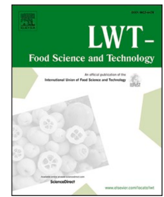
LWT - Food Science and Technology 180 (2023) 114687

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt



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

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A R T I C L E I N F O

Keywords:
By-products
Granola
Minerals
Pomegranate
Yeasts

A B S T R A C T

We aimed to develop a large-scale consumption food, added with fermented pomegranate matrices, to be able to meet consumer's needs. Chemical composition, antioxidant activity, and microbiota of pomegranate were analysed. Yeasts dominated (ca. 5 log cfu/g) the matrices. Among four *Hanseniaspora valbyensis* strains able to ferment pomegranate, S-L1 caused the highest increase in mineral in seeds (e.g., 162 mg of K/100 g of fermented seeds *vs.* 104 mg of K/100 g of unfermented seeds). Then, we designed a granola snack supplemented with pomegranate seeds flour (PSF) and compared: G-FS, granola containing fermented PSF; G-US, supplemented with unprocessed PSF; G-C, conventional granola. Compared to G-C, the use of PSF increased antioxidant activity of the snack (ca. 60% DPPH• scavenging activity *vs.* ca. 40% activity of G-C). The highest contents of minerals were expected in G-FS and G-C, compared to G-US, while lower energetic values were expected for the two fortified snacks. Fermentation of seeds with *H. valbyensis* S-L1 also improved acceptability of fortified snack (score on 1–9 scale: 6.3), making it more similar to the control (7.5) and more appreciated than G-US (4.9). The results could intrigue industries searching for novel foods, with improved nutritional quality and sustainability.

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, Karale, Jadhav, & Kad, 2014), variable depending on cultivar and environmental conditions (Venkitasamy, Zhao, Zhang, & Pan, 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, Grabež, Trchounian, & Arsić, 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, Balvardi, Akhavan, & Hajimohammadi-Farimani, 2022; Nuzzo et al., 2022; Sahraee, Ghanbarzadeh, & Falcone, 2022). Recently, juice and foods fortified with different part of pomegranate have been proven to have antioxidant, 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-Jaìuregui, & 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, Salama, Abul-Fadl, Akila-Hamza, & Emad, 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

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https://doi.org/10.1016/j.lwt.2023.114687
Received 28 October 2022; Received in revised form 2 March 2023; Accepted 20 March 2023
Available online 20 March 2023
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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 CO_2 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 and 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, Chiattoni, Mafaldo, Badiale-Furlong, & de Souza-Soares, 2021; Sabater, Ruiz, Delgado, Ruas-Madiedo, & Margolles, 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, Coda, De Angelis, & Gobbetti, 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. Material and methods

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. 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 5 min. To remove residual NaClO, arils were washed under tap water, at ca. 10 °C, for 2 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.

2.3. 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).

2.4. 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×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•), and determination of the ferric reducing antioxidant power (FRAP). The free radical scavenging activity against DPPH• was measured as described by Caponio et al. (2020). In details, 50 μl of each sample were added to 950 μl of DPPH• solution (0.0031 g/100 ml in ethanol). Blank and positive control containing 50 μl 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:

$$DPPH\bullet\ scavenging\ activity\ (\%) = [(blank\ absorbance - sample\ absorbance)\ /\ blank\ absorbance] \times 100.$$

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.

2.5. 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.

2.6. 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).

2.7. Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and genotypic identification 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 $MgCl_2$, 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 ™ 1 kb (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 $MgCl_2$, 2 mmol/l KCl, 0.04 U of GoTaq® G2 Flexi DNA polymerase (Promega), 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 and 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.

2.8. 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.

2.9. 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

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	-[a]	–	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

[a] Ingredient not added.

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.

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.

2.10. 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).

3. Results

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 (Fig. 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 (Fig. 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).

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).

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 macronutrients 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 (Fig. 2) and those of FRAP (Fig. 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 (Fig. 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) (Fig. 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.

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}	–*	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.

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) (Fig. 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) (Fig. 4a). On the contrary FRAP was higher (P < 0.05) in G-US than G-FS (Fig. 4b). Supplementary Table 1 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 (Supplementary Fig. 1 and Supplementary Table 2). 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.

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,

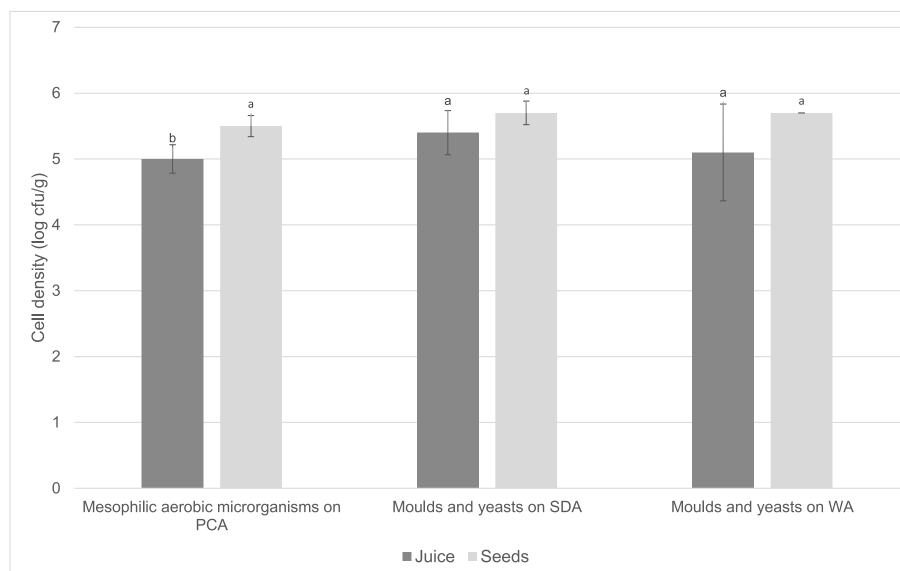

Fig. 1. Cell density (expressed as log cfu/g) of total mesophilic aerobic microorganisms (determined on Plate Count agar (PCA)), moulds and yeasts (determined on Sabouraud Dextrose agar (SDA) and Wort agar (WA)) in fresh pomegranate juice and seeds. For each microbial group, bars sharing one letter (a-b)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

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 (T0) 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	
	T0	T24	T0	T24
Hanseniaspora valbyensis J-L1	7.0 ± 0.2^b	7.6 ± 0.0^a	7.0 ± 0.1^b	7.9 ± 0.2^a
Hanseniaspora valbyensis J-L5	7.0 ± 0.0^b	7.9 ± 0.0^a	7.1 ± 0.0^b	7.9 ± 0.2^a
Hanseniaspora valbyensis J-L6	7.0 ± 0.0^b	7.8 ± 0.0^a	7.0 ± 0.2^b	8.0 ± 0.1^a
Hanseniaspora valbyensis 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

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

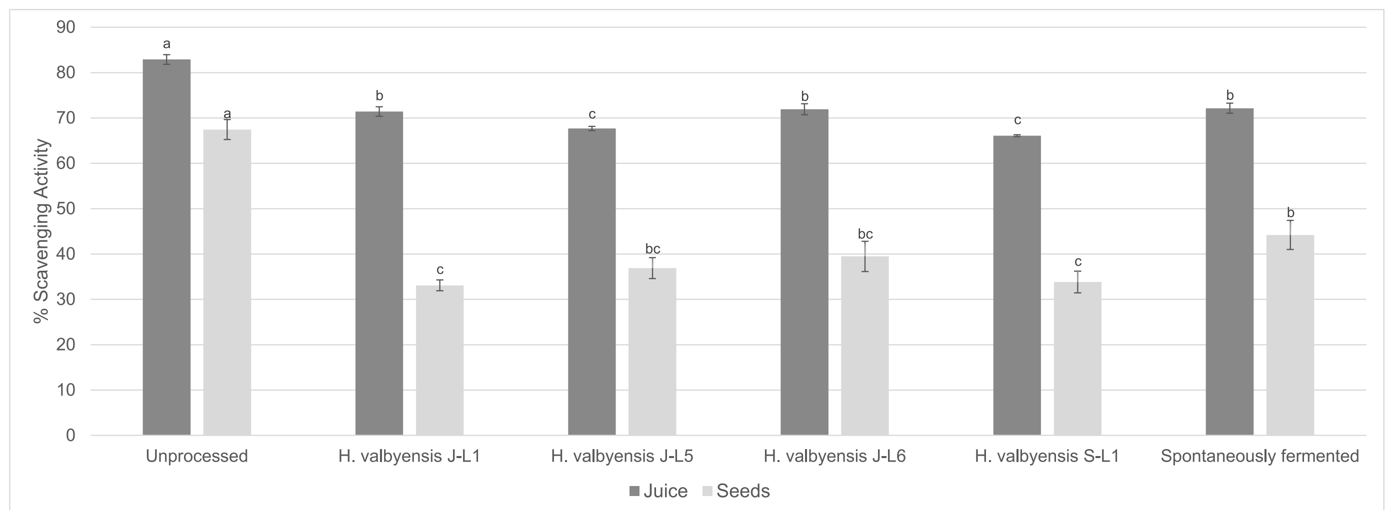

Fig. 2. DPPH• Scavenging activity (%) of pomegranate juices (dark grey) and seeds (light grey) unprocessed, fermented by *Hanseniaspora valbyensis* J-L1, *H. valbyensis* J-L5, *H. valbyensis* J-L6 and *H. valbyensis* S-L1 strains, and spontaneously fermented. Antioxidant activity was measured, in case 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.

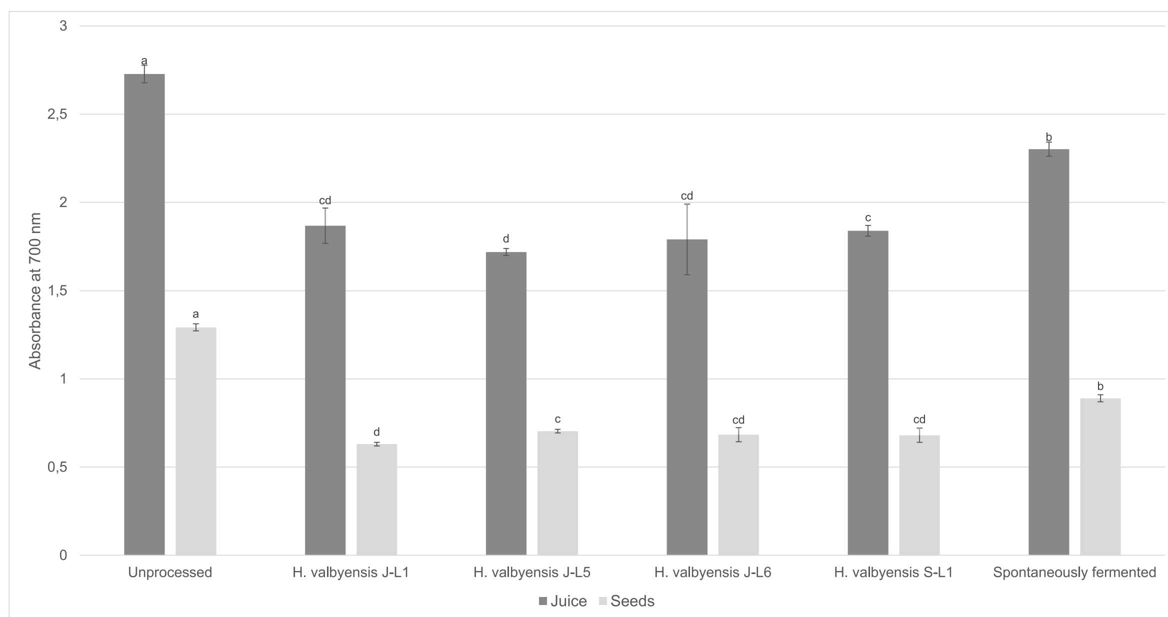

Fig. 3. Ferric reducing antioxidant power (FRAP) of pomegranate juices (dark grey) and seeds (light grey) unprocessed, fermented by *Hanseniaspora valbyensis* J-L1, *H. valbyensis* J-L5, *H. valbyensis* J-L6 and *H. valbyensis* S-L1 strains, and spontaneously fermented expressed as absorbance at 700 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.

(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

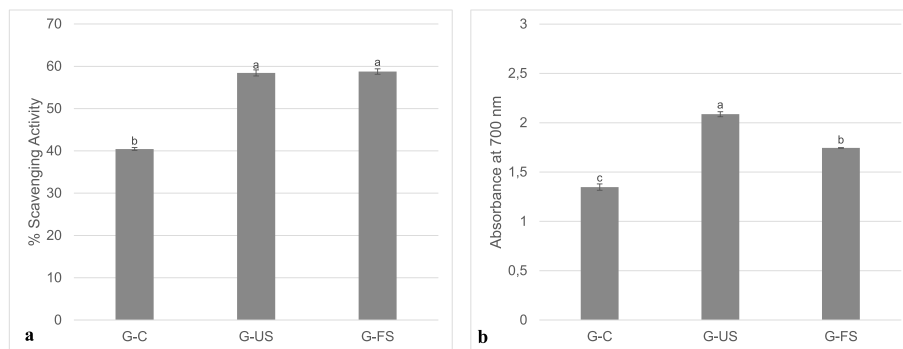

Fig. 4. Antioxidant activity on ethanolic extracts of *granola* snacks as assessed through determination of 2,2-DiPhenyl-1-PicrylHydrazyl (DPPH•) radical scavenging activity (panel a) and ferric reducing antioxidant power (FRAP) assay expressed as absorbance at 700 nm (panel b). *Granola* snack without 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 ($p > 0.05$).

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, Dingeo, Gobbetti, and 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.

In conclusion, 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.

Funding

This work was supported by the following projects: SYSTEMIC, "DAJS - Rigenerazione sostenibile dell'agricoltura nei territori colpiti da *Xylella fastidiosa* (DBA.002 CE.455)", and ON Foods. The project SYSTEMIC "an integrated approach to the challenge of sustainable food systems: adaptive and mitigatory strategies to address climate change and malnutrition", Knowledge hub on Nutrition and Food Security, has received funding from national research funding parties in Belgium (FWO), France (INRA), Germany (BLE), Italy (MIPAAF), Latvia (IZM), Norway (RCN), Portugal (FCT), and Spain (AEI) in a joint action of JPI HDHL, JPI-OCEANS and FACCE-JPI launched in 2019 under the ERA-NET ERA-HDHL (n° 696295). The project DBA.002 CE.455 was funded by the MIPAAF (Italy). Funder: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3 - Call for proposals No. 341 of 15 March 2022 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU; Award Number: Project code PE00000003, Concession Decree No. 1550 of 11 October 2022 adopted by the Italian Ministry of University and Research, CUP H93C22000630001, Project title "ON Foods - Research and innovation network on food and nutrition Sustainability, Safety and Security – Working ON Foods".

CRediT authorship contribution statement

Rosangela Limongelli: Investigation, Writing – original draft. **Fabio Minervini:** Conceptualization, Supervision, Writing – review & editing. **Maria Calasso:** Methodology, Data curation.

Declaration of competing interest

On behalf of all the authors, I hereby declare that there is no conflict of interest relevant to the study entitled "Fermentation of pomegranate matrices with *Hanseniaspora valbyensis* to produce a novel food ingredient" by Rosangela Limongelli, Fabio Minervini, and Maria Calasso, submitted for publication on LWT – Food Science and Technology.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank Prof. Giuseppe Ferrara for his stimulating advices, Alessia Lisi and Ph.D. Sonya Siragusa for technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114687.

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