**Development of Biotechnological Protocols for the Valorization of Alternative Plant Matrices as a Strategy for the Sustainability of Agri-Food Systems**

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This PhD thesis focused on the reuse of the food industry by-products using biotechnological approaches. In particular, i) red grape pomace and ii) flours obtained from carob pods without seeds were used to obtain yogurt-like prototypes and baked goods using selected microorganisms capable of inducing sensory, technological, nutritional and functional improvements.

**Sviluppo di protocolli biotecnologici per la valorizzazione di matrici vegetali alternative come strategia per la sostenibilità dei sistemi agro alimentari**

Questa tesi di dottorato ha riguardato il riutilizzo di sottoprodotti dell’industria alimentare attraverso l’uso di approcci biotecnologici. In particolare, i) vinacce derivanti da vinificazione in rosso e ii) sfarinati ottenuti da baccelli di carruba privati dei semi, sono stati utilizzati per ottenere bevande yogurt-like prototipali e prodotti da forno, utilizzando microrganismi selezionati in grado di indurre miglioramenti sia a livello sensoriale che tecnologico, nutrizionale e funzionale.

**Key words**: yogurt like, plant-based, bread, clean-label, biotechnological protocols, lactic acid bacteria.

# 1. Introduction

The Agenda 2030, signed on September 25, 2015, by the governments of the 193 member countries of the United Nations and approved by the UN General Assembly, consists of 17 Sustainable Development Goals (SDGs) to be achieved by 2030 in the environmental, economic, social, and institutional domains. The twelfth goal focuses on the implementation of sustainable production and consumption patterns. One of the implementation measures states, "Halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses" (source: https://www.agenziacoesione.gov.it/comunicazione/agenda-2030-per-lo-sviluppo-sostenibile/). The utilization of by-products from the food industry, including carob pods without the seeds and grape pomace, is necessary to avoid wastage. In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following three activities directed to:

A1) Use of grape pomace as an ingredient for fibre and antioxidant compounds fortification in bread

A2) Design of a plant-based yogurt like including red pomace grape-formulation and characterization of a new recipe

A3) Design of a high-fibre and “clean-label” plant-based yogurt like including carob flour -formulation and characterization of the new recipe

# 2. Materials and methods

## 2.1 Use of bioprocessed grape pomace as ingredient for fibre and antioxidant compounds fortification in bread

Once the composition of a pomace substrate had been optimized to be suitable for LAB (Lactic Acid Bacteria) fermentation, different starters, previously isolated from plant food matrices, were inoculated and incubated for 24 hours at 30°C: *Lactobacillus rossiae* T0A16, *Lactiplantibacillus plantarum* T0A10 and *Lactiplantibacillus plantarum* T6B10 isolated from quinoa flour (Rizzello et al., 2016) *Pediococcus acidilactici* 10MM0, *Lactiplantibacillus plantarum* 18S9 and *Leuconostoc mesenteroides* 12MM1 isolated from hemp (Nionelli et al., 2018), *Lactiplantibacillus plantarum* H22, *Pediococcus pentosaceus* H18 and *Lactiplantibacillus plantarum* H64 isolated from hops (Nionelli et al., 2018b), *Lactiplantibacillus plantarum* LB1 and *Lactobacillus rossiae* LB5 isolated from wheat germ and *Enterococcus faecium* CA16 isolated from carob. To select the best performing starter, total titratable acidity (TTA) values were measured before and after fermentation and lactic acid bacteria were enumerated by plate count. In addition, the pH of the optimized substrate was measured at defined time intervals during fermentation and the data were used to obtain acidification kinetics. Based on these results, the starter selected was used for obtaining type II sourdoughs (LNs), mixing wheat flour with grape pomace at 0% (LN0), 2.5% (LN2.5) and 5% (LN5) of the total weight of flour. The three LNs were incubated at 30 °C for 24 hours. Pre- (t0) and post-fermentation (t24) pH, TTA, total free amino acid (TFAA) concentration and antioxidant activity, using the DPPH assay, were measured for each of the sourdoughs. At the end of fermentation, LAB were also enumerated, and lactic and acetic acid quantified. Based on the results obtained, the best sourdough was selected to be used for bread production. Three types of experimental breads were compared: control bread leavened with bakers’ yeast (Ct LB), control bread with sourdough (Ct LN) and bread with the sourdough fortified with pomace (LNV). At the end of leavening, and thus before baking, volume increase, pH, TTA, acetic acid concentration and antioxidant activity were assessed. TFAA were quantified before and after baking using the automatic Biochrom 30+ Amino Acid Analyzer (De Pasquale et al., 2021). At the end of baking the structural characteristics of the loaves were determined, and the colour coordinates of the crust and crumb were measured. Finally, the sensory analysis of the breads produced was carried out.

## 2.2 Design and characterization of a yogurt-like and plant-based beverage fortified with red grape pomace

Red grape pomace, without separation of the grape seeds, was mixed with rice flour to obtain a yogurt-like (YL). Three different strains were then inoculated separately: *L. plantarum* T0A10, previously selected as able to grow in the pomace-enriched substrate and to increase antioxidant activity of plant-derived matrices (Rizzello et al., 2016); *Leuconostoc pseudomesenteroides* DMS20193, potentially able to produce exopolysaccharides in situ from sucrose (Montemurro et al., 2023); and *Lacticaseibacillus rhamnosus SP1*, a probiotic strain (Lorusso et al., 2018; Coda et al., 2011; Nionelli et al., 2014). An uninoculated control (Ct) was also prepared. After fermentation, the YL were characterized by enumerating the presumptive LAB and measuring their pH, TTA, viscosity, TFAA, organic acids, in vitro protein digestibility (IVPD), total polyphenols and antioxidant activity. A sensory analysis was also conducted for all fermented beverages. The analysis of pH, TTA, cell density and antioxidant activity were also repeated after 7 and 14 days of cold storage to assess the shelf-life of the fermented beverages.

## 2.3 Use of selected lactic acid bacteria and carob flour to produce a high-fibre and “clean label” plant-based yogurt-like

A new formulation of YL was designed using rice and carob flours. Six previously characterized LAB strains were used as starters to singly inoculate the rice/carob yogurt-like (YL). More specifically, *L. rhamnosus* SP1, chosen because already demonstrated optimal technological properties and high survival under refrigerated storage conditions (Lorusso et al., 2018; Coda et al., 2011; Nionelli et al., 2014), *L. plantarum T6B10* showed the best adaptation and highest acidification when used as starter in a quinoa YL (Lorusso et al., 2018), *Weissella cibaria* P9 and *L. pseudomesenteroides* DSM20193, chosen for their ability to produce exopolysaccharides (EPS) in several vegetable matrices (Montemurro et al., 2021; 2023) whereas *Levilactobacillus brevis* AM7 was selected due to its proteolytic activity and antimicrobial properties (Coda et al., 2008; Nionelli et al., 2020). Lastly, *E. faecium* CA16, isolated from carob pulp flour, was also used. A deep level of investigation in a case-by-case assessment should be provided before using such microorganisms as starters at industrial level (EFSA, 2007), since bacteria belonging to the genus Enterococcus are frequent opportunistic human pathogens and express adhesion factors, yet some strains are used as starter cultures in dairy products. At the end of fermentation cell densities of presumptive LAB, yeasts, molds, and enterobacteria were evaluated for all the YL. pH, TTA, organic acids, sugars, TFAA, water holding capacity (WHC), viscosity, and antioxidant activity were also determined. Biochemical, microbiological, technological, and functional characterizations were also repeated after 15 and 30 days of refrigerated storage to investigating the microbiological quality of the products and the survival of the inoculated strains under refrigerated conditions. Based on the previous results, the best rice/carob YL formulation was selected for subsequent nutritional and sensory analysis.

# 3. Results and discussion

## 3.1 Use of grape pomace as an ingredient for fibre and antioxidant compounds fortification in bread

Based on the ΔpH values, the 2,5 % LN supplemented with glucose and yeast extract was chosen to select the best starter for fermentation. At the end of fermentation, L. *plantarum* T0A10, T6B10, and LB1 exhibited a cell density higher than 9 log cfu/mL and greater antioxidant activity (70.63 ± 0.75%, 70.72 ± 1.80%, and 69.41 ± 3.40%, respectively). Among that strains, *L. plantarum* T0A10 was selected for further testing because it showed a short adaptation phase and, at the same time, a high final cell density, and antioxidant activity comparable to that of BHT (at 75 ppm). *L. plantarum* T0A10 was then used for obtaining type II sourdoughs added with 0% (LN0), 2.5% (LN2.5), and 5% (LN5) grape pomace. The starter was able to grow in all LNs. At the end of fermentation, the three different LNs had a pH of approximately 3.6. The antioxidant activity revealed that the addition of grape pomace already increased the antioxidant activity from about 7% (LN0) to 74% and 34% (LN5 and LN2.5, respectively) before fermentation. Fermentation further increased the antioxidant activity, which reached 89% and 48% respectively for LN5 and LN2.5 after 24 hours of incubation. An increase in TFAA was observed for all LNs compared to pre-fermentation values. The concentration of lactic acid and acetic acid was significantly lower in LN2.5 and LN5 compared to LN0. Based on these results, the LN enriched with 5% grape pomace was chosen for bread production (LNV). LNV was compared to control bread with bakers’ yeast (Ct LB) and control bread with sourdough (Ct LN). During proofing, the volume increase of the three doughs bread showed no significant differences. However, the final pH was lower in dough containing sourdough. The concentration of organic acids reflected what was found in the sourdoughs: the lactic acid in Ct LN and LNV was approximately 23-24 mmol/kg, with no significant differences between them, while acetic acid was significantly higher in Ct LN. Organic acids were only found in traces in Ct LB since both are associated with lactic fermentation (of *L. plantarum*) (Nuryana et al., 2019). The concentration of free amino acids was higher in bread containing LN compared to the control made with bakers’ yeast. As expected, the antioxidant activity was markedly higher than in the two breads without grape pomace. The analysis of FAA showed that the addition of grape pomace alone led to an increase in Cys and Tyr. The nutritional label (Tab.1), commissioned to an external laboratory, showed that the three breads differ in fiber content, which is 30% higher in LNV compared to Ct-LB; fats and ashes are slightly but significantly higher compared to the two breads made with wheat flour alone; carbohydrates (excluding fibers) are slightly but significantly lower in LNV. However, the observed differences are not significant enough to determine a substantial variation in the energy value of the three types of breads. It is important to note that the increase in fiber content is attributed not only to the addition of grape pomace but also to the potential contribution from resistant starch generated due to the biological acidification associated with sourdough fermentation.

**Table 1** *Nutrition labels of the experimental breads.*

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Ct LB** | **Ct LN** | **LNV** |
| Moisture (%) | 27.00 ± 1.00a | 27.00 ± 2.00a | 27.00 ± 1.00a |
| Proteins (%) | 8.68 ± 0.38a | 8.68 ± 0.48a | 8.71 ± 0.45a |
| Fat (%) | 0.87 ± 0.10b | 0.87 ± 0.08b | 0.95 ± 0.09a |
| Carbohydrates (%) | 61.63 ± 0.36a | 61.63 ± 0.42a | 61.05 ± 0.39b |
| Fibres (%) | 2.17 ± 0.14b | 2.27 ± 0.11b | 2.82 ± 0.19a |
| Ashes (%) | 0.52 ± 0.20b | 0.52 ± 0.10b | 0.60 ± 0.02a |
| Energy (kcal)  Energy (kJ) | 289.00 ± 3.00a  1209.00 ± 13.00a | 289.00 ± 2.00a  1209.00 ± 6.00a | 287.00 ± 3.00a  1203.00 ± 7.00a |

a-b values marked with a different superscript are significantly different (P<0.05)

Structural analysis indicated that the breads had comparable hardness, although the use of sourdough, especially when supplemented with grape pomace, resulted in lower cohesiveness. In contrast to what is typically found in fiber-enriched breads, the bread containing grape pomace exhibited higher springiness, which is indicative of elasticity. The chewiness parameter suggested that LNV bread may require more effort for chewing.

The inclusion of grape pomace caused significant color variations. Both the crumb and crust showed a clear and notable reduction in lightness, with the crust exhibiting a significantly higher value of the "a" parameter (green/red) compared to the other breads. Based on evaluations by 10 tasters, the main differences between the sourdough bread (LNV) and the other two breads were primarily associated with the color of the crust and crumb, which appeared significantly more intense in the bread containing grape pomace. The differences related to the presence of sourdough involve aroma and acidic taste, which are typical attributes of sourdough breads and are associated with the metabolic activity of lactic bacteria. Among the positive notes, it is noteworthy that the friability of LNV bread was perceived like the control bred made with bakers’ yeast, despite the higher fibers content. Astringency and herbaceous aroma/taste, attributes characteristic of grape pomace, were detected. However, the overall aroma of LNV bread received the highest rating among the three breads, indicating that it was not perceived as a defect.

## 3.2 Design and characterization of a yourt-like and plant-based beverage fortified with red grape pomace

At the end of fermentation, an increase in cell density of approximately 2 log cycles was observed for all the starters used (Tab. 2). In addition, intense acidification was observed, with lower pH values for DSM20193 (4.27) (Tab. 2). The lactic acid concentration was highest in DSM20193 and T0A10 (14.80 and 14.66 mmol/L, respectively). The latter also showed the highest concentration of acetic acid compared to the other samples (2.35 mmol/L) (Tab. 2). TFAA were analyzed at the end of fermentation to assess the proteolytic activity of the strains. A decrease in the concentration of free amino acids was observed in all samples (Tab. 2), with the greatest decrease for the DSM20193 sample (-62 %). The viscosity of Ct and SP1 was significantly similar (0.60 and 0.63 Pa x s, respectively), and lower than that measured for T0A10 and DSM20193. The latter, unlike expected, did not show particularly higher viscosity values than the other theses, suggesting no or limited exopolysaccharide synthesis under the test conditions (Tab. 2). A protocol mimicking *in vitro* digestion was used to estimate the IVPD of fermented beverages. It was observed that protein digestibility increased in all fermented samples compared to the control, especially in the sample fermented with T0A10 (49.59%) (Tab. 2). The total polyphenol concentration of the beverages was found to be in the range of 818-826 mg/l, with no significant differences between the samples (Tab. 2). The antioxidant activity of the three yogurt-like beverages, analyzed as radical scavenging activity on DPPH, is referred to a 1:3 dilution of the methanolic extract. The fermented theses all had significantly higher activity values than the control (Tab. 2). The results of the panel-test on the beverages shown that the fermentation reduced the perception of 'particulate' and 'earthy', which had higher scores in the Ct sample. The perception of acidity, which characterized all fermented theses as an intrinsic characteristic of yogurt, was positively evaluated. A reduction in adhesiveness was also recorded in the fermented theses, probably related to the effect of acidification on the gelatinized starch, which leads to a progressive release of water from the samples. The fermented samples appeared to be relatively similar, except for the greater perception of sweetness in the DSM20193 sample, probably related to the amount of sucrose added and not fully converted by the starter into exopolysaccharides and organic acids. At the end of fermentation, the samples were kept at 4 °C and analyzed after 7 and 14 days of cold storage. In all samples, post-acidification was observed, which was particularly intense in DSM20193. It can be assumed that the addition of sucrose also favored increased lactic acid production during both production and cold storage. Cell density remained high and always exceeded 8 log cfu/ml. Antioxidant activity was persistent throughout the monitored storage period.

**Table 2** *Characterization of the beverages at the end of incubation.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Ct | T0A10 | SP1 | DSM20193 |
| Cell Density of LAB (log cfu/g) | 2.70 ± 0.23b | 8.84 ± 0.04a | 8.56 ± 0.38a | 8.96 ± 0.04a |
| pH | 5.50 ± 0.00a | 4.39 ± 0.02 b | 4.48 ± 0.01b | 4.27 ± 0.08 b |
| TTA (mL NaOH 0,1 M) | 1.80 ± 0.15c | 4.20 ± 0.10a | 3.40 ± 0.15b | 4.60 ± 0.07a |
| Lactic acid (mmol/L) | 0.66 ± 0.00c | 14.66 ± 0.01a | 11.49 ± 0.02b | 14.80 ± 0.03 a |
| Acetic acid (mmol/L) | 0.45 ± 0.01c | 2.35 ± 0.01a | 0.59 ± 0.02b | 0.69 ± 0.05b |
| Free Total Aminoacid (mg/L) | 65.46 ± 3.44a | 35.82 ± 7.71 c | 48.53 ± 8.50 b | 24.94 ± 1.26d |
| Viscosity (Pa x s) | 0.60 ± 0.02b | 0.70 ± 0.02a | 0.63 ± 0.04b | 0.67 ± 0.01a |
| Total Phenols (mg/L) | 826.00 ± 49.49a | 819.00 ± 53.27 a | 823.00 ± 46.64 a | 818.00± 42.67 a |
| Radical Scavenging Activity on DPPH\* | 50.09 ± 0.03c | 58.77 ± 0.02a | 52.54 ± 0.03b | 53.94 ± 0.02b |
| In vitro protein digestibility (%) | 28.69 ± 8.36c | 49.59 ± 7.21a | 47.56 ± 4.88b | 40.99 ± 11.24b |

\*\*samples diluted 1:3

a-d values marked with a different superscript are significantly different (P<0.05)

## 3.3 Use of selected lactic acid bacteria and carob flour to produce a high-fibre and “clean label” plant-based yogurt-like

The pH of the rice-carob mixture employed as substrate for fermentation ranged from 5.5 to 5.6 (Tab. 3). After 16h of fermentation, all samples had pH values lower than 5.0, with only T6B10 reaching a pH lower than 4.5 (4.33). Despite thermal treatment, the rice-carob gelatinized mixture still contained low Enterobacteriaceae, yeasts and moulds. During incubation, all inoculated strains increased by approximately 2 log cycles, with cell densities ranging from 9.00 to 9.73, at the end of fermentation (tf). Moreover, Enterobacteriacee decreased significantly in chemically acidified control (Ct) and in YL fermented with DSM20193, SP1, T6B10 and AM7 compared to t0. Molds were not detected in inoculated samples at tf. The lactic acid concentration in all YL after fermentation ranged from 7.21-8.80 mmol/L with T6B10 having the highest concentration (20.17 mmol/L, Tab. 3). Acetic acid concentrations were low in all inoculated samples, with higher levels observed in P9 and 20193 (Tab. 3).

**Table 3** *Main characteristics of the YL after incubation at 30°C for 16h (tf). A control sample (Ct) corresponding to a not inoculated, but chemically acidified YL, was also characterized.*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Ct | CA16 | DSM20193 | SP1 | T6B10 | AM7 | P9 |
| pH | 4.50c | 4.91b | 4.56c | 4.71b | 4.33d | 4.68b | 4.87b |
| TTA (mL NaOH 0,1 M) | 3.40b | 3.00c | 4.00a | 3.60b | 4.40a | 3.40b | 3.00b |
| Lactic acid (mmol/L) | 12.07c | 7.21d | 11.69c | 13.54b | 16.17a | 13.98b | 8.80d |
| Acetic Acid (mmol/L) | n.d. | 0.21d | 1.28a | 0.65b | 0.50c | 0.61b | 1.36a |
| Glucose (g/L) | 1.66a | 1.01b | 0.43d | 0.47d | 0.54d | 0.80c | 0.45d |
| Fructose (g/L) | 2.14c | 2.41b | 2.79a | 2.53b | 2.83a | 2.44b | 2.57b |
| Maltose (g/L) | 0.52a | 0.45a | 0.46a | 0.50a | 0.56a | 0.50a | 0.55a |
| Sucrose (g/L) | 16.86a | 17.04a | 16.06c | 16.02c | 15.85c | 16.47b | 16.84a |
| Total Free Amino Acid (mg/L) | 159.00a | 76,00c | 113.00b | 159.00a | 112.00b | 123.00b | 156.00a |
| WHC (%) | 82.26c | 85.82c | 93.88a | 90.59b | 91.20b | 84.41c | 84.16c |
| Viscosity (mPa x s) | 8180.00d | 12760.00b | 9920,00c | 9720.00c | 9770.00c | 9800.00c | 12020.00b |
| Radical scavenging activity | 81.96a | 81.08a | 82.23a | 81.62a | 82.90a | 82.67a | 83.08a |

Nd = not detected

a-d values marked with a different superscript are significantly different (P<0.05)

Glucose concentration significantly decreased in all inoculated samples (-50/70%), except for CA16 (Tab. 3).

Significant decreases were also found for sucrose in AM7, SP1, DSM20193, and especially T6B10 (Tab. 3). No variation was observed in maltose concentration during incubation, while fructose concentration slightly increased in inoculated samples, particularly in DSM20193 and T6B10 (Tab. 3). Before fermentation, the yogurt-like substrate had a total TFAA concentration ranging from 159 to 163 mg/L (Tab. 3). Significant decreases in TFAA occurred during fermentation in CA16, T6B10, 20193, and AM7, while no significant decreases were found in SP1 and P9 (Tab. 3). The antioxidant activity was already high at the start of fermentation and remained unaffected by the process (Tab. 3). WHC increased significantly in three out of six inoculated samples (DSM20193, T6B10, and SP1, Tab. 3). Viscosity data showed a correlation with acidification during fermentation, with only CA16 and P9 having higher values than 12000 mPa x s at the end of fermentation. The other samples experienced decreases of approximately 30% compared to their initial values (Tab. 3). At the end of the 30 days of refrigerated storage, LAB cell density in YL decreased of ca. 1 log cfu/mL. Yeasts persisted in all the inoculated YL samples, while a significant increase was observed in Ct. No Enterobacteriaceae and molds were found in any samples. Furthermore, all samples showed a slight but significant decrease in pH compared to t15, accompanied by increases in lactic and acetic acids. A further decrease of sucrose also occurred. Regarding the TFAA, a significant increase characterized T6B10 (+87 and +46% compared to tf and t15 respectively). A significant (P<0.05) increase in TFAA was also observed in DSM20193 (+41% compared to tf and t15), although final concentration was markedly lower than that found for T6B10.

Based on these results, *L. plantarum* T6B10 was identified as the starter capable of the most intense and fast acidification of the substrate. Additionally, the YL-T6B10 was characterized by the highest concentration and better-balanced mixture of FAA. For these reasons YL-T6B10 was further characterized for its nutritional and sensory profiles. YL-T6B10 had the following nutritional label: proteins, 1.58 g/100g; fats, 0.36 g/100g; carbohydrates 15,41 g/100g of which 2.40 sugars; and 2.66 g/100g dietary fibres). The energy value was 76.34 Kcal/100g. The starch hydrolysis index (HI) of the two samples analysed were 53.47 ± 3.45 and 37,99 ± 1,01 % respectively for Ct and T6B10. Consequently, the pGI of the Ct was markedly and significantly (P<0.05) higher than that of T6B10 (69.07 ± 1,89 vs 60.57 ± 0,91).

Aiming at describing the sensory profile of the YL-T6B10 and highlighting the effect of the fermentation, a list of descriptors was selected during the preliminary sessions of the analysis. YL-T6B10 appearance was characterized by a very high score for uniformity and adherence to spoon. For both the attributes, values were significantly higher than control (chemically acidified substrate). Odour intensity and cocoa smell were also intensified by the fermentation. Sweet and bitter taste perceptions were lower in fermented samples compared to control, and astringent and earthy aftertaste resulted significantly lower in YL-T6B10.

# 4. Conclusions and Future Perspectives

These studies demonstrated the suitability of bioprocessed food industry by-products such as carob and grape pomace, to be used as ingredients in the formulation of innovative plant-based foods, such as yogurt-like and baked goods. Moreover, the results may be considered as the basis for future research for the development of novel ingredients through the use of lactic acid bacteria strains with specific functional features.

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