From Waste to Value: Unlocking the potential of red wine pomace and microalgae through tailored fermentation

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The first three activities of the PhD thesis project are described. Firstly, fermentation set-up of wine pomace and *Chlorella vulgaris* was optimized. Secondly, microbiological analysis, sugars, organic acids, ethanol, total protein, and peptides in fermented formulations were quantified. Thirdly, functional characterization of fermented substrates in terms of phenolic compounds, total free amino acids, and peptidomics profiling were demonstrated.

Dagli scarti al valore aggiunto: sfruttare il potenziale della vinaccia e delle microalghe attraverso la fermentazione guidata

Le prime 3 attività del progetto di tesi di dottorato sono di seguito descritte. In primo luogo, sono stati ottimizzati i parametri di fermentazione della vinaccia e di *Chlorella vulgaris*. In secondo luogo, le analisi hanno quantificato gli zuccheri, acidi organici, etanolo, proteine e peptidi totali dei campioni fermentati. Infine, sono state dimostrate le caratteristiche funzionali dei substrati fermentati considerando i composti fenolici, il profilo degli amminoacidi liberi totali e individuali e il profilo peptidico.

**Key words**: Microalgae, by-product, wine pomace, fermentation, phenolic compounds, peptidomics.

# **1. Introduction**

In accordance with the PhD thesis project previously described, this poster reports the main results of the first three activities concerning:

(A1) optimization of fermentation set-up of wine pomace and chlorella;

(A2) microbiological analysis and assessment of sugars, organic acids, ethanol, total protein, total peptides;

(A3) investigation of phenolic compounds, total free amino acids and peptidomics profile.

# **2. Materials and Methods**

(A1) *Chlorella vulgaris* powder and red wine pomace were chosen as starting substrates, whereas ten strains of lactic acid bacteria (LAB), including one fructophilic LAB (FLAB) and yeasts were selected for tailored fermentation. Three different mixtures were produced: *Chlorella vulgaris* (30:70), wine pomace (30:70) and a mixture of both (15:15:70). All mixtures were sterilized at 121° C for 15 min. Each mixture was then inoculated with single selected strains at an initial cell density of ca. 7 Log CFU mL-1 for LAB and 5 Log CFU mL-1 for yeasts. The samples were then incubated at 30° C for 72 hours. Raw sterile sample of each combination (stored at -20° C) and unstarted samples were considered as controls for each mixture. (A2) Microbiological analysis were evaluated and plated on agar media of de Man Rogosa and Sharpe agar (MRS), for LAB, Fructose Yeasts Extract Polypeptone agar (FYP) for FLAB, and Malt extract agar (MEA) for yeasts. Additionally, the pH of each sample was measured using a Foodtrode electrode (Hamilton, Bonaduy, Switzerland). Organic acids, sugars and ethanol were determined by High Performance Liquid Chromatography (HPLC), equipped with an Aminex HPX-87H column (ion exclusion, Biorad), a UV detector operating at 210 nm, and a Perkin Elmer 200a as the refractive index detector. Total protein quantification and total peptides quantification were determined using Bradford and o-phthalaldehyde (Opa) assays. (A3) Phenolic compounds were identified and quantified with targeted LC-MS/MS analysis using an UHPLC Dionex 3000 (Thermo Fisher Scientific, Germany) equipped with a Waters Acquity HSS T3 column (1.8 μm, 100 mm × 2.1 mm) (Milford, MA, USA) and coupled to a TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Germany) with an electrospray source. Total free amino acids were examined with the Biochrom 30 Amino acid analyzer equipped with a NA-cation exchange column measuring 20 by 0.46 cm in inner diameter. Peptides profile of the samples were investigated with ultra-high performance liquid chromatography/high-resolution tandem mass spectrometry.

# **3. Results and Discussion**

## **3.1 Determination of the microbiological and physico-chemical properties**

(A2) Focusing on the interaction between protein and phenolics, we target our attention on the combination of chlorella and pomace. Preliminary analyses were conducted to characterize the substrates and the results were consistent with the available literature. All LAB and yeasts strain demonstrated their capability to grow in the substrates, while LAB also showed their ability for acidification. HPLC analysis confirmed the conversion of available sugars into ethanol by yeasts and organic acids by LAB, with the exception of the synthesis of mannitol, which was observed only by *Ap. kunkeei* (BEE4) and *Leuc. mesenteroides* (S3d1) due to their specific metabolic pathways. Total protein content together with total peptides trend showed a common reduction during fermentation in different proportions among all the strains.

A picture containing screenshot, text, colorfulness, diagram

Description automatically generated(A3) LC-ESI-MS/MS analysis enabled the identification of 17 phenolic compound in all samples (Figure 1). Most of these compounds derive from pomace, whereas in chlorella only 4-hydroxybenzaldehyde was detected. Different trends were observed during fermentation when compared to non-fermented samples. Phenolics such as 4-hydroxybenzaldehyde, caftaric acid, hyperoside, *p*-coumaric acid, procyanidin B1, procyanidin B2 and petunidin 3-glucoside have been utilized by all the strains. The reduction of polyphenols is often associated to an increase of their bioavailability, thanks to the enzymatic activity of different microorganisms that modify their complex structure and potentially enhance their absorption in human body (Gibson et al.; 2006). On the contrary, isoquercetin, isorhamnetin, myricetin and quercetins seems to be enhanced significantly during fermentation. The increase of polyphenols is associated to improve sensory feature, anti-inflammatory and antioxidant properties (Zhu et al., 2022).The total and free amino acids were presented in the heat map (Figure 2). Among the amino acids analyzed, alanine was found to be the most abundant. Proline and asparagine showed an increase after fermentation with *Lactiplantibacillus. plantarum* AFI5 and *S. cerevisiae* KFAY3, while glutamic acid was degraded by these same strains. While on the one hand increase of the total free amino acids showed to improve nutritional profile and to be potential for the formation of bioactive compounds such as bioactive peptides, on the other their decrease is often associated to reduce the bitterness or astringency and to improve the texture of fermented foods. Notably, ornithine was only detected in the fermented samples with *Weissella cibaria* P9 and *Lc. Lactis* WSL2. UHPLC/HR-MS/MS investigated the peptide profile of the samples (data not shown). The screening of peptides with a molecular weight lower than 100 kDa identified a total of 7708 distinct peptides across the 14 analyzed samples. After fermentation, an overall increase in peptide levels was observed by all strains, especially by *W. cibaria* and *Leuc. Mesenteroides* (739 and 645 additional peptides, respectively). Furthermore, the length of the peptides was evaluated, focusing on lengths ranging from 6 to 11 amino acids, the most inclined to have bioactivity properties. In terms of total peptides, *Lb. plantarum* had the lowest amount within this range, with 928 peptides, followed by *Ap. Kunkeei* with 933 peptides. The highest amount was found in Spontaneous, *W. cibaria*, and *S. cerevisiae*, with 1043, 1047, and 1045 peptides, respectively. All 7708 identified peptides underwent cross verification and validation using the BIOPEP-UWM database (Minkiewicz et al., 2019), which comprises a comprehensive list of 4,670 known bioactive peptides. Unfortunately, none of the sequence of the identified peptides shared 100% homology in the amino acid sequence, with previously identified bioactive peptides indicating the absence of known bioactive peptides in the sample. However, the software utilized for this analysis is not able to detect the peptides of lee than 5 amino acids, leaving margin for further research. Further analysis will deal with the selection of the best-performing starters and the samples will be further characterized by bioactivity *in vitro* analyses.

**Figure 2** Abundance of total free amino acids(mg kg-1) by Biochrom 30 Amino acid analyzer among all the tested samples.

**Figure 1** Quantification of phenolic compounds (mg L−1 FW) by LC-ESI-MS/MS among all the tested samples.

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# **4. References**

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