Use of meta-omics approaches for characterization of microbiota isolated from different ecological niches

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In the present work, the first two activities of the PhD thesis are described. Firstly, pomegranate microbiota was investigated trough culture-dependent approach and potentially pro-technological microorganisms were isolated and identified. Secondly, fermentation process of pomegranate juices and seeds by microorganisms previously isolated were set up. New large-scale consumption foods were designed, on laboratory scale, and characterized.

Approcci meta-omici per la caratterizzazione del microbiota in diverse nicchie ecologiche

Nel presente lavoro sono descritte le prime due attività del progetto di tesi di dottorato. Il microbiota del frutto del melograno è stato studiato mediante approccio coltura-dipendente e i microrganismi potenzialmente pro-tecnologici sono stati isolati ed identificati. Successivamente, succhi e semi di melagrana sono stati fermentati con i microrganismi precedentemente isolati al fine di progettare, su scala di laboratorio, nuovi alimenti di largo consumo e caratterizzarli.

**Key words**: pomegranate, yeasts, by-products, granola, cider.

# **1. Introduction**

In accordance with the PhD thesis project previously described in Proceedings of the 26th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology (Rolle et al., 2022), this poster reports the main results of the first two activities concerning:

(A1) isolation and identification of potentially pro-technological microorganisms from pomegranate juice (PJ) and seeds (PS), able to ferment the same matrix;

(A2) exploitation, through fermentation, of pomegranate juice and seeds, to develop, on pilot plant, new large-scale consumption foods (soft drink from PJ and seeds flour from PS, to be added as an ingredient), in order to better meet consumer’s expectations.

# **2. Materials and Methods**

* 1. **Isolation and identification of pro-technological microorganisms from PJ and PS**

PJ and PS from 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). Both juice and water-seeds mixture were incubated at 30 °C for 16 h. Spontaneously fermented juice and seeds were then analysed to isolate lactic acid bacteria and yeasts, using serial dilutions method. After incubation of plates, only yeast colonies were found. Therefore, yeasts were isolated by picking up colonies from the Sabouraud Dextrose (SD) agar plates inoculated with the highest diluted samples. Yeasts DNA was extracted using the Wizard® Genomic DNA purification kit, according to the manufacturer’s instructions. Yeasts were biotyped by RAPD-PCR analysis singly using the primers M13m and RP11 (Del Bove et al., 2009). Yeasts were identified upon partial sequencing of 26S rRNA gene, amplified using the primers NL-1 and NL-4 (Kurtzman and Robnett, 1998). PCR products were sequenced by Macrogen Europe BV and the DNA sequence homology (higher than 99%) was determined through pair-wise sequence alignments, using BLAST within the NCBI nucleotide collection database.

* 1. **Fermentation of PJ and PS and characterization of the fermented products**

Commercial pasteurized 100% natural PJ were used in the second part of this study. Commercial PJ differed in sugar content (8.9 and 12.2 for low sugar (LS) and high sugar (HS) respectively). PS were obtained from chemically sterilized arils by squeezing them through a lab blender mixer in a stomacher bag with lateral filter for three cycles of 3 min each.

Yeast strains isolated from pomegranate matrices were singly used as starters for fermenting PJ and PS (seeds:water ratio of ca. 4:1). When used for fermentation, yeasts were cultivated at 30 °C (24 h or 48 h, respectively for PJ or PS) in SD 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. 5.5 log cfu/g in juices and 7.0 in seeds). A commercial yeast (CY) was used as a control to ferment PJ. All the juices were fermented at 15 °C for seven days, to obtain pomegranate cider. In addition, PS were spontaneously fermented without the addition of starters at 30 °C for 24 h. At the end of fermentations, pomegranate cider was stored at 4 °C; PS were dried in an oven at 60 °C overnight, ground through a coffee mill and the flours obtained were used as an ingredient for production of *granola* snack. Cell density of presumptive yeasts, chemical analyses, determination of antioxidant activity were carried out along all the production process and at the end of it. Moreover, volatile compounds produced during ciders fermentation will be analyzed.

# **3. Results and Discussion**

## **Isolation and identification of pro-technological microorganisms from PJ and PS**

Pomegranate microbiota was dominated by yeasts, even after spontaneous fermentation. This microbial group, probably environment-borne, typically contaminates fruit peels (Kalia and Gupta, 2006). Notwithstanding the use of selective or elective culture media 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 and D'Souza, 2013; Reddy et al., 2007)*.* Therefore, we isolated yeasts from both spontaneously fermented PJ and PS. After biotyping through RAPD-PCR, twelve isolates were grouped into four clusters (I-IV). 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 molecular identification. All the isolates were identified as *Hanseniaspora valbyensis*, a non-conventional yeast often found as one of the microbial drivers of balsamic vinegar and cider fermentations (Bellut et al., 2018). Strains of *H. valbyensis* were singly used as starter for driven fermentation of PJ and PS.

* 1. **Fermentation of PJ and PS and characterization of the fermented products**

The four representative autochthonous strains of *H. valbyensis* were able to drive the fermentation of pomegranate matrices, causing modifications of the chemical composition. Among the four autochthonous yeasts, we selected *H. valbyensis* J-L6 and S-L1 for the fermentation of juices and seeds respectively, because besides being able to grow in the pomegranate matrices, they caused the best increase in mineral content. Therefore, we tried to design a pomegranate cider from PJ and a fortified snack (*granola*) supplemented with PS each one fermented with a different strain (S-L1 for PS, J-L6 for cider) of *H. valbyensis*. After three days of fermentation of PJ, cell density of moulds and yeasts increased significantly (p < 0.05) in all the thesis (LS-CY, LS J-L6, HS-CY, HS J-L6); however, the highest increase (about three logarithmic cycles) was observed in HS e LS juices fermented by *H. valbyensis* J-L6. No significant differences (p > 0.05) of cell density of yeast were observed after seven days with respect to day 3, except for LS and HS juice fermented by commercial yeast, wherein cell density increased significantly (p < 0.05). Despite this noticeable growth, yeast cell density was still significantly higher in PJ fermented by J-L6. Chemical analyses, including mineral composition, sugar (glucose, fructose, sucrose), alcohol and acetic acid concentration, profile of volatile compounds, and antioxidant activity of ciders will be investigated. PS flour from unfermented seeds and from seeds fermented by *H. valbyensis* S-L1, were used as additional ingredient for the production of *granola* snack (G-US and G-FS respectively). Compared to *granola* control (G-C) obtained without adding pomegranate seeds flour (PSF), the use of PSF, as an additional ingredient of *granola*, increased *in vitro* antioxidant activity of the snack. The expected contents of calcium, iron, potassium, and zinc were higher in G-FS and G-C, compared to G-US. Finally, we expect that the two *granola* snacks fortified with PSF would have lower (ca. 15%) energetic values than control *granola*. 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 unfermented seeds.

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