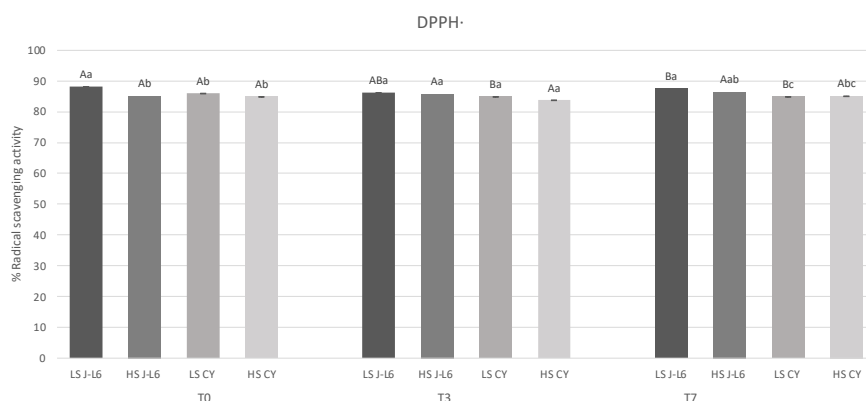


Figure 6. DPPH· scavenging activity (%) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C. For each thesis, bars sharing one capital letter (A-C) and for each time of fermentation, bars sharing one lower-case letter (a-c) indicate not significantly ($p > 0.05$) different values.

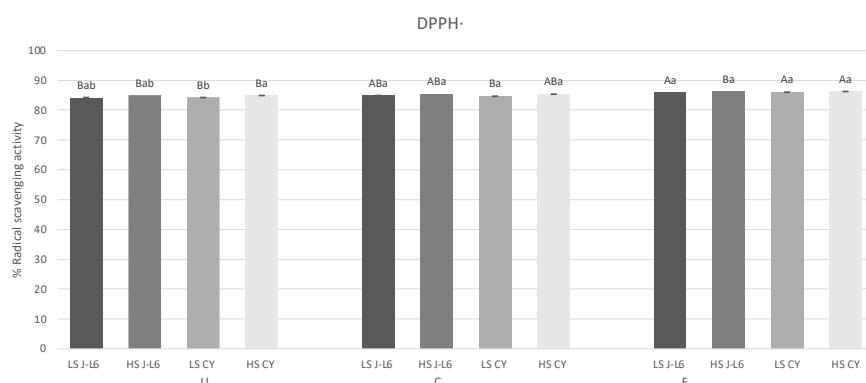


Figure 7. DPPH· scavenging activity (%) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented for seven days by cider commercial yeast (CY)

Saccharomyces cerevisiae Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), subsequently clarified through centrifugation (C) or filtration (F) or matured “as-is” (untreated, U) at 4 °C, for seven days. For each thesis, bars sharing one capital letter (A-C) and each treatment (U, C or F), bars sharing one lower-case letter (a-c) indicate not significantly ($p > 0.05$) different values.

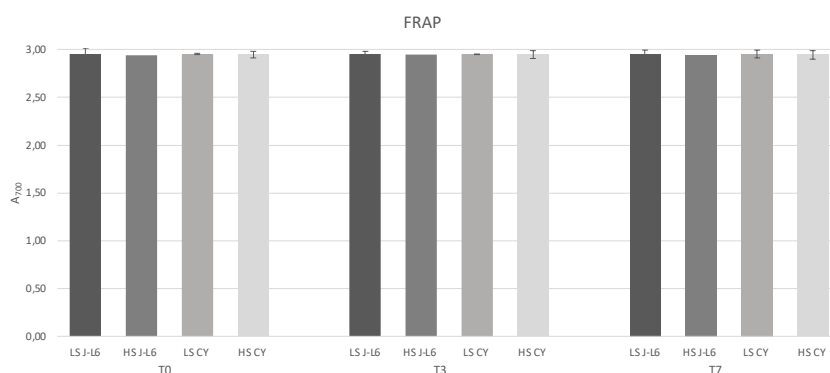


Figure 8. Ferric reducing antioxidant power (FRAP) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), after inoculation (To), and after three (T3) and seven (T7) days of fermentation at 15 °C. No significant differences between time and between juices.

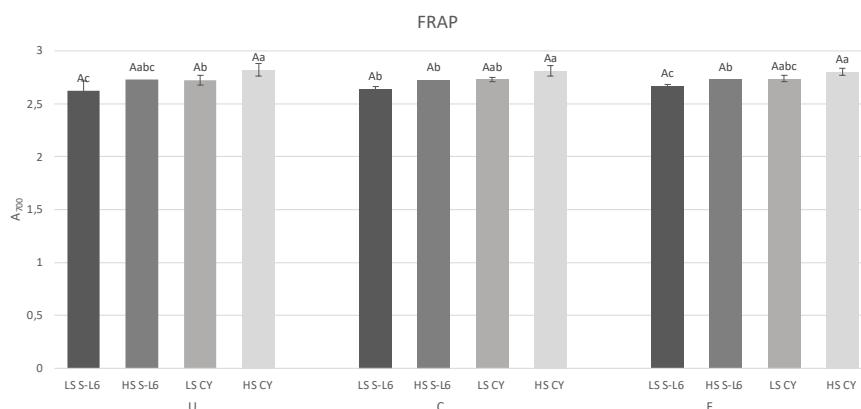


Figure 9. Ferric reducing antioxidant power (FRAP) of “low” sugar (LS) and “high” sugar (HS) pomegranate juices fermented for seven days by cider commercial yeast (CY) *Saccharomyces cerevisiae* Mo2 or by *Hanseniaspora valbyensis* J-L6 (J-L6), subsequently clarified through centrifugation (C) or filtration (F) or matured “as-is” (untreated, U) at 4 °C, for seven days. For each thesis, bars sharing one capital letter (A-C) and each treatment (U, C or F), bars sharing one lower-case letter (a-c) indicate not significantly ($p > 0.05$) different values.

4.4 Discussion

Cider is typically obtained from apple juice, fermented by commercial strains of *S. cerevisiae* (Lorenzini *et al.*, 2019). Previously, other authors scouted the potential of pomegranate juice and *S. cerevisiae* to produce alcoholic beverages (Cardinale *et al.*, 2021; Kokkinomagoulos *et al.*, 2020). In particular, the ethanol concentrations reported by Kokkinomagoulos *et al.* (2020) could fall within the range envisaged for cider. Besides using conventional starters, cider was produced with non-*Saccharomyces* species. Xu *et al.* (2006) used *H. valbyensis*, alone or in co-culture with *S. cerevisiae*, showing that the use of non-conventional yeast led to an improved VOCs profile. In the current study, we used pomegranate juice and compared the effects on cider characteristics, antioxidant properties and flavor profile, attributable to the use of two different starters for alcoholic fermentation: the commercial yeast *S. cerevisiae* Mo2 and the non-conventional autochthonous yeast *H. valbyensis* J-L6. Both the strains were singly used to ferment two types of pomegranate juices, which, although produced by the same company, had slightly different chemical compositions in terms of sugars. Both starters were able to drive the fermentation of pomegranate juice, but the autochthonous yeast reached the highest cell density four days before the commercial yeast. This behaviour could be attributed to the higher adaptability of the autochthonous *H. valbyensis*, compared to the commercial yeast *S. cerevisiae*, as reported in previous studies (Corbo *et al.*, 2017). However, it has to be considered that the autochthonous yeast was added to juice as fresh biomass, whereas the commercial yeast was added as dried and rehydrated biomass, which somehow could have determined the slower growth of the latter. Dominance of *Hanseniaspora* spp. during early (spontaneous) fermentation of cider was previously reported by Valles *et al.* (2007), who also showed that *Saccharomyces* spp. tended to replace *Hanseniaspora* spp. after four days. Our results were in contrast with previous research that showed a more rapid growth of *S. cerevisiae*, compared to *H. valbyensis*, during the fermentation of apple cider (Xu *et al.*, 2006). As expected, we found that filtration, used as clarification treatment, caused a stronger decrease in yeast cell density compared to centrifugation, in agreement with Stevens & Jaykus (2004). To the best of our knowledge, no previous studies had monitored the microbial population of cider after clarification and maturation.

The concentration of some mineral elements varied during fermentation and maturation, depending on the yeast used as starter, the type of juice, as well as the clarification treatment, when applied. Studies reported in the literature about the effects of fermentation on the mineral elements composition of ciders refer mainly to apple cider (Zuo *et al.*, 2019) and, only in one case, to pomegranate juice (Limongelli, Minervini, & Calasso, 2023). Variation of the mineral elements profile during fermentation is an important indicator not only of the nutritional value of cider, but also of the maintenance of an adequate metabolism of yeasts. Indeed, K, Ca, Fe, Zn and other elements are essential nutrients for yeasts, acting as enzyme cofactors, and contributing to balance both the ionic strength and the pH during fermentation (Zuo *et al.*, 2019). The total elemental concentrations measured in the liquid fraction of pomegranate juices or ciders revealed an increase in K and Ca levels during fermentation. Since the fermentation was conducted in a closed system, with no addition nor loss of mineral elements or water, the rising of K and Ca concentrations might be attributed to the increased solubilization of these elements during the fermentation. Indeed, it is well known that yeast cells can hydrolyze macromolecules, such as proteins and cell wall polysaccharides, facilitating the release of K from cytosol and Ca from cell walls. Conversely, Fe and Cu concentrations generally decreased during fermentation. This behavior might be attributed to chelation of these two metals by tannins and other phenolic compounds, which pomegranate is particularly rich in, and their subsequent precipitation (Orak, Yagar, & Isbilir, 2012), as occurs during wine fermentation (Bimpilas *et al.*, 2015). Iron and Cu uptake by yeast cells cannot also be excluded. When clarification by centrifugation or filtration preceded the maturation of ciders, a strong reduction of micronutrient concentrations (Cu, Zn and, in several cases, also Fe and Ni) was observed compared to the untreated mature ciders. As discussed before, these metals can be complexed or adsorbed by tannins or suspended solids present in cider, as well as they can be adsorbed or taken up by microbial cells, and then can be removed by centrifugation or filtration. Clarification did not affect Ca concentration of ciders, suggesting that this element was mainly distributed in the liquid fraction of the ciders. As for K content, it remained almost unvaried in clarified ciders compared to untreated ciders when *S. cerevisiae* was used, whereas it decreased in clarified ciders (especially in the

filtrated ones) fermented with *H. valbyensis*. The latter result deserves further investigation.

In agreement with the dynamics of yeasts, we found that the use of autochthonous *H. valbyensis* as starter led to a more rapid consumption of monosaccharides, compared to commercial *S. cerevisiae*. The dynamic of monosaccharides consumption approached that previously described by Kokkinomagoulos *et al.* (2020). In agreement with these authors, we found that mature pomegranate ciders did not contain monosaccharides, therefore appearing stable beverages in view of further storage. Pomegranate ciders obtained through *H. valbyensis* contained higher acetic acid and lower ethanol concentrations than those obtained through *S. cerevisiae*. These results confirm previous reports about *H. valbyensis* as strong and weak producer of acetic acid and ethanol, respectively (van Wyk *et al.*, 2024). Overall, both the clarification treatments applied in the current study caused a decrease of acetic acid and ethanol in pomegranate ciders. This may be explained considering that when subjected to either filtration or centrifugation, manipulation of cider might have caused a loss of the two main fermentation metabolites, which are volatile.

VOCs profile of pomegranate ciders was dominated by alcohols, acids, but also esters, in agreement with previous studies (Medina *et al.*, 2020; Perestrelo *et al.*, 2019). It was affected by the time of fermentation and type of starter used. When the commercial starter drove pomegranate juice fermentation, seven days were necessary to reach a VOCs profile well differentiated from the initial juice. On the contrary, three days of fermentation with the autochthonous starter were sufficient to reach the final VOCs profile. These results agreed with both yeast cell density dynamic and monosaccharides consumption during fermentation.

Some VOCs, such as ethyl acetate, acetic acid, and esters, characterized pomegranate cider obtained with the autochthonous yeast *H. valbyensis*. This yeast species is indeed reported as capable of generating relatively high amounts of ethyl acetate (Xu *et al.*, 2006), one of the typical VOCs for cider (Medina *et al.*, 2020), conferring fruity aroma (Sumby, Grbin, & Jiranek, 2010). According to Xu *et al.* (2006), during fermentation *Hanseniaspora* spp. produces significant amounts of acetate and esters of organic acids. Esters of organic acids, ethyl esters (Sumby, Grbin, & Jiranek, 2010), methyl esters (El Hadi *et al.*, 2013) of straight chain fatty acids, and acetates are largely responsible for the fruity aroma (Sumby, Grbin, &

Jiranek, 2010). In this study, pomegranate cider obtained with commercial *S. cerevisiae* was mainly characterized by hexanoic acid, associated with sweet odour (Bellincontro *et al.*, 2016), and octanoic acid, associated with rancid odour, when present at relatively high concentration (Lukić *et al.*, 2008). After maturation, pomegranate ciders obtained with *H. valbyensis* showed a more complex VOCs profile, compared to *S. cerevisiae*. Overall, when clarification was obtained by filtration, the VOCs profiles of the corresponding ciders became simpler than those observed for centrifuged or untreated ciders. Previously, maturation of cider, without clarification, was reported as an important step to reach the typical flavour of this fermented beverage (Madrera, Lobo, & Alonso, 2010).

During fermentation, we found a slight increase of lightness (L index), which could be attributed to a decrease in the concentration of monomeric anthocyanins, as reported in Kwaw *et al.* (2018). On the opposite, lightness tended to decrease after maturation. Colour parameters, including L index, can be influenced by several factors during cider maturation, including an eventual clarification treatment, maturation conditions, and chemical reactions involving pigmented molecules (Fan *et al.*, 2019; Li *et al.*, 2020; Zhang *et al.*, 2020). The panel test carried out only for centrifuged pomegranate ciders did not result in significant differences for any of the descriptors used. However, in agreement with the results of VOCs profiles, panellists generally perceived ciders obtained through *H. valbyensis* as more complex in terms of odour and taste, with respect to those obtained through *S. cerevisiae*.

Overall, the antioxidant activity of pomegranate ciders was always high and not affected by fermentation, type of starter, and maturation. During fermentation of pomegranate juice, the antioxidant activity can increase, remain unchanged or even decrease (Gumienna, Szwengiel, & Górna, 2016). In a recent study, we found that fermentation of pomegranate juices at 30 °C for 24 h, using *H. valbyensis*, caused a decrease of antioxidant activity, probably caused by an acetification process (Limongelli, Minervini, & Calasso, 2023).

4.5 Conclusions

In conclusion, we showed the differences, in terms of fermentation performance and quality of pomegranate cider, resulting from the use of autochthonous non-conventional *H. valbyensis* as starter, compared to a commercial strain of *S. cerevisiae*. Some results obtained during this research, such as variations in mineral elements concentration and VOCs profile, as affected by the type of starter and maturation, would need further investigation to be understood more in depth. Although it is not currently possible to use *H. valbyensis* as a starter for fermented beverages, because it lacks the “Qualified Presumption of Safety/Generally Recognized As Safe” labels, this research paves the way for better understanding the fermentation capability and impact of this non-conventional yeast on food quality.

4.6 References

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Enhancing nutritional and sensory properties of plant-based beverages: a study on chickpea and Kamut® flours fermentation using *Lactococcus lactis*

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This chapter represents an extended version of that published in *Frontiers in Nutrition*, 2024 (11, 1269154)

5.1 Introduction

The European market share of plant-based food and beverage is growing exponentially with projections indicating a Compounded Average Growth Rate (CAGR) of 9–10% over the next 5–7 years (Mordor Intelligence, 2022). This surge is attributed to the increased consumers interest regarding the environmental impact of food production, the health implications of dietary choices and the significant venture capital investment in plant-based alternatives to animal-derived products (dairy products, meat, etc....) (Mefleh *et al.*, 2023). In particular, the consumers request for plant-based beverages (PBB) continues to move upward (Gorman *et al.*, 2021) with an anticipated annual market growth rate of 12.7% from 2022 to 2030 (Grand View research, 2022).

Despite the growing interest, consumers' concerns reside in: (i) the poor nutritional profile (especially protein content) of PBB when compared with the conventional products (milk and fermented dairy beverages) and; (ii) the generally long list of added ingredients, employed to improve texture and flavor of the end-product (Boukid *et al.*, 2023, Mefleh *et al.*, 2022d). Traditional and familiar plants used as protein sources, such as cereals, legumes, pseudocereals, and nuts, are still preferred by consumers over novel protein sources (e.g., fungi, insects, and algae) (Mefleh *et al.*, 2023). The cultivation of old wheat varieties and legumes is gaining traction for their environmental sustainability and adaptability to marginal areas (Calles, 2016). Notably, old durum wheats predominantly grown in the Mediterranean region are recognized for their higher protein content compared to many other wheat varieties (Mefleh, Boukid, & Fadda, 2022a; Mefleh *et al.*, 2021; Mefleh *et al.*, 2020; Bonke, Sieuwerts, & Petersen, 2020). Additionally, among the

traditional plant sources, legumes confer the highest protein content to PBB, but they are deficient in certain sulfur amino acids (e.g., methionine and cysteine) which can be supplemented by cereal proteins (Bonke, Sieuwerts, & Petersen, 2020).

However, legumes and other plant sources contain antinutritional factors (ANF) and off-flavors. Some ANFs, such as phytic acids and raffinose, are thermostable but could be reduced significantly through biotechnological processes, including fermentation. This process has been showed to lower the ANF content, enhancing protein digestibility, mineral bioavailability, and overall sensory properties of the plant-based food and beverages (Adebo *et al.*, 2022; Chaturvedi & Chakraborty, 2022; Pontonio *et al.*, 2020). The main microorganisms used in fermentation are usually from the *Lactobacillales* order, commonly referred to as lactic acid bacteria (Cichońska & Ziarno, 2021). Specifically, *Lactococcus lactis* is one of the main microorganisms used in fermented dairy products and is available as both commercial starter and a component of natural starters (Kelly, Ward, & Leahy, 2010; Mills, Ross, & Coffey, 2011).

Lactococcus lactis strains have been isolated from a variety of environmental sources (e.g., raw milk and many vegetables and fruits) (Alemayehu *et al.*, 2014; Mefleh *et al.*, 2022b). However, it is believed that the industrial strains used in dairy productions originally inhabited plants and have been subsequently adapted to milk over time (Kelly, Ward, & Leahy, 2010; Cavanagh, Fitzgerald, & McAuliffe, 2015). Plant-derived lactococci have showed efficacy in improving texture, flavor, and other sensory properties, thanks to their ability to produce many desirable compounds, in both dairy and plant-based products (Mefleh *et al.*, 2022c; Wels *et al.*, 2019).

In alignment with the United Nations' Sustainable Development Goal 2 ("Zero hunger") for 2030, the present study aimed to set up a protocol of production and characterize a sustainable and clean-label PBB. This beverage, created by blending chickpea and the old wheat *Triticum turanicum* (Kamut®) flours and fermented using a commercial *L. lactis* starter, was evaluated from nutritional, microbiological, textural, shelf-life, and sensory perspectives.

5.2 Materials and methods

5.2.1 Materials

Chickpeas (*Cicer arietinum*) flour was provided by Molini Bongiovanni S.P.A (Cambiano, Torino, Italy), whereas Kamut® (*Triticum turgidum* subsp. *uranicum*, cultivar Khorasan) flour was provided by Fior di Loto (Bentivoglio, Bologna, Italy). *Lactococcus lactis* ssp. *lactis* (Lyofast VMO 01) was purchased from the company Sacco S.r.l (Cadorago, Italy). Organic dark chocolate powder (Alce Nero) and table sugar was purchased from a local market. Culture media and supplements for microbiological analyses were purchased from Oxoid (Dublin, Ireland).

5.2.2 Protocol of preparation of the fermented PBB

Tap drinkable water was slowly added (ratio 4:1) to the chickpeas flour while pre-heating and slowly mixing. The slurry (1) was heated for 30 min at 70°C (73°C being the temperature of gelatinization as observed in preliminary rheological analysis) while stirring continuously. Likewise, water was slowly added (ratio 4:1) to the Kamut® flour. The slurry (2) was heated for 30 min at 60°C (62°C being the temperature of gelatinization as observed in preliminary rheological analysis) while stirring continuously. The temperatures for heating (70 and 60°C, respectively) had been preliminarily chosen based on the desired consistency of the slurries, namely a creamy texture similar to that of a conventional plain yogurt. The two slurries (1 and 2) were then mixed (in the ratio 1:1), stirred and homogenized through pulsed ultrasounds treatment (Sonoplus HD 3200, Bandelin, Berlin, Germany) (Guimarães *et al.*, 2018). In detail, aliquots of 80 mL of mixed slurries were placed in containers of 100 mL and treated with the probe (KE 76, Type 3200 UW 24 kHz) with nominal power ultrasound of 100 W, a constant amplitude of 100%, maximum temperature set at 25°C, and with pulsed sonication (pulse duration of on-time: 10 s; pulse duration off-time: 5 s), for a total treatment time of 10 min. The probe, with a tip of 2 mm in diameter, was immersed in the slurry (direct sonication). During treatment, the slurry was held in ice bath to prevent any rise in temperature.

Afterward, the homogenized slurry was divided into two aliquots: a control (CTRL), not inoculated, and a thesis (LL-PBB) that was inoculated (0.05%, w/vol, corresponding to ca. 7 log CFU/mL) with *L. lactis* (Mefleh *et al.*, 2022c). Both these

were incubated at 30°C for 16 h and then stored at 4°C. Analyses were carried out at the beginning (T_0) and end (T_1) of fermentation. In addition, LL-PBB was analyzed after 10 (T_{10}) and 40 (T_{40}) days of storage at 4°C.

5.2.3 Chemical analyses

Moisture content was determined using a thermal balance (MAC 110/NP, Radwag, Radom, Poland) and water activity (a_w) was determined with Aqua Lab 4TE (Meter Group Inc., Pullman, WA, United States). The lipid content was determined by the Soxhlet method (AACC International, 2000c). Saturated fat content was determined in agreement with EEC 2568/91 (Regulation EEC 2568/91). The ash content was determined following AACC method 08-0.1.01 (AACC International, 2000b). Protein content ($N \times 6.00$) was determined by the Kjeldhal method (AACC International, 2000e). Essential amino acids content was determined after acid hydrolysis with 6 N HCl for 24 h at 110°C, as described by White, Hart, & Fry (1986) and using the Water Pico-tag system. Calories from proteins were calculated using the Atwater conversion factors and expressed in kilocalories (Merill & Watt, 1973). For determination of mono- and di-saccharides (fructose, galactose, glucose, lactose, maltose, and sucrose), samples were analyzed by high-performance liquid chromatography coupled with refractive index detector on an Agilent apparatus (Agilent, Santa Clara, CA, United States) equipped with a Spherisorb Amino (NH_2) column 80 Å, 5 mm, 4.6 mm $\times$ 250 mm (Waters, Milford, MA, United States). The separation was done under isocratic conditions as reported by Trani *et al.* (2017), using acetonitrile–water (70, 30, v/v) as mobile phase at a constant flow rate (1.8 mL min⁻¹). In addition, total carbohydrates were determined by difference, based on the results of moisture, lipid, protein, and ash, as follows:

$$\text{Total carbohydrates (\%)} = 100 - (\text{moisture} + \text{lipid} + \text{protein} + \text{ash})$$

Total dietary fiber was determined following the AACC method 32-05.01 (ACC International, 2000d). Minerals contents (phosphorus, sodium, iron, nickel, calcium, magnesium, manganese, potassium, and selenium) were analyzed as described by Mefleh *et al.* (2022d).

pH was evaluated by means of a pH meter (Edge HI2002, Hanna Instruments, Columbus, OH, United States). Total titratable acidity (TTA) was measured according to the AACC 02-31.01 method (AACC International, 2000a). Briefly, 1 mL of PBB was mixed with 9 mL of distilled water and 3–5 drops of phenolphthalein

0.1% (w/v). The titration was carried out with a solution of NaOH (0.1 N) and TTA% was calculated by converting the volume (V) of NaOH (0.1 N) using the following equation:

$$\text{TTA (\%)} = (V_{\text{NaOH}} \times 0.1 \text{ N} \times 0.009 \times 10) \times 100$$

wherein V_{NaOH} was the volume of NaOH in mL used for titration, 0.009 was the correction factor of NaOH, and 10 was the dilution factor.

Phytic acids and raffinose were determined using the kit K-PHYT 05/07 and Raffinose/D-Galactose Assay, respectively, following the manufacturer's (Megazyme Ltd., Bray, Ireland) instructions.

5.2.4 Culture-dependent analysis of microbiota

Microbiological analyses were carried out on 1 mL of PBB, after serial dilutions in peptone water solution (8.5 g/L NaCl and 1 g/L peptone). Dilutions were plated using the following agar media and incubation conditions: Plate Count (PCA) (30 °C, 48 h, for total mesophilic aerobic microorganisms), de Man Rogosa Sharpe (MRS) agar with cycloheximide (0.01% w/v) (30 °C, 48 h, for mesophilic rod-shaped lactic acid bacteria, LAB), M17 agar supplemented with glucose solution (10% w/v) and cycloheximide (0.01%) (30 °C, 48 h, for mesophilic coccus-shaped LAB), Violet Red Bile Glucose (VRBGA) (37 °C, 24 h, for *Enterobacteriaceae*), Baird Parker supplemented with egg yolk-tellurite emulsion (37 °C, 48 h, for staphylococci), Slanetz and Bartley (37 °C, 48 h, for enterococci), and Sabouraud Dextrose agar (SDA) (30 °C, 48 h, for molds and yeasts). For the enumeration of LAB, *Enterobacteriaceae*, molds and yeasts, pour plate technique was performed; instead, spread plate technique was carried out for the enumeration of staphylococci and enterococci. Cell densities were averaged and expressed as Log Colony Forming Units (CFU)/g of beverage.

5.2.5 Culture-independent analysis of bacterial biota

Total DNA was extracted from PBBs after spontaneous (CTRL) or driven (LL) fermentation, as well as from LL-PBB after 10 and 40 days of storage. Before extraction, 10 g of PBBs were homogenized with 90 mL of 0.1 mol/L sodium phosphate buffer (pH 7), through a lab blender mixer (Bag Mixer 400 P, Interscience International, France) for 3 min and centrifuged at $200 \times g$, for 5 min, at 4°C. Supernatant was collected and centrifuged at $20,000 \times g$, for 15 min, at 4°C; then supernatants were discarded and pellets used for DNA extraction.

DNA was extracted starting from 500 mg of pellet, using the FastDNA Spin Kit for Soil (MP Biomedicals, Illkrich, France), according to the manufacturer's instructions (Lattanzi *et al.*, 2013).

Next generation sequencing experiments, including quality control and primary bioinformatics analysis, were performed by Genomix4life S.R.L. (Baronissi, Salerno, Italy). DNA quality control was carried out by using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, United States) and Qubit Fluorometer 1.0 (Invitrogen Co., Carlsbad, CA, United States). DNA amplification was performed with the following primers: 5'-AGA GTT TGA TYM TGG CTC AG-3' (Forward) and 5'-ATT ACC GCG GCT GCT GG -3' (Reverse) (Walker *et al.*, 2015), which target the hypervariable V₁ and V₃ region of the 16S rRNA gene. Each PCR reaction was assembled according to Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA, United States). A negative control is included in the workflow, consisting of all reagents used during sample processing (16S amplification and library preparation) but not containing the sample, to exclude contamination. Libraries were quantified using Qubit fluorometer (Invitrogen Co., Carlsbad, CA, United States) and pooled to an equimolar amount of each index-tagged sample to a final concentration of 4 nM, including the Phix Control Library. Pooled samples were subjected to cluster generation and sequenced on MiSeq platform (Illumina, San Diego, CA, United States) in a 2 × 300 paired-end format. The generated raw sequence files (fast files) underwent quality control analysis by means of FastQC tool.

Unidentified, contaminant (plastidic and mitochondrial) and singletons OTUs were eliminated. The bioinformatic taxonomic classification of 16S rRNA targeted amplicon reads was carried out through a high-performance implementation of the Ribosomal Database Project (RDP) Classifier, described in Wang *et al.* (2007). The Chao₁ indices were calculated using the open-source metagenomics RAST server (Meyer *et al.*, 2008).

The 16S rRNA gene sequences are available in the Sequence Read Archive of NCBI (accession number PRJNA1010704).

5.2.6 Rheological analysis and color measurements

Plant-based beverages were gently stirred five times prior to rheological analyses that were carried out on PBBs maintained at 4 °C. Flow curves were obtained using Mars iQ Air HAAKE, molecular advance rheometer system fitted with a Couette

measuring geometry with a diameter of 25 mm. The shear rate varied from 0.00185 to 116 s⁻¹ (Hassan *et al.*, 2003) and shear stress was registered at increasing shear rate. Continuous shear was applied with a delay time of 5 s between measurements at a given shear rate. Strain oscillation frequency sweep was also performed. Frequencies ranged from 0.01 to 9 Hz and the applied strain was 0.00412, which fell within the linear viscoelastic region previously determined by running a strain sweep test (Hassan *et al.*, 2003). The elastic and viscous modulus (G' and G'') and loss tangent ($\tan \delta$) were registered as function of frequency. All results were processed using HAAKE RheoWin data manager, an internal software. Instrumental color evaluations of lightness (L^*), green-red (a^*), and blue-yellow (b^*) were performed using a CM-600d Konica Minolta (Chiyoda, Tokyo, Japan) colorimeter.

5.2.7 Consumer test

Forty-seven people, aged between 18 and 64 years, were randomly selected and asked to participate in the test, performed at the E. Quagliariello Campus at the University of Bari, Italy. Participants were asked to fill in a questionnaire and to try two products: (1) plain LL-PBB, with no added ingredients and (2) sweet LL-PBB, with added chocolate paste. The latter was prepared as follow: 17% of sugar and 8% of dark chocolate powder were added to the LL-PBB, and mixed until well homogenized. The two products were prepared at the plant of Vallefiorita (Ostuni, BR, Brindisi). Participants were given information about the study aims and the products to try. All participants acknowledged an informed consent statement in order to participate in the test. 22 participants were BSc students, 12 were PhD students, and 13 were researchers/professors. Before tasting, participants had to respond to four questions (Figure 1) in order to understand better their dietary choices. Samples of the two products were served chilled at 5°C in small plastic cups. Participants assessed acidity, viscosity, color, and aroma of the products, based on nine-point hedonic face scale (Gámbaro & McSweeney, 2020) where 1 is “terrible,” 2 is “very bad,” 3 is “bad,” 4 is “just a little bad,” 5 is “maybe good or maybe bad,” 6 is “just a little good,” 7 is “good,” 8 is “very good,” and 9 is “great.” Subsequently, participants were asked to express, on a 1–9 range scale, their overall acceptability and interest to buy the product. They were asked to start with the plain LL-PBB and then to taste the sweet LL-PBB.

No human ethics committee or formal documentation process is available. We confirm that appropriate protocols for protecting the rights and privacy of all participants were utilized during the consumer test, a verbal consent of participants was obtained, and participants could withdraw from the study at any time.

Questions	Answers
What type of diet do you follow?	a- Mediterranean b- Vegan c- Vegetarian d- Flexitarian e- Other
Do you consider buying “healthy” product when doing the grocery?	a- Always b- Frequently c- Rarely d- Never
Do you consider buying “vegan” product?	a- Always b- Frequently c- Rarely d- Never
How do you rate your legumes consumption?	a- Once per month b- Twice a month c- Once per week d- Twice or more per week

Figure 1. Questionnaire given to the participants of the consumer test.

5.2.8 Statistical analysis

All the data were collected from two replicate analyses carried out on three different batches of PBBs. Data were subjected to one-way ANOVA, followed by Tukey’s HSD test at $\alpha = 0.05$, using the R software (R Core Team, 2014). Data from culture-dependent microbiological analyses were subjected to the Student’s T-test at $p = 0.05$.

5.3 Results

5.3.1 Nutritional composition of plant-based beverages

Fermentation of PBB with *L. lactis* (LL-PBB) led to lower ($p < 0.05$) pH, moisture, and a_w , and to higher ($p < 0.05$) TTA than the spontaneously fermented PBB (CTRL) (Table 1). At day 10, pH and a_w of LL-PBB did not significantly vary ($p > 0.05$), whereas TTA decreased ($p < 0.05$) and moisture increased ($p < 0.05$). At day 40, pH, TTA, and moisture did not significantly vary ($p > 0.05$), whereas a_w decreased ($p < 0.05$) (Table 1).

Table 1. Moisture, water activity (a_w), total titratable acidity (TTA), and pH of CTRL (spontaneous fermentation) and LL plant-based beverage (PBB) after 16 h of fermentation (T1), and after 10 (T10-LL) and 40 (T40-LL) days of storage at 4 °C. Values (mean $\pm$ standard deviation) in the same row flanked by different letters (a-c) are significantly different ($p \leq 0.05$) based on Tukey's test.

	T1-CTRL	T1-LL	T10-LL	T40-LL
Moisture (g/100 g)	78.8 $\pm$ 0.33b	76.0 $\pm$ 0.14c	79.8 $\pm$ 3.76a	79.1 $\pm$ 0.19ab
a_w	0.995 $\pm$ 0.00a	0.990 $\pm$ 0.00b	0.994 $\pm$ 0.00ab	0.984 $\pm$ 0.00c
TTA (%)	0.540 $\pm$ 0.00c	0.925 $\pm$ 0.035a	0.700 $\pm$ 0.03b	0.815 $\pm$ 0.06ab
pH	5.14 $\pm$ 0.02a	4.42 $\pm$ 0.02b	4.38 $\pm$ 0.03b	4.39 $\pm$ 0.02b

Maltose was the highest sugar among mono- and di-saccharides found in both PBBs (CTRL and LL-PBB). Fructose, galactose, and glucose were found at significantly ($p < 0.05$) lower concentrations in the LL-PBB than in the CTRL-PBB (Table 2). No differences ($p > 0.05$) were found for lipids, saturated fat, proteins, fiber, total carbohydrates and all the minerals, except for phosphorous and calcium, which were found at higher ($p < 0.05$) concentrations in the LL-PBB than in the CTRL-PBB. In addition, LL-PBB showed higher ($p < 0.05$) concentration of ash than CTRL-PBB. Both PBB had 20% of their caloric values from proteins (Table 2).

Table 2. Concentrations of mono-saccharides, di-saccharides, lipids, saturated fat, ash, total carbohydrates, total digestible fiber, proteins, calculated calories from proteins, and concentrations of minerals in CTRL-PBB (spontaneous) and LL-PBB after 16 h of fermentation (T1).

	T1-CTRL	T1-LL
Fructose (g/100g)	0.56±0.00a	0.010±0.00b
Galactose (g/100g)	0.055±0.01a	0.033±0.00b
Glucose (g/100g)	0.100±0.002a	0.040±0.02b
Maltose (g/100g)	4.410±0.00a	4.560±0.10a
Sucrose (g/100g)	0.031±0.00a	0.033±0.00a
Lipids (g/100g)	0.20±0.00a	0.20±0.01a
Saturated fat (g/100g)	0.10±0.00a	0.10±0.00a
Ash (g/100g)	0.52±0.00b	0.83±0.00a
Total carbohydrates (g/100g)	15.0±0.00a	15.3±0.07a
Total digestible fiber (g/100g)	1.99±0.20a	2.12±0.30a
Proteins (g/100g)	3.84±0.01a	3.93±0.02a
Calories from proteins (Kcal)	20±0.00a	20±0.00a
Phosphorus (mg/kg)	331±0.35b	478±0.35a
Calcium (mg/kg)	117±3.54b	165±4.24a
Iron (mg/kg)	9.20±0.17a	9.35±0.12a
Nickel (mg/kg)	0.31±0.00a	0.30±0.00a
Magnesium (mg/kg)	203±10.2a	208±7.30a
Manganese (mg/kg)	2.65±0.54a	2.70±0.74a
Potassium (mg/kg)	1,661±40.3a	1,682±19.6a

Values (mean±standard deviation) in the same row flanked by different letters (a-b) are significantly different ($p < 0.05$) based on Tukey's test.

The essential amino acids (histidine, isoleucine, leucine, lysine, methionine plus cystine, phenylalanine plus tyrosine, threonine, and valine) were quantified in the LL-PBB and to have a score higher than 1, except, for the limiting amino acids lysine (0.64) and methionine plus cysteine (0.32) (Table 3).

Before fermentation, concentrations of phytic acid and raffinose in the PBBs were 0.19 and 5.72 g/100 g, respectively. At the end of fermentation, both PBBs contained lower ($p < 0.05$) concentrations of both antinutritional compounds. In detail, concentration of phytic acid in the CTRL-PBB was 0.14/100 g, not significantly different ($p > 0.05$) from the value found in the LL-PBB (0.13/100 g). On the other hand, LL-PBB contained a lower concentration ($p < 0.05$) of raffinose (5.03/100 g) than the CTRL-PBB (5.51/100 g).

Table 3. Concentrations of essential amino acids contents in mg g⁻¹ of proteins in the PBB after fermentation with *Lactococcus lactis* (T1-LL), the essential amino acids requirements in mg g⁻¹ Proteins for adults (FAO/WHO/UNU, 1991) and the calculated amino acids score. Tryptophane was not analyzed.

	T1-LL	Reference (mg/g protein) - older child, adolescent, adult	Amino Acids Score
Histidine	37.66	16	2.35
Isoleucine	37.66	30	1.26
Leucine	75.32	61	1.23
Lysine	30.79	48	0.64
Methionineplus cystine	7.38	23	0.32
Phenylalanineplus tyrosine	97.71	41	2.38
Threonine	30.28	25	1.21
Valine	45.80	40	1.15

5.3.2 Culturable microbiota of PBBs

Before fermentation (T₀), culturable microbiota of the CTRL-PBB was largely dominated by presumptive LAB, whereas presumptive staphylococci were found at low cell density (Table 4). However, *ca.* 90% of the colonies grown on the MRS and glucose-M17 plates inoculated with CTRL-PBB diluted at 10⁻⁵ were catalase-positive (data not shown), suggesting that (strictly or facultatively) aerobic bacteria grew on media that are elective for LAB. After spontaneous fermentation (T₁), all the microbial groups, included undesirable *Enterobacteriaceae* and staphylococci, increased ($p < 0.05$). Yeasts were not found at detectable levels both before and after fermentation. Therefore, based on the cell densities of undesired bacteria, the spontaneously fermented beverage (CTRL-PBB) was not stored for 40 days at 4 °C, unlike LL-PBB.

Table 4. Cell densities (log CFU/g) of different microbial groups in the formulated beverage before (T₀) and after (T₁) 16 h of spontaneous fermentation.

Microbial group	T ₀	T ₁
Total mesophilic aerobic microorganisms	7.0±0.0b	9.0±0.1a
Mesophilic rod-shaped lactic acid bacteria	7.2±0.2a	7.6±0.1a
Mesophilic coccus-shaped lactic acid bacteria	7.1±0.0b	8.0±0.0a
Enterococci	<1	3.0±0.0a
<i>Enterobacteriaceae</i>	<1	7.1±0.1a
Presumptive staphylococci	1.3±0.0b	4.8±0.0a
Presumptive yeasts	<1	<1

Values were the average (± standard deviation) of three biological replicates analyzed in duplicate. Mean values in the same row flanked by different letters (a-b) are significantly different ($p < 0.05$) based on Student's T-test

Before fermentation of PBB with *L. lactis* (LL-PBB, T₀), the culturable microbiota was dominated by LAB, as expected, whereas presumptive staphylococci were found at much lower cell density (Table 5). After fermentation (T₁), cell densities of LAB and mesophilic aerobic microorganisms increased ($p < 0.05$) by *ca.* two log cycles, whereas all the other microbial groups, including staphylococci, were not detectable. After 10 days of storage at 4 °C (LL-PBB, T₁₀), cell densities of presumptive coccus-shaped LAB and mesophilic aerobic microorganisms did not vary ($p > 0.05$), whereas the number of presumptive rod-shaped LAB decreased ($p < 0.05$). No enterococci, enterobacteria, staphylococci, and yeasts were found (Table 5). After 40 days of storage (LL-PBB, T₄₀), rod-shaped LAB further decreased ($p < 0.05$) to 5.7 log CFU/g; coccus-shaped LAB and mesophilic aerobic microorganisms also decreased ($p < 0.05$), reaching an order of magnitude of 5 log CFU/g. At this time-point of shelf-life, enterococci and enterobacteria were detected as subdominant bacterial populations, whereas staphylococci and yeasts were still not detectable (Table 5).

Table 5. Cell densities (log CFU/g) of different microbial groups in the formulated beverage before (T₀), after 16 h of driven fermentation (T₁), and during 10 (T₁₀) and 40 (T₄₀) days of storage at 4 °C.

Microbial group	T ₀	T ₁	T ₁₀	T ₄₀
Total mesophilic aerobic microorganisms	7.3±0.0b	9.1±0.1a	9.0±0.1a	5.5±0.1c
Presumptive mesophilic rod-shaped lactic acid bacteria	7.3±0.0c	9.1±0.0a	8.4±0.1b	5.2±0.5d
Presumptive mesophilic coccus-shaped lactic acid bacteria	7.3±0.0b	9.0±0.1a	8.9±0.0a	5.7±0.1c
Presumptive enterococci	<1	<1	<1	2.9±0.0a
Presumptive Enterobacteriaceae	<1	<1	<1	2.0±0.0a
Presumptive staphylococci	1.9±0.0a	<1	<1	<1
Presumptive yeasts	<1	<1	<1	<1

Values were the average (± standard deviation) of three biological replicates analyzed in duplicate. Mean values in the same row flanked by different letters (a-d) are significantly different ($p < 0.05$) based on Student's T-test

5.3.3 Bacterial biota of PBBs described through culture-independent analysis

The metagenetic analysis of the two fermented PBBs (i.e., CTRL and LL) through 16S allowed to observe a higher species richness (number of identified species and values of Chao1 and Shannon index) in CTRL-PBB (spontaneously fermented), compared to LL-PBB (fermented by *L. lactis*) (Table 6). CTRL-PBB microbiota was dominated by *Bacillus* sp. and *Alphaproteobacteria* (Class), which were also found, at lower percentage of relative abundance in LL-PBB (Table 7). LL-PBB microbiota was largely dominated by *L. lactis* which, however, was detected as sub-dominant OTU also in CTRL-PBB. Moreover, the number of identified species and values of Chao1index tended to increase during shelf-life of LL-PBB (Table 6). However, during 40 days of shelf-life, the relative abundances of bacterial OTUs found in the LL-PBB did not vary with respect to day 1 (Figure 2).

Table 6. Alpha diversity indexes of *Bacteria* (16S rRNA) found in plant-based beverage (PBB) after 16 h of spontaneous (T1-CTRL) and driven (T1-LL) fermentation, and in PBB-LL after ten (T10) and forty (T40) days of storage at 4 °C. a and b indicate two technical replicates.

Sample	Number of reads	Number of identified species	Chao1	Shannon
T1-CTRL_a	193,838	386	1167	1.011
T1-CTRL_b	193,813	412	824	1.046
T1-LL_a	132,488	26	37	0.636
T1-LL_b	131,176	34	40	0.646
T10-LL_a	149,545	30	44	0.650
T10-LL_b	152,303	43	34	0.614
T40-LL_a	148,074	63	46	0.645
T40-LL_b	155,912	43	46	0.636

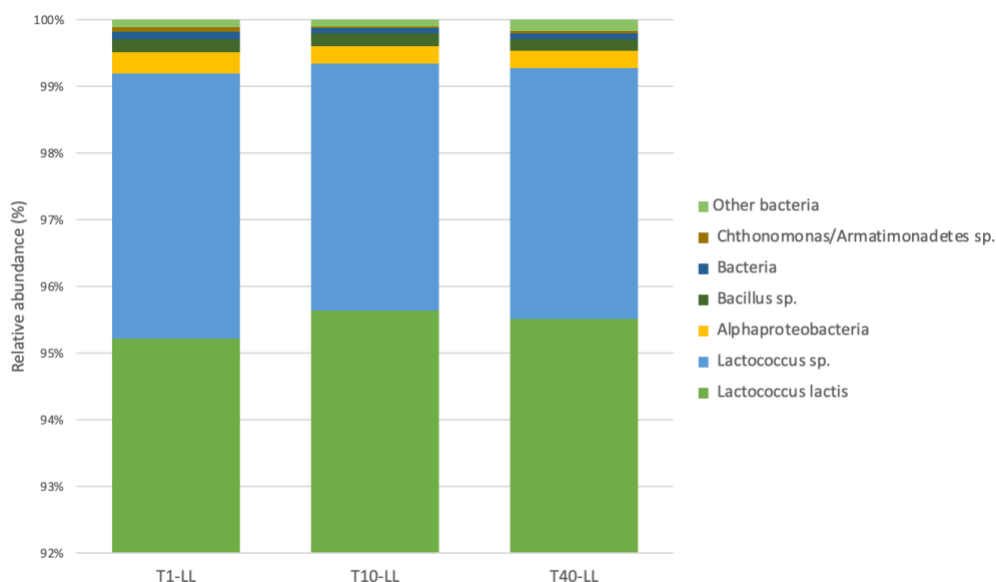


Figure 2. Relative abundance (%) of bacterial OTUs found in plant-based beverage after 16 h of driven (T1-LL) fermentation, and after ten (T10-LL) and forty (T40-LL) days of storage at 4 °C.

Table 7. Relative abundance (%) (mean of two replicates $\pm$ standard deviation) of bacterial OTUs, with number of reads ranged from ca. 131,000 to ca. 194,000 per sample, found in plant-based beverages after 16 h of spontaneous (T1-CTRL) and driven (T1-LL) fermentations.

Bacterial OTUs	T1-CTRL	T1-LL
<i>Bacteria</i> (Kingdom)	6.18 $\pm$ 0.44	0.12 $\pm$ 0.03
<i>Chthonomonas/Armati</i>	4.28 $\pm$ 0.08	<0.1
<i>monadetes</i> sp.	0.16 $\pm$ 0.01	*
<i>Bacillales</i> (Order)		-
<i>Bacillus</i> sp.	45.62 $\pm$ 3.14	0.19 $\pm$ 0.03
<i>Alkalilactibacillus</i>	0.32 $\pm$ 0.01	<0.1
<i>ikkensis</i>		
<i>Lactobacillus</i> sp.	0.71 $\pm$ 0.00	<0.1
<i>Lactococcus</i> sp.	0.60 $\pm$ 0.16	3.97 $\pm$ 0.41
<i>Lactococcus lactis</i>	3.28 $\pm$ 2.36	95.22 $\pm$ 0.54
<i>Proteobacteria</i>	0.90 $\pm$ 0.09	-
(Phylum)		
<i>Alphaproteobacteria</i>	33.70 $\pm$ 1.66	0.32 $\pm$ 0.04
(Class)		
<i>Maricaulis</i> sp.	0.15 $\pm$ 0.00	-
<i>Pleomorphobacterium</i>	0.85 $\pm$ 0.01	<0.1
<i>xiamenense</i>		
<i>Ralstonia</i>	0.16 $\pm$ 0.02	-
<i>solanacearum</i>		
<i>Neisseria</i> sp.	0.27 $\pm$ 0.03	-
Other bacteria**	3.16 $\pm$ 0.01	0.18 $\pm$ 0.03

*OTU not found

**Sum of relative abundances of those bacterial OTUs found at less than 0.1%

5.3.4 Texture and color measurements

Plant-based beverage fermented with *L. lactis* was characterized by significantly ($p < 0.05$) higher values for all the viscosity parameters (except for the loss tangent, ratio G''/G' , which was not significantly different), compared to CTRL-PBB (Table 8). During storage of LL-PBB, viscosity decreased by 30% after 10 days and by 45% after 40 days. Both G' and G'' decreased ($p < 0.05$) after 10 days and then, after 40 days, they did not vary ($p > 0.05$).

Table 8. Rheological measurements and color parameters of CTRL-PBB (spontaneous) and LL-PBB after 16 h of fermentation (T₁), and after 10 (T₁₀-LL) and 40 (T₄₀-LL) days of storage at 4°C.

	CTRL	LL - D ₁	LL - D ₁₀	LL - D ₄₀
Viscosity Pas	0.231±0.00d	0.473±0.00a	0.344±0.00b	0.260±0.00c
Shear Pa	26.6±0.36d	54.5±0.48a	39.7±1.10b	30.0±0.33c
G' Pa	109±10b	265±68a	85.8±2.92ab	113±5.78b
G'' Pa	31.5±4.20b	80.6±7.76a	22.0±0.91b	26.9±0.48b
G''/G'	0.29±0.07a	0.31±0.05a	0.26±0.02a	0.23±0.02a
L*	58.6±0.04c	74.3±0.05a	74.5±0.08a	70.7±0.01b
a*	0.65±0.28a	0.39±0.17b	0.31±0.06b	0.28±0.03b
b*	16.1±0.32b	19.2±0.39a	19.3±0.11a	16.7±0.11b

Mean values in the same row flanked by different letters (a-d) are significantly different ($p < 0.05$) based on Tukey's test. L*: lightness, a*:red/green; b*:yellow/blue.

Regarding the color indexes, LL-PBB showed significantly ($p < 0.05$) higher values of L* (lightness) and b* (yellow), and lower value of a* (red) compared to CTRL-PBB (Table 8). The differences in terms of color indexes between the *L. lactis* fermented and the spontaneously fermented PBBs could be clearly perceived in the Figure 3. After 10 days of storage of LL-PBB, no significant ($p > 0.05$) differences were found in the values of color indexes, with respect to the end of fermentation, whereas after 40 days L* and b* significantly ($p < 0.05$) decreased.



Figure 3. Appearance of PBBs after 16 h of driven (*L. lactis*, A) or spontaneous (CTRL, B) fermentation.

3.3.5 Consumer test

Participants who claimed to follow a vegan and flexitarian diets were 2 and 6%, respectively. 53% of participants reported to rarely buy vegan food and 32% reported to never buy them. 90% of participants expressed their interest in buying

healthy food (data not shown). More than 50% of participants rated positively (score ≥ 6) the viscosity and color of the plain LL-PBB, but only 38% of participants gave positive scores to acidity. When participants tasted the sweet (added with chocolate paste) LL-PBB, all the attributes were rated positively by at least 62% of participants. In detail, the percentages almost doubled for acidity and flavor, with respect to those observed for the plain LL-PBB (Table 9).

Table 9. Percentage (%) of participants that rated from 6 to 9 (where 6 is “just a little good”, 7 is “good”, 8 is “very good” and 9 is “great”) the acidity, viscosity, color and flavor of the PBB fermented by *Lactococcus lactis* tasted in its plain (PBB-LL) and sweet (Sweet PBB-LL) version.

	PBB-LL (%)	Sweet PBB-LL (%)
Acidity	38	62
Viscosity	52	70
Color	86	100
Flavor	44	82

Acceptability of the plain LL-PBB was rated positively by 46% of participants (Figure 4A); the percentage increased to 88% for the sweet LL-PBB (Figure 4C). 58% of the participants expressed good interest in buying the plain LL-PBB (Figure 4B), and this percentage increased to 78% for the sweet LL-PBB (Figure 4D).

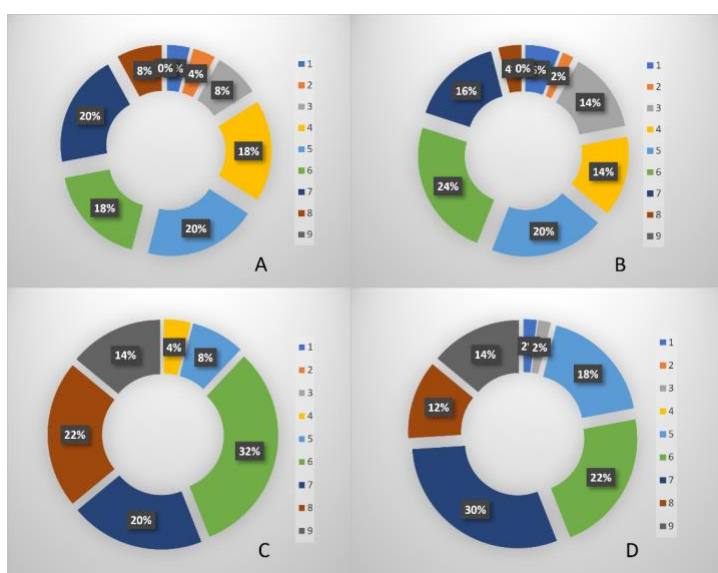


Figure 4. Pie charts describing, on a scale from 1 to 9, acceptability for the plain PBB (panel A) and the sweet PBB (panel C), and interest in buying the plain PBB (panel B) and the sweet PBB (panel D).

5.4 Discussion

The use of the commercial starter *L. lactis* ssp. *lactis* VMO 01 in this study markedly influenced various parameters, notably acidification, water activity, nutrients concentrations, and raffinose levels, compared to the spontaneously fermented beverage. The pH levels observed in the LL-PBB align with those reported in other legumes-based fermented beverages (Mefleh *et al.*, 2022c, Boeck *et al.*, 2022). Distinctively, LL-PBB demonstrated a lower pH and higher total titratable acidity (TTA) compared to the control (CTRL-PBB). This was evidenced by the reduced residual concentrations of monosaccharides, likely consumed more extensively via lactic acid fermentation by the lactococcal starter. The resultant higher acidity was found to be pleasantly balanced when chocolate paste was added, enhancing the overall taste profile. The lower moisture found in the LL-PBB is consistent with a study on soymilk fermentation (Obadina *et al.*, 2013) and could be explained by the increase in dry matter content observed as lactic acid fermentation proceeds. Contrasting with the results reported by Mefleh *et al.* (2022c), the LL-PBB demonstrated higher ash concentrations compared to the CTRL-PBB, paralleling the observed increases in phosphorus and calcium levels. While an initial hypothesis was that the higher concentration of those two minerals in LL-PBB could be attributed to reduced levels of phytic acid, a known antinutritional factor that limits mineral bioavailability (Verni *et al.*, 2022), our results did not indicate significant differences in the reduced phytic acid between the CTRL and the *L. lactis* fermented beverages. This phenomenon might be explained by the activation of endogenous phytases in the flour at the reduced pH levels, as cereal phytases show the highest activity at pH 5.5, but remain active even at lower pH values (Leenhardt *et al.*, 2005). In order to explain the higher concentration of minerals found in the PBB fermented by *L. lactis*, we may propose two hypotheses: (i) the fermentation by *L. lactis* might have released higher amount of lactic acid than spontaneous fermentation, thus improving mineral solubility (Sharma *et al.*, 2020); (ii) minerals might be bound to ANF other than phytic acid, such as tannins and raffinose (Verni *et al.*, 2022). Compared to the CTRL, a lower concentration of raffinose, a potential cause of gastrointestinal disturbances if consumed in a high quantity (> 15 g/day) (Verni *et al.*, 2022), was noted in the LL-PBB. This could be potentially due to the raffinose-degrading capability (through α -galactosidase activity) of *L. lactis* strains (Zartl *et al.*, 2018),

especially in those isolated from vegetables, such as the commercial strain used in this study. Therefore, we may hypothesize that the commercial starter used could have decreased raffinose concentration. In addition to these findings, the LL-PBB displayed several interesting nutritional traits: fat free ($<0.5/100$ g), source of fiber (>1.5 g of fiber/100 Kcal) (REGULATION (EC) no 1924/2006), and a protein content similar to that of a conventional yogurt (minimum of $2.7/100$ g) (Codex Alimentarius, 2011). The formulation, which includes Kamut® and chickpeas, achieved a complete Essential Amino Acid (EAA) profile, meeting 100% of the amino acid requirements as per guidelines of FAO/WHO/UNU (1991), with the exception of methionine + cysteine and lysine. Given that casein, commonly found in dairy products, is also deficient in cysteine and methionine, the *L. lactis*-fermented PBB could be considered as a valuable alternative to fermented dairy beverages. As expected, fermentation of PBB differently drove the microbial community depending on the use of the *L. lactis* commercial starter. In the LL-PBB, LAB were the only microbial population detected, whereas in the CTRL, LAB and other bacterial groups were found, including undesirable *Enterobacteriaceae* and staphylococci at critical values (ca. $5 \log$ cfu/g or higher) of cell density. It is likely that the pH reached in the LL-PBB, lower than 4.5, inhibited the growth of undesired microorganisms. The role of LAB as tools to guarantee safety of food is well known, when used as either starters or protective cultures. In detail, *L. lactis* is used in bio-preservation because some groups of this species synthesize the bacteriocin nisin. In addition, the antibacterial activity of this species could be due to its ability to release organic acids, hydrogen peroxide, and diacetyl (Khelissa *et al.*, 2021). The values of cell density of LAB in the LL-PBB fell in the range found in previous studies on legume-based beverages fermented with LAB (Boeck *et al.*, 2022, Jiménez-Martínez, Hernández-Sánchez, & Dávila-Ortiz, 2003; Zannini *et al.*, 2018). In agreement with the results from culture-dependent analyses, the use of the *L. lactis* commercial starter simplified the bacterial community compared to the CTRL, as shown by the lower values of all the alpha diversity indexes. As shown by 16S metagenetics analysis, the CTRL was dominated by undesirable bacterial groups. Among those groups, the spore-forming *Bacillus* sp., which includes species able to cause food-borne diseases or food spoilage (Crossley, Jordan, & Walker, 2008; Jessberger *et al.*, 2020), represented the most abundant of all the bacterial OTUs detected in the CTRL-

PBB. Probably, the heat treatment (60–70°C for 30 min) carried out on the flours as the first step of the protocol of preparation and the following direct sonication did not inactivate bacterial spores which, in absence of the commercial starter, could have the possibility to germinate and the resulting vegetative cells to multiply (Sperber, 2009). On the other hand, the very high relative abundance of an OTU classified as *L. lactis* in the LL-PBB confirmed the dominance of the starter, as suggested by the results of culture-dependent analysis. It is probable that in the LL-PBB the *L. lactis* strain used as starter inhibited the growth of undesired microorganisms, presumably because of release of lactic acid and other antimicrobial compounds (Durango-Zuleta *et al.*, 2022; Montemurro *et al.*, 2021). In our findings, the *L. lactis*-fermented PBB exhibited a notably improved viscosity compared to the control (CTRL-PBB), a result that aligns with previous research on beverages fermented with LAB (Mefleh *et al.*, 2022c; Looijesteijn *et al.*, 1999). A plausible explanation for this increased viscosity is the synthesis of exopolysaccharides (EPS) by the lactococcal starter (Harper *et al.*, 2022). Indeed, EPS are known for their natural gelling and thickening capacity (Jurášková, Ribeiro, & Silva, 2022; Sørensen *et al.*, 2022). At the end of fermentation, the measurements of the storage modulus (G') and loss modulus (G'') of LL-PBB were comparable to those observed in a yogurt manufactured using EPS-producing strain of *S. thermophilus* (Xu *et al.*, 2021). This similarity underscores the potential of EPS-producing lactococcal strains in enhancing the textural attributes of plant-based fermented beverages, mirroring the effects traditionally achieved in yogurts.

The LL-PBB was subjected to a consumer test to provide insights into consumer preferences and market potential for two variants of PBBs (Gorman *et al.*, 2021). The LL-PBB was presented to consumers in two variants: “plain” (without additional ingredients) and “sweet” (with added chocolate paste). The inclusion of chocolate paste was hypothesized to potentially elevate consumer appeal, especially considering the presence of chickpea flour (approximately 50% of the total flour blend) in the formulation which is known to impart a thermoresistant beany flavor not well appreciated (Mefleh *et al.*, 2022d). Both acidity and viscosity were appreciated in both variants, likely due to the pH reduction and the presumed synthesis of EPS by the lactococcal starter (Pontonio *et al.*, 2020). Notably, the “sweet” LL-PBB variant, with added chocolate paste, showed higher acceptability and purchase interest scores than the “plain” variant. This finding

suggests that the “sweet” LL-PBB could be considered as a valuable and appealing alternative to traditional flavored yogurts, particularly in segments of the market seeking novel plant-based options. Throughout its shelf-life, notable changes were observed in the LL-PBB. Although no post-acidification was observed, LAB populations, especially mesophilic lactobacilli decreased. This observation aligns with previous studies on fermented PBBs reporting a decreased viability of LAB after 30 days of storage at 4°C (Coda *et al.*, 2011; Setyawardani, 2022). It is postulated that a combination of storage at low temperature and low pH environment possibly induced a decline phase in the LAB populations, as supported by findings in similar studies (Setyawardani, 2022, Costa *et al.*, 2013). Additionally, the viscosity of LL-PBB decreased over time. These results are essential for understanding the storage and distribution potential of this product. The change in viscosity could be due to partial hydrolysis of the starch-protein matrix, facilitated by both endogenous and microbial enzymes. Moreover, the acidic conditions prevailing during storage may have also contributed to the hydrolysis of the EPS matrix, further influencing the beverage’s viscosity. Such interactions between microbial activity, enzymatic processes, and storage conditions underscore the dynamic nature of the LL-PBB’s textural properties over its shelf-life.

5.5 Conclusion

This study successfully developed an environmentally friendly, clean-label PBB using flours from chickpea and the old wheat Kamut®, fermented with a commercial *L. lactis* starter. This research enhances our understanding of using *L. lactis*, a traditional dairy fermenter, in PBB production. Notably, fermentation with *L. lactis* improved the nutritional value of the beverage, increasing the phosphorus and calcium concentrations, and reducing the raffinose content. Additionally, the LL-PBB was high in fiber and had a protein content, similar to conventional yogurt, aligning with the rising consumer interest in nutritious and sustainable plant-based foods. After fermentation with *L. lactis*, the beverage mirrored the texture qualities of milk-based fermented foods. However, during its shelf-life, the beverage experienced a decline in LAB populations and in viscosity, underscoring the need for further research to optimize storage conditions and extend the shelf-life, while maintaining desirable sensory and textural properties. We believe that conducting shelf-life analyses at only three intervals over 40 days (on days 1, 10, and 40) was limiting. More frequent assessments would have provided a more comprehensive understanding of the beverage's stability and dynamic changes over time. Sensory evaluation and consumer acceptability and interest in purchasing revealed that LL-PBB with added chocolate paste held market promise as an alternative to traditional flavored yogurts, especially for consumers seeking more plant-based options.

Overall, this study contributes to the growing body of knowledge on plant-based fermented foods and the consumer behavior toward innovative plant-based foods. Future research could focus on extending the shelf-life and exploring the scalability of this production protocol, which may pave the way for the introduction of this innovative PBB into the wider market. Furthermore, a larger and more diverse group of participants in a consumer test could provide more robust insights into consumer preferences and market potential.

5.6 References

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Overall conclusions and perspectives

Conventional food production is no longer sustainable and places significant strain on the environment. With the world's population projected to reach nearly 10 billion by 2050, feeding this growing population while preserving natural ecosystems presents a critical challenge. To address this, drastic changes are necessary, including a shift towards healthy diets, regenerative agricultural practices, and waste valorization. In this context, new or alternative crops and food by-products present promising opportunities for sustainable food production. Furthermore, innovations—understood as new products, processes, or services—are recognized as essential tools for companies in the food industry to better meet consumer needs by producing more sustainable products with enhanced nutritional value. This thesis explored innovative approaches to the development of more sustainable fermented food products, focusing on the use of local matrices such as chickpea, Kamut, pomegranate and its by-products. A key aspect of the studies was the selection of fermentation starters, emphasizing the potential of non-conventional autochthonous microorganisms—isolated from the matrix itself and used as starters—due to their better adaptability to the matrix. This approach enabled the conversion of food by-products, such as pomegranate seeds, into valuable nutritional enhancers and led to the formation of more complex volatile organic compound profiles in pomegranate ciders. In particular, this research offered valuable insights into the fermentation capabilities of the non-conventional autochthonous yeast *H. valbyensis* (isolated from pomegranate), focusing on its metabolism of glucose and fructose. The yeast's ability to grow on fructose and the identification of potential fructose transporters present exciting opportunities for further exploration of this relatively understudied species, which seems to be promising for novel applications in food fermentation. Moreover, this thesis contributes to a growing understanding of plant-based fermented foods and consumer behavior toward innovative products. Future research should prioritize scaling production and extending the shelf life of these foods, while broadening consumer testing to provide more detailed insights into market potential. The findings of these studies could support the development of new plant-based fermented foods that meet the increasing consumer demand for sustainable and nutritious options.

Appendix

First author publications

Limongelli, R., Minervini, F., & Calasso, M. (2023). Fermentation of pomegranate matrices with *Hanseniaspora valbyensis* to produce a novel food ingredient. *LWT*, 180, 114687.

Other publications

Mefleh, M., Omri, G., **Limongelli, R.**, Minervini, F., Santamaria, M., & Faccia, M. (2024). Enhancing nutritional and sensory properties of plant-based beverages: a study on chickpea and Kamut® flours fermentation using *Lactococcus lactis*. *Frontiers in Nutrition*, 11, 1269154.

Publications in Progress

Limongelli, R., Apa, C.A., Porfido, C., De Angelis, M., Gattullo, C.E., Minervini, F., Terzano, R., Celano, G. Impact of autochthonous non-conventional *Hanseniaspora valbyensis* on fermentation and Quality of pomegranate cider. Article submitted to Applied Food Research Journal, December 2024.

Limongelli, R., Minervini, F., Morrissey, J. Characterization of *Hanseniaspora valbyensis* and identification of potential fructose transporters. Article in progress.

Author contribution

1. Fermentation of pomegranate matrices with *Hanseniaspora valbyensis* to produce a novel food ingredient

Limongelli R., carried out carried out all the analysis and related data elaboration. She also wrote the original draft of the manuscript.

2. Characterization of *Hanseniaspora valbyensis* and identification of potential fructose transporters

Limongelli R., collaborated in the conception of the work and the design of the experimental plan, carried out all the analysis and related data elaboration. She also wrote the original draft of the manuscript.

3. Impact of autochthonous non-conventional *Hanseniaspora valbyensis* on fermentation and Quality of pomegranate cider

Limongelli R., collaborated in the conception of the work and the design of the experimental plan, carried out the production of ciders, microbiological analysis, Determination of mono-, di-saccharides, acetic acid, and ethanol, preparation of the samples for GC-MS analysis, colour and sensory analysis, determination of antioxidant activity, and related data elaboration (except for VOCs). She also wrote the original draft of the manuscript.

4. Enhancing nutritional and sensory properties of plant-based beverages: a study on chickpea and Kamut® flours fermentation using *Lactococcus lactis*

Limongelli R., carried out microbiological analyses, determination of raffinose and related data elaboration. She also contributed to writing the original draft of the manuscript and reviewed the final version.