UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Environmental Sciences

UNIMONT

XXXVIII CYCLE

CHARACTERIZATION AND VALORISATION OF MOUNTAIN PLANT GENETIC RESOURCES

BIOS-01/C

DR. DAVIDE PEDRALI

SUPERVISOR: PROF. ANNAMARIA GIORGI
CO-SUPERVISOR: PROF. VERA LAVELLI

ACADEMIC YEAR 2024/2025

PHD PROGRAMME IN ENVIRONMENTAL SCIENCES
Department of Environmental Science and Policy

UNIVERSITÀ DEGLI STUDI DI MILANO

PHD COURSE IN ENVIRONMENTAL SCIENCES
XXXVIII CYCLE

DIPARTIMENTO DI SCIENZE E POLITICHE AMBIENTALI

CHARACTERIZATION AND VALORISATION OF MOUNTAIN PLANT GENETIC RESOURCES

BIOS-01/C

DAVIDE PEDRALI
MATR. R13983
ORCID N. 0000-0002-5522-0866

TUTOR: PROF.SSA ANNAMARIA GIORGI
COTUTOR: PROF.SSA VERA LAVELLI

PHD COODINATOR: PROF.SSA MARCELLA GUARINO

2024-2025

INDEX

Abstract

Nowadays, there has been a significant global loss of agrobiodiversity, with an estimated 75% decline. Plant agrobiodiversity encompasses wild relatives, landraces, and modern cultivars of agricultural and food interest. Landraces, in particular, are traditional, locally adapted crop varieties that can hold immense value in terms of agronomic traits, phytochemical-nutritional profiles, and resilience to climate change. Many of them exhibit unique nutritional and phytochemical characteristics, especially in terms of secondary metabolites, antioxidants, and micronutrients, making them highly relevant for crop improvement, functional food development, and human health. However, only a limited number of these varieties are currently studied and conserved either *in situ* (on-farm) or *ex situ* (in germplasm banks) leaving many at risk of genetic erosion.

To address these concerns, the European Union (EU) has implemented strategies such as the EU Biodiversity Strategy 2020, the National Recovery and Resilience Plan (PNRR), and the 2030 Agenda for Sustainable Development, all of which emphasize the need for innovative solutions to safeguard agrobiodiversity. These policies encourage the conservation and valorization of traditional cultivars, while promoting circular economy models to counteract genetic erosion in agriculture. In line with these directives, Italy has established the National Register of Agrobiodiversity, an innovative policy tool designed to protect local genetic resources of plant, animal, or microbial origin that are threatened by extinction or erosion.

Italy is rich in agrobiodiversity; in 2020 more than 1600 traditional local cultivars were registered and most of them cultivated in the mountain and submountain areas. Thought, many of these varieties are little or not at all scientifically characterized and, due to their characteristics (biological, ecological and phytochemical), can constitute important resources for the sustainable development of mountain areas. In particular, they could be considered raw materials for the creation of innovative agro-food systems.

The research presented in this thesis focused on the scientific characterization and valorization of four plant genetic resources: the “Copafam” bean, “Carciofo di Malegno” and “Grano Siberiano Valtellinese” landraces and the Italian saffron.

This work is the result of the research activities realized at UNIMONT, the University of Milan’s Hub located in Edolo (BS) dedicated to mountain research, in collaboration with the Department of Agricultural and Environmental Sciences-Production, Landscape and Agroenergy (DISAA) and at the Department of Food, Environmental and Nutritional Sciences (DeFENS) of the University of Milan and at the Department of Nutrition and Food Science of the University of Madrid.

The four case studies presented in this thesis demonstrate the potential of mountain landraces and high-value crops as strategic resources for agrobiodiversity conservation and valorization. The “Copafam” bean (*P. coccineus*), a traditional landrace from the Brescia Pre-Alps, was

characterized by high dietary fiber, the richest phenolic content, strong antioxidant activity, and low levels of antinutritional factors, confirming its potential as a functional food ingredient. Multivariate analyses highlighted its unique profile, while technological trials demonstrated its suitability for bakery products.

Valorization of processing by-products focused on cooking water, which proved to be a source of proteins and phenolics. Protein recovery reached 47% at pH 6, while phenolic retention was markedly higher, up to 85% in enriched systems. Moreover, 97% of externally added rutin was encapsulated, confirming the potential of alginate hydrogels for developing polyphenol-enriched functional ingredients.

In parallel, "Copafam" bean pods was explored, which are typically discarded after seed harvest. Pods, initially rich in insoluble fiber, were effectively transformed through high hydrostatic pressure (HHP) combined with enzymatic hydrolysis, leading to the release of soluble fiber and prebiotic oligosaccharides (raffinose, verbascose, cellobiose). HHP treatments also enhanced the availability of phenolic compounds, with moderate pressures (200 MPa) improving antioxidant potential and higher pressures (400 MPa) releasing bound hydroxycinnamic acids.

Overall, "Copafam" demonstrated value both as a food crop and through its by-products, supporting sustainable valorisation pathways for mountain agrobiodiversity.

The "Carciofo di Malegno" was morphologically different from commercial cultivars; it resulted belong to the "spinosi" group. Phytochemical characterization revealed appreciable levels of chlorogenic acid, cynarine, luteolin, and apigenin, comparable to those of commercial varieties, with edible capitula showing the highest concentrations. Interestingly, stems and outer bracts, usually discarded as waste, were also found to contain relevant amounts of these compounds, highlighting opportunities for circular economy valorization. Ecological niche modeling predicted an upward altitudinal shift in cultivation suitability under future climate scenarios, raising concerns for in *situ* conservation in the traditional lowland areas but also identifying new opportunities for mountain cultivation. On the valorization side, the landrace was submitted for inclusion in the Italian National Register of Agrobiodiversity, securing its legal recognition and protection. In parallel, participatory innovation initiatives, such as the development of leaf-based spirit prototypes in collaboration with local stakeholders and their successful sensory evaluation, demonstrated the potential of this landrace to generate added value through new agri-food products. These results underline the dual cultural and economic importance of the "Carciofo di Malegno", showing how scientific characterization, conservation tools, and community-driven valorisation can converge to strengthen biodiversity conservation and support rural development in mountain regions.

The "Grano Siberiano Valtellinese" showed a distinctive nutritional and phytochemical profile, with high protein, fiber, and quercetin content. Extraction studies confirmed that hydroalcoholic solvents maximized flavonol recovery, particularly quercetin and rutin, while aqueous systems favored polar phenolic acids due to rutinosidase activity. Antioxidant assays (Folin, FRAP, ORAC) reinforced the strong bioactive potential of this landrace. Valorization of its by-product (straw) demonstrated that high hydrostatic pressure combined with enzymatic hydrolysis enhanced the release of soluble fibers and bound phenolic acids, improving antioxidant activity. These findings highlight the potential of this neglected crop both as a functional food and as a source of value-added ingredients in circular economy models.

Finally, Italian saffron quality analysis confirmed an overall excellent quality according to ISO 3632 classification (93% of samples were found to be of the first quality category). Non-destructive approaches, such as NIR spectroscopy combined with chemometric models, also proved highly effective in predicting quality parameters, offering valuable tools for rapid and sustainable monitoring. Ecological niche modelling projected a future upward shift of suitable cultivation areas, indicating that saffron will increasingly become an exclusive crop of mountain regions. For the valorization aspects, a new first category ISO 3632 subclassification ("premium", "superior", "high-quality") was proposed to better reward excellence. Valorization was further supported by participatory networks, producer engagement, and educational initiatives, reinforcing both competitiveness and sustainability of this niche mountain crop.

These results highlight how scientific characterization, advanced analytical tools, and participatory valorization strategies can transform underutilized landraces and crops into key drivers for sustainable rural development, functional food innovation, and the long-term conservation of mountain agrobiodiversity.

1. Introduction

1.1 *Agrobiodiversity and the Strategic Role of Mountain Landraces*

One of the world's greatest challenges in the 21st century is to secure access to adequate supplies of healthy, safe, and high-quality food for all, while ensuring that agricultural systems operate within the limits of environmental sustainability. This challenge is made more urgent by the intertwined crises of biodiversity loss, climate change, and the degradation of natural resources. Achieving this goal requires placing the sustainable management of natural capital at the core of agri-food strategies, ensuring that agricultural production not only feeds populations but also safeguards ecosystems and the biodiversity they host.

Agrobiodiversity, defined as the variety and variability of animals, plants, and microorganisms used directly or indirectly for food and agriculture, is a critical component of this natural capital (FAO, 1999). It encompasses diversity at the genetic, species, and ecosystem levels, and underpins the resilience of agro-ecosystems to biotic and abiotic stresses. Beyond ecological considerations, agrobiodiversity plays a pivotal role in cultural identity, dietary diversity, and rural livelihoods. However, this vital resource is under severe threat. According to the FAO, over the last century, more than 75% of global agrobiodiversity has been lost, and today, three-quarters of the world's food supply relies on only twelve plant species and five animal species (Esquinas-Alcázar, 2005; FAO, 2010). Similarly, the IUCN has estimated that around 70% of plant species are at risk of extinction (Vié et al., 2019).

Within plant genetic resources for food and agriculture, landraces (also referred to as local varieties or traditional cultivars) are the most vulnerable components. Landraces are dynamic populations of cultivated plants with a historical origin, distinct identity and no formal breeding. These are typically adapted to the local environmental conditions through farmer selection over generations (Villa et al., 2005; Spataro & Negri, 2013) and they often exhibit high genetic diversity, resilience to biotic and abiotic stresses making them valuable for use in breeding programs. Their erosion represents not only a loss of ecological resilience but also endangers traditional knowledge systems and cultural heritage associated with these traditional crops cultivation (Losa et al., 2025).

Many landraces possess traits that make them particularly relevant in the current context of climate change. Their ability to thrive in marginal environments, tolerate climatic extremes, and display unique nutritional or phytochemical profiles (including bioactive compounds with antioxidant or health-promoting properties) makes them highly valuable for the development of functional foods and high-value niche products. Beyond their dietary value, diversified practices based on landraces can contribute to soil conservation, provide habitats for pollinators, and sustain both ecological services and the socio-economic vitality of rural territories, especially in mountain and marginal areas.

In fact, mountain regions are recognized as important reservoirs of agrobiodiversity. Their environmental heterogeneity, relative isolation, and the persistence of traditional small-scale farming systems have allowed the survival of many locally adapted cultivars that were lost in lowland areas due to industrial agriculture, urbanization, and land-use change (Giorgi & Scheurer, 2015; Giupponi et al., 2020, 2021; Falcione et al., 2022). Despite this potential, the survival of mountain landraces is increasingly threatened by the abandonment of traditional farming, the expansion of standardized commercial varieties, the socio-economic marginalization of rural areas, and the additional pressures of climate change. In the Alps and Apennines, rural depopulation and generational turnover have reduced cultivated plant diversity and depleted local knowledge (Cislaghi et al., 2019; Didonna et al., 2024).

Italy is rich in agrobiodiversity, particularly in mountain and sub-mountain areas. However, many of these varieties remain poorly studied or undocumented, and only a limited number have been effectively conserved (Giupponi et al., 2021). Recent census activities have registered more than 1,600 traditional local varieties, underscoring the scale of this heritage but also the urgent need for deeper scientific characterization and valorization strategies.

In addition to staple food crops, officinal plants (cultivated for their medicinal, aromatic, or spice properties) represent another valuable component of mountain agrobiodiversity. Their cultivation generally requires low inputs, can be integrated into diversified farming systems, and generates high-value products for niche markets in herbal medicine, cosmetics, and gastronomy. Saffron (*Crocus sativus* L.), for example, is a high-value spice that thrives in several Italian mountain and marginal areas (Caser et al., 2018), where it contributes to multifunctional farm models that integrate agriculture with agritourism and local cuisine. Like landraces, saffron production can be linked to territorial identity and marketed as a premium product.

Preserving and enhancing these mountain plant genetic resources (landraces and officinal plants) is therefore not only a matter of ecological or cultural heritage but also a strategic pathway towards sustainable rural development. By maintaining a diversified agricultural base, these territories can respond more effectively to climate variability, diversify income sources, and sustain rural populations through niche market opportunities.

1.2 *From Conservation to Valorisation: A Multidisciplinary Pathway*

The conservation of agrobiodiversity has been recognized as a priority by numerous international, European, and national policy frameworks. Two main strategies are recognized: *in situ* conservation (on farm), which maintains varieties within their traditional farming systems, and *ex situ* conservation, which preserves genetic material in seed banks, field collections, or botanical gardens. While *ex situ* approaches are important for safeguarding germplasm, *in situ* conservation is critical for maintaining the dynamic evolution of landraces in response to environmental and socio-economic changes.

The EU Biodiversity Strategy, the 2030 Agenda for Sustainable Development, and the European Green Deal's Farm to Fork strategy all highlight the importance of protecting genetic resources, diversifying agricultural production and supports *in situ* conservation encouraging the role of "custodian farmers" (EU Commission, 2010; United Nations, 2015). In Europe, specific tools such as the European Register of Conservation Varieties aim to halt the loss of genetic resources and support their sustainable use (EU Commission, 2023).

Italy has complemented these efforts with Law No. 194/2015, which created the National Register of Agrobiodiversity (Ministerial Decree 2019/39407; https://rica.crea.gov.it/APP/anb/). This register was established in December 2019 with the objective of protecting and promoting agricultural and food resources from the risk of genetic erosion and extinction. It contains detailed information (morphological, agronomic, and historical documentation, as well as evidence of cultivation in the traditional area) on landraces submitted by the regions.

Registration provides formal recognition and legal protection, creating opportunities for promotion and funding. However, the system remains underutilized only 11 of Italy's 20 regions have recorded conservation varieties (traditional cultivars), and most entries are tree species; herbaceous landraces, particularly in mountain areas, are still poorly represented (Giupponi et al., 2020). This highlights the need for a better evaluation of the effectiveness and accessibility of such regulatory tools in the coming years and increase the awareness of citizens on agrobiodiversity conservation connecting researchers, population, seed savers and farmers (Didonna et al. 2024; Bocci et al. 2025)

An essential step for the registration of landraces at both national and European levels is the provision of a detailed varietal description following internationally recognized standards. The UPOV (International Union for the Protection of New Varieties of Plants) guidelines, which define Distinctness, Uniformity, and Stability (DUS) descriptors, provide the official framework and form the basis for the "Technical Questionnaire" required for registration and crucial to distinguish landraces from other varieties and to guarantee their traceability within conservation programs.

However, conservation alone is not sufficient: valorization is essential to ensure that landraces remain viable in modern agri-food systems. Without economic relevance, conservation efforts risk becoming isolated and unsustainable. Valorisation means transforming landraces from underutilised genetic resources into products and services that generate income, sustain rural livelihoods, and reinforce cultural identity but this requires a multidisciplinary approach integrating scientific research, farmer participation, and consumer engagement.

From this perspective, the transition from conservation to active use is key. Landraces should be integrated into viable value chains, new products should be developed to enhance their unique traits, and their profiles aligned with current consumer trends toward functional foods and sustainable diets (Firoozzare et al., 2024). To do this, Through European Rural Development Programmes (RDPs), training schemes, and cooperative networks, young farmers can be encouraged to engage in landrace cultivation, counteracting depopulation and ensuring the intergenerational transmission of traditional knowledge.

At the same time, in Italy some farmers have formed cooperatives, associations or consortia to conserve and promote traditional cultivars (Negri 2003; Piergiovanni et al., 2010; Montesano et al. 2012; Raggi et al. 2022; Colombo et al. 2022). Examples include the successful recovery of landraces like “Nero Spinoso maize” (Cassani et al., 2017) or “Sponcio maize” (Fenzi et al., 2022). These networks not only promote conservation but also foster social cohesion and local development by enabling the exchange of seeds, knowledge, and marketing strategies. These collective structures have proven particularly valuable because most traditional varieties are still maintained by elderly hobby farmers, who often cultivate them on a very small scale and outside any formal network (Negri, 2003; Colombo et al., 2022).

In addition, these traditional cultivars also could serve as key instruments for the valorisation of mountain territories, since their unique sensory and nutritional profiles are strongly shaped by the environments in which they evolved and the conditions under which they are cultivated. They therefore carry a distinctive "environmental fingerprint," making them unique and closely tied to their territories of origin.

Ex situ conservation tools, such as seedbanks and genebanks, complement on-farm efforts by safeguarding germplasm for future use. In Italy, organisations like “Rete Semi Rurali” maintain collections of traditional cultivars and can redistribute them to farmers committed to their cultivation in purity (Alicandri et al. 2024; Bocci et al. 2025). Linking *ex situ* resources with on-farm conservation creates a dynamic conservation model that maximises both security and adaptability (Didonna et al. 2024).

Ultimately, conservation and valorisation are not separate processes but two sides of the same strategy. Scientific characterisation generates the data required for registration and market promotion, farmer involvement ensures continuity of cultivation, and consumer demand drives sustainability. For mountain landraces, this integrated approach can transform them from underutilised resources into strategic assets for resilient, sustainable agri-food systems.

1.3 *Bibliography*

Alicandri, E., Paolacci, A. R., Coluccia, L., Marcomeni, M., Taviani, P., & Ciaffi, M. (2024). Exploring molecular, morphological, and biochemical diversity of Phaeolus vulgaris landraces cultivated in the Aniene Valley (Lazio region, Italy). Current Plant Biology, 39, 100374. https://doi.org/10.1016/j.cpb.2024.100374

Bocci, R., Bartha, B., Maierhofer, H., Arndorfer, M., & Salvan, M. (2025). Community seedbanks in Europe: Their role between ex situ and on-farm conservation. Genetic Resources, S2, 147–161. https://doi.org/10.46265/genresj.OHNK3179

Caser, M., Victorino, Í. M. M., Demasi, S., Berruti, A., Donno, D., Lumini, E., Bianciotto, V., & Scariot, V. (2018). Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds. Agronomy, 9(1), 12. https://doi.org/10.3390/agronomy9010012

Cassani, E.; Puglisi, D.; Cantaluppi, E.; Landoni, M.; Giupponi, L.; Giorgi, A.; Pilu, R. Genetic studies regarding the control of seed pigmentation of an ancient European pointed maize (Zea mays L.) rich in phlobaphenes: The "Nero Spinoso" from the Camonica valley. Genet. Resour. Crop Evol. 2017, 64, 761–773

Cislaghi A, Giupponi L, Tamburini A, Giorgi A, Bischetti GB (2019) The effects of mountain grazing abandonment on plant community, forage value and soil properties: observations and field measurements in an alpine area. CATENA 181:104086. https://doi.org/10.1016/j. catena.2019.104086

Colombo, F., Franguelli, N., Licheri, G., Ghidoli, M., Cassani, E., Castelli, L., Pasquali, M., Bresciani, A., Marti, A., Dell'Anno, M., Rossi, L., De Negri, I., Landoni, M., & Pilu, R. (2022). Agriculture in Marginal Areas: Reintroduction of Rye and Wheat Varieties for Breadmaking in the Antrona Valley. Agronomy, 12(7), 1695. https://doi.org/10.3390/agronomy12071695

Didonna, A., Bocci, R., Renna, M., & Santamaria, P. (2024). The Conservation Varieties Regime: Its Past, Present and Future in the Protection and Commercialisation of

Vegetable Landraces in Europe. Horticulturae, 10(8), 877. https://doi.org/10.3390/horticulturae10080877

Esquinas-Alcázar, J. (2005). Protecting crop genetic diversity for food security: Political, ethical and technical challenges. Nature Reviews Genetics, 6(12), 946–953. https://doi.org/10.1038/nrg1729

EU Commission (2017) Region Innovation Monitor Plus. https://ec.europa.eu/growth/tools-databases/regional-innovation-monitor/base-profile/lombardy. Accessed 11 April 2024

EU Commission. EUROPE 2020 a Strategy for Smart, Sustainable and Inclusive Growth. 2010. Available online: https: //eurlex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52010DC2020 (accessed on 11 September 2024).

Falcione, M., Simiele, M., Renella, A., Scippa, G. S., Di Martino, P., & Trupiano, D. (2022). A Multi-Level Approach as a Powerful Tool to Identify and Characterize Some Italian Autochthonous Common Bean (Phaseolus vulgaris L.) Landraces under a Changing Environment. Plants, 11(20), 2790. https://doi.org/10.3390/plants11202790

FAO. Agricultural biodiversity. Background Paper 1. In Proceedings of the FAO/Netherlands Conference: “The Multifunctional Character of Agriculture and Land”, Maastricht, The Netherlands, 12–17 September 1999.

FAO (2010) Second Report on the State of the World’s Plant Genetic Resources for Food and Agriculture. Commission on Genetic Resources for Food and Agriculture; Food and Agriculture Organization of the United Nations: Rome, Italy, 2010.

Fenzi, M., & Couix, N. (2022). Growing maize landraces in industrialized countries: From the search for seeds to the emergence of new practices and values. International Journal of Agricultural Sustainability, 20(3), 327–345. https://doi.org/10.1080/14735903.2021.1933360

Firoozzare, A., Boccia, F., Yousefian, N., Ghazanfari, S., & Pakook, S. (2024). Understanding the role of awareness and trust in consumer purchase decisions for healthy food and products. Food Quality and Preference, 121, 105275. https://doi.org/10.1016/j.foodqual.2024.105275

Giorgi, A., & Scheurer, T. (2015). Alpine Resources: Assets for a Promising Future—Conclusions from the ForumAlpinum 2014. Mountain Research and Development, 35(4), 414–415. https://doi.org/10.1659/MRD-JOURNAL-D-15-00061

Giupponi, L., Pedrali, D., Leoni, V., Rodari, A., & Giorgi, A. (2021). The Analysis of Italian Plant Agrobiodiversity Databases Reveals That Hilly and Sub-Mountain Areas Are Hotspots of Herbaceous Landraces. Diversity, 13(2), 70. https://doi.org/10.3390/d13020070

Giupponi, L., Pilu, R., Scarafoni, A., & Giorgi, A. (2020). Plant agro-biodiversity needs protection, study and promotion: Results of research conducted in Lombardy region (Northern Italy). Biodiversity and Conservation, 29(2), 409–430. https://doi.org/10.1007/s10531-019-01889-3

Losa, A., Sala, T., Toppino, L., Fricano, A., Rossi, G., Gipli, V., & Landoni, M. (2025). Genetic Diversity and Distinctiveness of Common Beans (Phaseolus vulgaris L.) Between Landraces and Formal Cultivars Supporting Ex Situ Conservation Policy: The Borlotti Case Study in Northern Italy. Agronomy, 15(4), 786. https://doi.org/10.3390/agronomy15040786

Montesano, V., Negro, D., Sarli, G., Logozzo, G., & Spagnoletti Zeuli, P. (2012). Landraces in Inland areas of the Basilicata region, Italy: Monitoring and perspectives for on farm conservation. Genetic Resources and Crop Evolution, 59(5), 701–716. https://doi.org/10.1007/s10722-011-9712-7

Negri, V. (s.d.). Landraces in central Italy: Where and why they are conserved and perspectives for their on-farm conservation.

Pedrali, D., Zuccolo, M., Giupponi, L., Sala, S., & Giorgi, A. (2024). Characterization and Future Distribution Prospects of “Carciofo di Malegno” Landrace for Its In Situ Conservation. Plants, 13(5), 680. https://doi.org/10.3390/plants13050680

Piergiovanni, A. R., & Lioi, L. (2010). Italian Common Bean Landraces: History, Genetic Diversity and Seed Quality. Diversity, 2(6), 837–862. https://doi.org/10.3390/d2060837

Raggi, L., Pacicco, L. C., Caproni, L., Álvarez-Muñiz, C., Annamaa, K., Barata, A. M., Batir-Rusu, D., Díez, M. J., Heinonen, M., Holubec, V., Kell, S., Kutnjak, H., Maierhofer, H., Poulsen, G., Prohens, J., Ralli, P., Rocha, F., Rubio Teso, M. L., Sandru, D., … Negri, V. (2022). Analysis of landrace cultivation in Europe: A means to support in situ conservation of crop diversity. Biological Conservation, 267, 109460. https://doi.org/10.1016/j.biocon.2022.109460

Spataro, G., & Negri, V. (2013). The European seed legislation on conservation varieties: Focus, implementation, present and future impact on landrace on farm conservation. Genetic Resources and Crop Evolution, 60(8), 2421–2430. https://doi.org/10.1007/s10722-013-0009-x

United Nations. Transforming Our World: The 2030 Agenda for Sustainable Development. 2015. Available online: https: //undocs.org/A/RES/70/1 (accessed on 11 September 2024).

Villa, T. C. C., Maxted, N., Scholten, M., & Ford-Lloyd, B. (2005). Defining and identifying crop landraces. Plant Genetic Resources, 3(3), 373–384. https://doi.org/10.1079/PGR200591

EU Commission (2017) Region Innovation Monitor Plus. https://ec.europa.eu/growth/tools-databases/regional-innovation-monitor/base-profile/lombardy. Accessed 11 April 2024

EU Commission. EUROPE 2020 a Strategy for Smart, Sustainable and Inclusive Growth. 2010. Available online: https: //eurlex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52010DC2020 (accessed on 11 September 2024).

United Nations. Transforming Our World: The 2030 Agenda for Sustainable Development. 2015. Available online: https: //undocs.org/A/RES/70/1 (accessed on 11 September 2024).

Cislaghi A, Giupponi L, Tamburini A, Giorgi A, Bischetti GB (2019) The effects of mountain grazing abandonment on plant community, forage value and soil properties: observations and field measurements in an alpine area. CATENA 181:104086. https://doi.org/10.1016/j. catena.2019.104086

2. Aims

The main aim of this thesis was to scientifically characterize neglected plant genetic resources from mountain areas, for which little or no data were previously available. By adopting a multidisciplinary approach, the research sought to document their unique morphological, nutritional, and phytochemical traits shaped by the specific pedoclimatic conditions of their environments. This characterization provides the basis for their valorization, conservation, and potential registration in national and European agrobiodiversity registers, while emphasizing their role as distinctive expressions of the territories where they evolved.

More specifically, the work focused on:

- Copafam bean (*Phaseolus coccineus* L.): to characterize both the seeds and by-products (pods and cooking water) from nutritional and phytochemical perspectives, and to explore innovative valorisation strategies such as high hydrostatic pressure, enzymatic hydrolysis, and alginate encapsulation, with the dual aim of enhancing functional properties and supporting circular economy models.
- Carciofo di Malegno (*Cynara cardunculus* subsp. Scolymus): to provide a morphological and phytochemical characterization of this rare artichoke landrace and to test its potential through the development of innovative food products, combining scientific data with local gastronomy and participatory approaches to foster community-based valorisation.
- Grano Siberiano Valtellinese (*Fagopyrum tataricum* Gaertn.):to assess the nutritional composition, dietary fibre fractions, and polyphenolic profile of this underutilized buckwheat landrace, and to evaluate the application of green processing technologies for the release of bioactive compounds, thereby promoting its recovery and integration into local agri-food chains.
- Saffron (*Crocus sativus* L.): to evaluate the quality traits of Italian saffron through ISO 3632 classification and NIR spectroscopy, and to model its ecological niche under climate change scenarios, while strengthening producer networks as a tool for both market valorization and in situ conservation.

Through this integrated framework, the thesis aimed to demonstrate that scientific characterization, when coupled with valorization strategies, can ensure the effective conservation of mountain landraces, generating new knowledge that support their registration in official databases, their sustainable use, and their role in the socio-economic development of rural and marginal territories.

This work is the result of the research activities realized at UNIMONT, the University of Milan's Hub located in Edolo (BS) dedicated to mountain research, in collaboration with the Department of Agricultural and Environmental Sciences-Production, Landscape and Agroenergy (DISAA) and at the Department of Food, Environmental and Nutritional Sciences

(DeFENS) of the University of Milan and at the Department of Nutrition and Food Science of the University of Madrid.

3. Thesis organization

The body of the thesis has been divided into four main chapters (study cases) related to the main research lines followed during the doctoral activites:

I. "Copafam" Bean landrace
II. "Carciofo di Malegno" landrace
III. "Grano Siberiano Valtellinese" landrace
IV. Italian saffron

Each chapter opens with an introduction that contextualizes the specific topic, followed by three main sections: 1) Characterization, presenting the results and discussion related to the morphological, agronomic, nutritional, and/or phytochemical characterization of the landrace or plant considered; 2) Valorization, reporting the results and discussion of the research activities aimed at enhancing its utilization and potential applications promoting its conservation and 3) Conclusion and future activities, which summarize the main findings and outline possible directions for further research and valorization strategies. Each chapter concludes with its bibliography and a collection of the candidate's publications and/or original data generated during the Ph.D. activities.

The study case I is composed of the following articles/original data:

- Article 1: The bean (*Phaseolus* spp.) landraces of the Lombardy Alps (Northern Italy): characterization and prospects for their valorization. (page 65)
- Article 2: Nutritional Characterization and Novel Use of "Copafam" Bean (*Phaseolus coccineus* L.) for the Sustainable Development of Mountains Areas. (page 107)
- Article 3: Binary Alginate-Whey Protein Hydrogels for Antioxidant Encapsulation. (page 127)
- Original data 1: Recovery proteins and phenolics of Phaseolus aquafaba in alginate microbeads – A circular approach to develop innovative food applications of a Copafam bean landrace. (In draft)
- Original data 2: Valorization of "Copafam" Bean (*Phaseolus coccineus* L) landrace byproduct by High Hydrostatic Pressure and Enzymatic Hydrolysis: A Circular Approach to Functional Ingredient Development. (In draft)

The study case II is composed of the following paper:

- Article 4: Characterization and Future Distribution Prospects of "Carciofo di Malegno" Landrace for Its *In Situ* Conservation (page 154)

The study case III is composed of the following paper:

- Original data 3: Combined Strategy Using High Hydrostatic Pressure and Enzymatic Hydrolysis for the Release of Soluble Fibre and Phenolics from landrace By-Products: the case of "Grano Siberiano Valtellinese". (In draft)

The study case IV is composed of the following papers:

- Article 5: Progress in quality assessment of italian saffron. (page 194)
- Article 6: The species distribution models reveal that mountain areas will be the most suitable for *Crocus sativus* L. in Italy. (page 205)

4. Materials and methods

4.1 Case study I: "Copafam" Bean

Article 1

Plant material

Based on the census of Lombardy landraces by Giupponi et al. (2020), 16 bean landraces were identified in Lombardy (Northern Italy; Latitude N: 47°1.122'; Longitude E: 12°20.553') between 2020 and 2022.

These landraces have been cultivated in mountainous areas for over 30 years by local farmers, mainly hobbyists, primarily for self-consumption. Twelve landraces were *P. vulgaris* L. while only four were *P. coccineous* L. Plant identification was conducted using the dichotomous keys of Pignatti (2017) and the landrace data collected was verified to ensure it matched the characteristics outlined by Camacho Vila et al. (2005).

In 2023, the seeds of the 16 landraces were cultivated again in their original fields, following traditional farming practices under the supervision of researchers. This approach aimed to prevent potential hybridizations or phytopathological issues and allowed for the collection of morphological and agronomic data for each landrace. Approximately 500 grams of each landrace were allocated for genetic analysis, and for morphometric and nutritional assessments of the seeds. For comparison, two commercial *P. vulgaris* varieties (Cannellino and Borlotto), purchased at a local market, were cultivated in two experimental fields.

Genetic analysis

The genetic analysis was performed on the DNA extracted from the leaves of the 18 varieties (16 landraces and the two commercials' beans) using 20 molecular markers SSRs.

For each variety, two seeds were germinated in the laboratory under controlled conditions and their leaves were sampled and crushed and then used for DNA extraction following the Steve1 method modified (Ghidoli et al., 2024).

Out of 20 SSRs we used 15 markers (PV128, PV131, PV167, BM153, and BM187 were discarded) that showed the most robust amplification throughout all the work.

PCR reactions were performed in a thermocycler, where the amplification conditions consisted of an initial denaturation cycle at 94°C for 2' followed by 35 denaturation cycles for 1' at 94°C and extension for 1' at 72°C, with a final extension cycle for 5' at 72°C.

By electrophoresis, the amplified fragments were fractionated using 4% agarose gel and stained with ethidium bromide. The amplifiers were outsourced to BMR Genomics for sizing. The results obtained were used to compile the binary data matrix used to generate the Principal Coordinate Analysis (PCoA) using the GenAlEx 6.5 program. Finally, a cluster analysis using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm was performed to evaluate the similarity among landraces and commercial varieties.

Ecological and agronomic analysis

The analysis of the competitor, stress-tolerator, ruderal (CSR) functional strategy of Grime (Grime 2001) of all 18 genotypes was performed according to the method proposed by Pierce et al. (2017). In detail, 10 fully expanded leaves of each genotype were collected in the experimental fields during July 2023. The leaf samples were collected from different plants considering those without disease. All the 10 leaf samples collected were wrapped in moist paper and stored in a refrigerator at 4 °C for one night. Leaf fresh weight was measured from these saturated organs using an analytical weight scale (Precisa XB 220A, 0.0001 g). The beans leaves were digitized with a digital scanner (Samsung X3280NR) and ImageJ software (Lattanzio et al., 2009; Spararo et al., 2013;Schneider et al., 2012) was used to calculate their leaf area. Beans dry weight was measured after oven drying (105 °C for 24 h). CSR values and functional strategy were determined using 'StrateFy' spreadsheet (Pierce et al., 2017) and were plotted in the CSR ternary graph using the 'ggplot2' package of R software (R Core Team 2024).

In field, phenotypic data were collected for all landraces through the completion of UPOV morphological descriptors. These forms provide essential information for the potential registration of these varieties in official biodiversity or conservation registers.

Geometric morphometric analysis

All landraces seed samples and those of the two commercial beans collected from local markets were used for the elliptical Fourier descriptors analysis (Giupponi and Giorgi 2019). For each genotype, 30 seeds were randomly selected. Each seed sample was placed on a white support table and photographed using a Canon EOS 2000D digital camera positioned perpendicular to the support surface. The images of the beans were processed using Adobe Photoshop software: the shadows were removed, and the images were transformed into black and white. The outline coordinates were extracted with Momocs 1.4.0 (Bonhomme et al., 2014; Claude, 2008) in an R environment (R Core Team 2024) and converted into Fourier coefficients considering 8 harmonics that gathered at least 99% of the total harmonic power (Bonhomme et al., 2014). In order to control left/right asymmetry, the samples were flipped in the same direction, and then a landmark was defined at their base as a starting point for importing outline coordinates. The contours were centered, and the outline analysis was performed without numerical normalization. Principal Component Analysis (PCA) was conducted on the matrix of coefficients to reduce dimensionality, and the samples were plotted on the first two axes (principal components). Linear discriminant analysis of principal components (DAPC)

(Jombart et al., 2010) was performed. The mean shape of the beans of each genotype was reconstructed using the MSHAPES function of Momocs and multivariate analysis of variance (MANOVA) was performed to evaluate the differences in beans shape.

Proximate composition of the seeds

Approximately 300g of seeds collected from the field were milled into flour for nutritional analysis. Bean whole flours were obtained by finely grinding the seeds with a commercial blender (IKA A10 basic, Werke GmbH & Co.KG, Staufen, Germany). All plant material was collected in compliance with national and international mandatory regulations.

The total Starch content was measured according to the AACC 76-13.01 method; values were expressed as g/100 g of flour.

The protein content was determined with the Kjeldahl method in according to International Standard ISO 20483:2013. The conversion factor used to transform nitrogen into protein was 6.25 and the results were expressed as g/100g.

Lipid content was determined by extracting 0.5 g of flour with 5 mL of pentane under agitation at 37 °C for 2.5 hours. Following extraction, samples were placed in an oven at 40 °C overnight to ensure complete solvent evaporation. Lipid content was calculated using the following equation:

$$\% \, lipids = \frac{mi - mf}{mi} \times 100 \tag{1}$$

Where mi is the initial mass, and mf is the final mass.

The method was inspired by gravimetric lipid determinations described in AOAC protocols (AOAC 920.39), with pentane used as an alternative solvent.

Trypsin inhibitor activity (TIA) was performed following ISO 14902:2001 assay. The inhibition percentage of the sample extract solution was calculated as:

$$I = \frac{(At - Abt) - (As - Abs)}{(At - Abt)} \tag{2}$$

where I is the inhibition percentage, At is the absorbance of the solution with trypsin only, Abt is the absorbance of the blank trypsin only, As is the absorbance of the solution with the sample and Abs is the absorbance of the blank sample.

The trypsin inhibitor activity is calculated as:

$$TIA = \frac{(I \times mt \times ds)}{(100 \times ms)}$$

(3)

where *mt* is the mass of trypsin used in the assay, in milligrams, *ds* is the dilution factor of the sample and *ms* is the mass of the test sample used for the assay.

The content of phytic acid and Raffinose Series Oligosaccharides (RSO) were determined using the Megazyme assay kit (K-PHYT and K-RAFGL, respectively; Megazyme International Ireland Ltd, Wicklow, Ireland). The results were expressed as mg of phytic acid and g of RSO per g of bean flour.

Total phenolic content and antioxidant assessment

The total polyphenol content was analyzed using the Folin-Ciocalteau spectrophotometric method (Singleton et al. 1999) Briefly, 0.1 g of flour was suspended in 2 ml of methanol (ratio 1:20) and stirred for 20 minutes at room temperature. After the extraction, it was centrifuged at 2000 rpm for 10 minutes at 20°C. The procedure involved mixing 0.2 mL of the extract with 0.2 mL of Folin reagent and 3 mL of distilled water. Subsequently, 0.75 mL of sodium carbonate was added. The mixture was incubated at room temperature for 8 minutes. Afterwards, 0.85 mL of distilled water was added, followed by homogenization and incubation in the dark for 2 hours at room temperature. The absorbance was then measured at 765 nm and the results were expressed as mg GAE (Gallic Acid Equivalent)/g of flour.

The determination of total flavonoids was performed by aluminium chloride test (Alcázar-Valle et al., 2020). The procedure involved mixing 0.05 mL of extract (described above), 0.7 mL of distilled water, and 0.25 mL of $AlCl3$ (133 mg of $AlCl3$ and 400 mg of sodium acetate in 100 mL of methanol/water/acid acetic (140:50:10 v /v/v)). The absorbance was measured at 415 nm, and the results were expressed as mg of catechin/g of flour.

The antioxidant activity of samples was evaluated using the Fe (III)/tripyridyltriazine complex (FRAP), following the procedure of Benzie and Strain (1996). Briefly, 1 g of flour is suspended in 20 mL of extraction buffer, consisting of 80% ethanol (1:20 ratio). The mixture is stirred for 24 hours at room temperature. After extraction, it is centrifuged at 2370 rpm at 4°C for 30 minutes. Firstly, the FRAP reagent was prepared by mixing 300 mM acetate buffer pH 3.0 (sodium acetate+ acetic acid), 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, and 2.5 ml of 20 mM $FeCl3$ for a final volume of 20 ml. 10 µl of extract was added to 30 µl of water, and 300 µl of FRAP reagent, previously warmed to 37°C. A sample blank was also prepared by adding 10 µl of extraction buffer instead of the sample. Absorbance was read at 593 nm and taken after 0.5 s and every 15 s during the monitoring period. The reaction was monitored for up to 8 minutes, but the 4-minute reading were selected to calculate FRAP values. Ferric sulfate ($FeSO4$) concentrations in the range 100-1000 µmol/l were used for calibration. Results were expressed in µmolFe2+/g based on the calibration curve.

Data of proximate analysis and phytochemical profile were analysed using one-way ANOVA with Tukey post-hoc test. The assumptions of homogeneity of variances and normality of group data were verified using the Levene and Shapiro-Wilk's test, respectively. The data were expressed as mean ± standard deviation (SD) of three replicates, and differences were considered statistically significant when $P < 0.05$.

For further methodological details, see Article 1 (page 65).

Article 2

Plant materials

"Copafam" beans were cultivated and collected in licensed agricultural fields directly from farmers of Camonica Valley (Brescia Province, Northern Italy) in 2021. Three commercial beans (Bianco di Spagna, Cannellino and Borlotto) were included as comparison; these beans were bought at a local market. Bean whole flours were obtained by finely grinding the seeds with a commercial blender (IKA A10 basic, Werke GmbH & Co.KG, Staufen, Germany). All plant material was collected complying with national and international mandatory regulations.

Proximate composition

Moisture content of beans flour was determined by oven-drying method at 105°C according to the AOAC method n° 945.40.

Total nitrogen content was analyzed by the Kjeldahl procedure (AOAC method n° 979.09), the conversion factor used to transform nitrogen into protein was 6.25.

Ash content was determined by incineration at 550 °C in a microwave muffle (ZE muffle furnace, Ettore Pasquali s.rl., Milano, Italy) followed the AACC Method.

Lipid content was determined using the chloroform–methanol extraction method (AOAC 983.23), based on the Folch procedure. Five grams of flour were extracted in 30mL chloroform/methanol (2:1, v/v) with agitation at 0°C three times. The extract was vacuum filtered through filter paper on a Büchner funnel. This filtrate was dried in a rotary evaporator at 35°C. The residue was dissolved in 10mL of chloroform/methanol (2:1, v/v) after the addition of 2mL of KCL 0.75%; mixed vigorously and put in a separatory funnel attending the phase separation. The chloroform layer was removed, dried over anhydrous $Na2SO4$, filtered and concentrated by rotary vacuum evaporator at 35°C. The extracted lipids were weighed to determine the lipid content.

Dietary fibre was determined by the enzymatic-gravimetric assay (AOAC method No 991.43). Dietary fibre was isolated from the alcohol-insoluble residue with different enzymes (Sigma–Aldrich, St Louis, MO, USA): Termamyl (pH 6, 100 ◦ C, 30 min), protease (pH 7.5, 60 ◦ C, 30 min) and, finally, amyloglucosidase (pH 4.5, 60 ◦ C, 30 min). The residues (n = 6) obtained were filtered separating the filtrate liquid to analyse soluble dietary fibre (SDF) and the solid residue to deter- mine insoluble dietary fibre (IDF). In both residues, SDF and IDF, the contents of protein, ash and dietary fibre were calculated.

The determination of total starch, phytic acid and RSO content were performed by the Megazyme assay kit (K-TSTA-100A, K-PHYT, K-RAFGL, respectively) following the manufacturer instructions.

The TIA was performed follow ISO 14902:2001.

Assessment of Bioactive Compounds and Antioxidant Activity

Extraction

The sample extraction was performed accordingly to the procedure reported by Al-cázar-Valle (2020). Briefly, 10 g beans flour (dry weight) was mixed overnight with 100 mL of acetone solvent mixture (acetone, water and acetic acid, 70:29:0.5 v/v/v). Then the extracts were centrifuged a 4000 rpm for 15 min (Hermle z300, HERMLE Labortechnik GmbH, Wehingen, Germany), washed with the solvent mixture once and the supernatant was retained. Finally, the extract was concentrated using a rotary evaporator at 45°C (LABOROTA 4000eco, Heidolph InstrumentsGmbH & Co., Schwabach, Germany).

The antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method reported by Brand-Williams. Shortly, 0.3 mL of beans extracts was mixed with 2.7 mL of a DPPH solution 6×10^{-5} M. This mixture and a DPPH blank were incubated 30 min in the dark at room temperature. After this time, the absorbance was measured with a UV/Vis spectrophotometer at 517 nm (Varian Cary 50 scan, Agilent, 5301 Stevens Creek Blvd, Santa Clara, CA, USA). The antioxidant activity was calculated as the percentage of RSA (radical scavenging activity) using the formula:

$$RSA = \left[\frac{AB - AA}{AB}\right] * 100$$

where AB is the absorbance of the DPPH solution and AA is the absorbance of the sample solution.

Folin-Ciocalteu and aluminium chloride test were used to analyze total polyphenols and total flavonoids content as previous described.

The anthocyanins were performed following to the procedure reported by Abdel-Aal (1999). 3 g of beans flour were mixed in 24mL ethanol (0.15% HCL 0.1N). The mixture was shaken for 30 min and centrifuged for 15 min. The supernatant was poured into a 50 mL volumetric flask and made up to volume. The solution was filtered and absorbance was measured at 535 nm against a reagent (solvent) blank. Quantification was expressed as of cyanidin 3-glucoside equivalents per kg of beans flour (mg Cy3G/kg dw).

Extraction for HPLC

The sample extraction was performed according to Rodríguez Madrera (2021). Briefly, 1.5 g of bean flour was extracted with 30 mL ethanol 46 % (0.1 formic acid) overnight. After extraction, the solids were separated by centrifugation and the supernatant was dried in a rotary vacuum evaporator at 50 °C and reconstituted with 2 mL of methanol 20 % (0.1 formic acid).

Basic hydrolysis

The basic hydrolysis was performed following to the procedure reported by Lin (2008). Briefly, 1 mL of extract was dried and dissolved in 0.3 mL of NaOH 4N and magnetic stirred 18 hours at room temperature. 0.15 HCL 12N and 0.55 mL of methanol were added. The solution was filtered through a 0.2 µm pore size membrane filter prior to chromatographic analysis.

The High-Performance Liquid Chromatography (HPLC) system used was a LC Agilent series 1200 (Waldbronn, Germany) consisting of a degasser, a quaternary gradient pump, an auto-sampler and a MWD detector (Waldbronn, Germany). A Luna® C18 (150 x 4.6mm) column (Phenomenex, Santa Clara, USA) at 25 ◦C was used for the chromato-graphic separation. 10 µL of samples were used for injections. The run time was 50 min, with no post run time. Solvent (A) was HCOOH 0.1% while solvent (B) was acetonitrile; a constant flow rate of 0.8 mL/min was used. The gradient used was: 0 min 95% A; 5 min 95% A; 10 min 85% A; 40 min 60% A; 42 min 5% A; 45 min 5% A, 50 min 95% A. and absorbance wavelength was set at 310 nm.

Individual stock solutions of each standard were prepared using absolute ethanol and stored at -20°C. The working standard mixture solutions were made by diluting the appropriate amount of each stock standard solution to obtain 5 calibration levels (range concentration of p-cumaric and sinap acid was 2.5-100 mg/mL while the range concentration of ferulic acid was 5-500 mg/mL). The retention times of all the standards were confirmed by individual standard injections. A fortification of random samples was used to check further the retention factors. A standard mixture to check the retention times was injected each working day. LOD (0.5 µg/mL) and LOQ (1 µg/mL) was calculated ac-cording S/N ratio 3 and 10, respectively.

Statistical analysis

Data were analysed using one-way ANOVA with Tukey post-hoc test. The assumptions of homogeneity of variances and normality of group data and were verified using the Levene and Shapiro-Wilk's test respectively. The data were expressed as mean ± standard deviation (SD) and differences were considered statistically significant when $P < 0.05$. The samples were ordered using principal component analysis (PCA) performed by Stat-graphics 5.1 (STCC Inc.;

Rockville, MD, USA). Hierarchical clustering heatmap and dendrogram of was performed on nutritional and phytochemical trades.

Participants of Consumer study

Eighty subjects (40% men; mean age: 39 ± 15 years) were recruited among students and employees of the Centre of Applied Studies in the Sustainable Management of the Mountain Environment (Ge.S.Di.Mont.) of the University of Milan and among the population of the neighboring municipalities. This study was approved by the Ethics Committee of the University of Milan ad was conducted in compliance with the principles laid down in the Declaration of Helsinki. All participants signed a written informed consent to be involved in the study.

Samples

A biscuit control sample (ST) was developed with the following ingredients: 250 g type 1 wheat flour, 85 g brown sugar, 85 g butter, 20 g of eggs, 4 g baking powder, 50g water, 2 g of baking soda and 1 g common salt. In addition to the control, other six experimental samples were prepared by replacing wheat flour with 25% and 50% of borlotto flour (Bor25 and Bor50), cannellino flour (Can25 and Can50) and copafam flour (Cop25 and Cop50), respectively. The biscuits samples were baked at 180◦C for 15 min. All samples were prepared by a local bakery "Forneria Pasticceria Salvetti" (Malonno, BS, Italy).

Liking assessment and sensory descriptive evaluation

Subjects were asked to taste the biscuits and to express their liking using a 10 cm visual analogue scale (VAS) anchored by the extremes "extremely disliked" (rated 0) and "extremely liked" (rated 10).

In order to obtain a sensory description of the samples, a focus group was preliminary performed to define and select the appropriate attributes (Ares et al., 2010; Jaeger et al., 2015) For each sample, subjects had to evaluate their overall liking and perform a sensory descriptive analysis by means of the check-all-that-apply (CATA) methodology.

Food neophobia evaluation

The validated Italian version of the food neophobia questionnaire (Laureati et al., 2018) was applied to investigate the level of the reluctance to try and eat unfamiliar foods. The questionnaire consists of 10 statements evaluated using a 7-point Likert scale ranging from "I strongly disagree" (score 1) to "I strongly agree" (score 7). The food neophobia level was calculated, after reversing the negatively worded statements, as a sum of the responses yielding a range of 10–70. Each of the 10 statement was. Higher scores reflected higher food neo-phobia levels.

Data analysis

ANOVA model was performed on overall liking scores considering samples (ST, Bor25, Bor50, Can25, Can50, Cop25 and Cop 50) as factor. When a significant difference ($p < 0.05$) was found, the Tukey's HSD test was performed as a multiple comparison test.

Cochran's Q test was applied to identify which sensory attributes were discriminating among samples comparing the frequency of mention for each term of the CATA questionnaire. The relationship between samples and sensory attributes was evaluated by means of correspondence analysis (CA).

The internal consistency reliability of the food neophobia scale was evaluated by means of Cronbach's alpha. To investigate the relationship between food neophobic traits and biscuits liking, subjects were categorized (based on 25th and 75th percentile) according to their neophobia scores into the following three groups: Neophilic (score <19); Neutral (20≤ score ≤33); Neophobic (scores >33). ANOVA model was performed on liking data con-sidering food neophobia traits as factor.

All analyses were performed using IBMSPSS Statistics for Windows, Version 24 (IBMCorp., Armonk, NY, USA) and XLSTAT (Version 2019.2.2, Addinsoft., Boston,MA, USA).

For further methodological details, see Article 2 (page 97).

Article 3

Literature Study

The literature review was conducted using two major databases: Scopus and Web of Science. The search focused on the keywords "alginate" and "whey". Studies describing the development of encapsulation technologies for antioxidants were included, whereas those focused on the encapsulation of microorganisms, proteins, or enzymes were excluded.

Original data 1- valorisation activity

Plant Materials and Chemicals

Cooking water was obtained from two Phaseolus species: the "Copafam" bean and the borlotto bean, a commercially available cultivar purchased from the market. All chemicals and reagents were of analytical grade and purchased from Merck (Milan, Italy).

AQ Recovery

Different batches of dry beans (approx. 50 g) of the "Copafam" and Cranberry varieties were soaked in water at a ratio of 1:33 (w/w) at room temperature for 16 h. The soaking water was then discarded while the soaked seeds were weighed and rinsed with distilled at a ratio of 1:75 (w/w) in a sealed glass bottle. After cooking for 90 min, the bottles were cooled to room temperature and AQ was separated from cooked beans using a centrifuge.

Preparation of AQ - alginate microbeads at different pHs

Microbeads were obtained by a vibrating nozzle method using a Buchi B-390 encapsulator (Buchi Italia, Milan) as described by Lavelli & Sri Harsha (2019).

The inlet solution was prepared by dissolving sodium alginate at a final concentration of 1.6%, in 100 mL of AQ and mixing carefully by a T 25 Ultraturrax (IKA Werke, Staufen, Germany). As hardening solutions, 0.2 M CaCl2 was used. Before gelation, the pH of both the inlet and hardening solutions was adjusted to 3.0, 4.0, 5.0 or 6.0 with 0.1 N HCl.

For preparation of the microbeads, operating conditions were as follows: 300 μm nozzle, nitrogen pressure to deliver the inlet solution to the nozzle: 0.5 bar; vibration frequency used to break up the laminar liquid: 1000 Hz; voltage used to create an electrostatic field between the nozzle and the hardening solution to prevent the coalescence of the microdrops:1400 V; distance between the nozzle and the hardening solution: 20 cm.

The recovered microbeads were allowed to stir in the hardening solution for 10 min for complete hardening. Then, the microbeads were filtered with a Whatman n. 4 paper filter and the filtered hardening solution was recovered. The microbeads were finally washed with water. A total of three replicates were run in each pH condition. For every replicate, the inlet solution, hardening solution and washing solution volumes were 50 mL, 150 mL and 100 mL, respectively and 20.69 ± 4.93 g of microbeads were obtained.

Protein and total phenolics content of AQ

Total phenolic content in AQ samples and hardening solutions was determined by the Folin–Ciocalteu assay, as previously described (Article 1), with minor modifications: 0.5 mL of sample was mixed with 6.0 mL distilled water, 0.5 mL Folin–Ciocalteu reagent, and 3 mL of 10% Na_2CO_3, followed by a 90 min incubation (instead of 2 h). Protein content was also determined according to the previously described Kjeldahl method (ISO 20483:2013; Article 1); in this case, results were expressed as g/100 g of beads or hardening solution.

Encapsulation of rutin in AQ – alginate microbeads

Both the 1.6% alginate and the 0.2 M CaCl2 solution solutions were prepared in the AQ of the "Copafam" variety before gelation and adjusted to pH 6.0 with 0.1 M HCl. In parallel, 1.6%

alginate and 0.2 M $CaCl_2$ solutions were prepared in water and adjusted to pH 6.0 with 0.1 M HCl as a control. Rutin (2.6 g/L) was dissolved in the 1.6% alginate solution in AQ and in 1.6% alginate solution in water. AQ – alginate – rutin and alginate – rutin microbeads were obtained using the same operation conditions as described previously.

Rutin content by spectrophotometric analysis and HPLC

Rutin content was analyzed in the hardening solution of the AQ – alginate – rutin and alginate – rutin microbeads and encapsulated rutin was calculated by difference.

HPLC determination was performed using a model Shimadzu LC-20 AD pump coupled to a model Shimadzu SPD-M20A photodiode array detector (DAD) and an RF-20 AXS operated by Labsolution Software Shimadzu, Kyoto, Japan). A 2.6 µm Kinetex C18 column (150 × 4.6 mm; Phenomenex, Bologna, Italy) was used for the separation, at a flow-rate of 1.5 mL/min. The column was maintained at 40 °C. The separation was performed by means of a linear gradient elution. Eluents were: (A) 0.1% H_3PO_4; (B) acetonitrile. The gradient was as follows: from 6% B to 20% B in 18 min; from 20% B to 60% B in 7 min; from 60% B to 90% B in 19 min; 90% B for 10 min and then 6% B for 5 min. DAD analysis was carried out in the range of 200 – 600 nm. Rutin was identified using pure standard and quantified by the DAD set at 354 nm. Results were expressed as milligrams of rutin per liter of AQ.

Spectrophotometric analyses were performed using a UV–Vis spectrophotometer (Varian Cary 50). Absorbance measurements of the hardening and control solutions were recorded at 360 nm, and quantification was carried out using a rutin standard calibration curve (30–3.75 µg/mL).

Statistical analysis

Data were analysed using one-way ANOVA with Tukey post-hoc test. The data were expressed as mean ± standard deviation (SD) of three replicates, and differences were considered statistically significant when $P < 0.05$.

Original data 2- valorisation activity

Plant materials

Dried “Copafam” bean pods were collected directly from a local farmer (Andrea Messa) at Nasolino municipality located in Lombardy region (Northen of Italy) an altitude of 750m a.s.l. Approximately 500g of bean pods were milled with a commercial blender (IKA A10 basic, Werke GmbH & Co.KG, Staufen, Germany) into flour for nutritional analysis. All plant material was collected in compliance with national and international mandatory regulations.

Nutritional analysis

The moisture, protein and the ash contents of samples were determined according to the AOAC Methods as previous described. Lipid was extracted with diethyl ether solvent in a Soxhlet system (AOAC 963.15) while available carbohydrates were measured using anthrone method (Southgate, 1969). Dietary fibre was determined by the same assay as article 2.

Polyphenol and water-soluble carbohydrates extraction

Polyphenols and water-soluble carbohydrates were extracted from 0.25 g of powdered "Copafam" bean pod, following protocols adapted from Saura-Calixto (1998). For polyphenols, a two-step extraction was performed using 5 mL of acidified (pH 2) methanol–water (50:50, v/v) followed by 5 mL of acetone–water (70:30, v/v) (both solvents from Scharlab, S.L., Sentmenat, Barcelona, Spain). For carbohydrates, the sample was extracted twice with 5 mL of distilled water under stirring (1 h each), with centrifugation at 6000 rpm for 20 min after the first step. Extracts were filtered through syringe filters (45 µm for polyphenols; 0.22 µm for carbohydrates) and stored at −20 °C until analysis.

Total phenolic content and antioxidant assessment

The total phenolic content of the extracts was analysed by Folin–Ciocalteu assay adapted from the methodology reported by Singleton (1999) as previous described with some modifications. Concisely, Folin-Ciocalteu reagent (15 µL) was added to gallic acid standards (0.01–0.28 mg/mL) or samples (15 µL) and they were mixed with 120 distilled water, 30 µL of Na2CO3 and other 120 µL of distilled water. Absorbance readings were performed at 750 nm after 90 minutes of incubation, and results were expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g).

The "Copafam" bean pod extracts were subjected to FRAP and Oxygen Radical Absorbance Capacity (ORAC) assays to assess their antioxidant capacity.

FRAP methodology was performed as mentioned before with same differences: 0,9 µ L of Trolox standards (0.1–1 mmol/L and samples were combined with 26 µ L of distilled water and with 265 µL of FRAP reagent. FRAP reagent was freshly prepared as follows: 25 mL Acetic/acetate buffer 0.3 mol/L pH 3.6, 2.5 mL TPTZ solution 10 mmol/L, and 2.5 mL of Iron (III) chloride solution 0.03 mol/L. Furthermore, it was warmed at 37 C. Absorbance reading were carried out at 595 nm after 30 min of incubation and results were expressed as millimoles of Trolox equivalents (TE) per gram of pod (mmol TE/g).

Oxygen radical absorbance capacity (ORAC) was carried out according to Serra (2011)(Serra et al., 2011). Antioxidant scavenging ability of the different compounds of the sample was evaluated. The method measured the activity against peroxyl radicals (ROO·) generated from AAPH (Sigma-Aldrich Química S.A, Madrid, Spain) using disodium fluorescein (Merck,

Darmstadt, Alemania). Results of antioxidant capacity were expressed as micromoles of Trolox equivalents per gram of pod (µmol TE/g).
All phytochemical measurements were developed on a Synergy™ HTX Multi-Mode microplate reader (Bio-Tek Instruments, Winooski, USA).

HHP treatment assisted by Ultimase® L or Pectinex Yieldmax® L

"Copafam" bean pod was hydrated (4g/40 mL) in water before HHP –aided or not by food-grade enzymes treatments. For the HHP (100, 200 and 400 MPa) plus Ultimase® and Pectinex Yieldmax® (Novozymes Spain, S.A., Pozuelo de Alarcon, Madrid, Spain) procedure, the enzyme was added to the sample in a ratio of 1:40 enzyme: substrate (v/w) and the analyses were performed for 5 min. Control was the sample at atmospheric pressure 0.1 MPa. "Copafam" bean pod samples, without or with added enzyme, were placed in vacuum-sealed plastic bags (200 × 300 mm CRYOVAC, Ref. BB3255; Mam Envases Alimentarios, S.L., Madrid, Spain) following the procedure of De la Pena-Armada (2020). The HHP treatment was applied in a laboratory-scale high-pressure vessel (Stansted SFP 7100:9/2C equipment) sited in ICTAN (CSIC). After the HHP treatment, the samples were centrifugate at 6000 rpm for 20 minutes, filtered through 0.22 µm diameter syringe filters, and stored at -20 °C.

Water soluble carbohydrates analysis

Polysaccharides (100–5.94 kDa MW) oligosaccharides (0.83–0.50 kDa MW) and simple sugars (0.34–0.18 kDa MW) were quantified by HPLC-RID. The column was eluted with ultrapure water (Resistivity 18.2 M Ω cm at 25 ◦C; Milli-Q ® Advantage A10 Water Purification System from Millipore, Merck KGaA, Darmstadt, Germany) at 25 ± 0.1 ◦C temperature with a flow rate of 0.6 mL/min. Standards and samples were filtered through 0.22 µm (CLARIFY-NY 25 mm Syringe Filters, Non-Sterile, Luer/Slip, Phenomenex) and injected (15 µL) into the HPLC.

Characterization of phenolic compounds by Chromatography and Mass Spectrometry analysis

HPLC-ESI-QTOF equipment was used for a Chromatography and Mass Spectrometry analysis in order to characterize the extracted phenolic compounds. The HPLC unit (Agilent Technologies, Waldbroon, Germany) comprised of a quaternary pump (G1311A) with integrated degasser (G1322A), an autosampler (G1367B), a thermostatted column compartment (G1316A) a diode array detector (DAD) (G1315B) and a hybrid mass spectrometer quadrupole-time of flight via an electrospray ionization source (ESI) with JetStream technology (Agilent Accurate Mass QTOF LC-MS, Waldbronn, Germany) in series in the same chromatographic line. The used column was a Zorbax Eclipse XDB C18 993,967-902 Agilent 150 mm × 5 µ m x 4.6 mm. The gradient elution was carried out with a binary system consisting of 0.1% formic acid Sigma (St. Louis, MO, USA) in water (solvent A) and 0.1% acetonitrile (Labscan Ltd.

Dublin, Ireland) in aqueous formic acid (solvent B). The following gradient was applied at a flow rate of 1 mL/ min: 0 min, 95% (A); 30 min, 70% (A); 40 min, 50% (A); 45 min, 95% (B); 50 min, 95% (B). The injection volume was 50 µ temperature was 40 ◦ L, and the column C. Mass spectra were acquired with electrospray ionization and the TOF mass analyser in negative (rutin, quercetin, ferulic acid, chlorogenic acid, gallic acid, catechin, caffeic acid, procyanidin dimer 1, procyanidin dimer 2, epicatechin, quercetin- arabinoside, quercetin-rhamnoside, quercetin-galactoside, quercetin- glucoside, coumarolyquinic and cyanidin-galactoside) and positive (cyanidin glucoside) mode, over the range m/z: 100–1000. The quantification of the phenolic compounds was performed using the calibration curve of commercial standards (Sigma, St. Louis, MO, USA), namely, rutin, quercetin, ferulic acid, chlorogenic acid, gallic acid, catechin, caffeic acid, and cyanidin-glucoside. In addition, tentative compounds were also included, specifically, procyanidin dimer 1, procyanidin dimer 2, epicatechin, quercetin-arabinoside, quercetin-rhamnoside, quercetin-galactoside, quercetin-glucoside, coumarolyquinic acid, and cyanidin-galactoside.

Statistical analysis

Data were analyzed using one-way ANOVA with Tukey post-hoc test. The assumptions of homogeneity of variances and normality of group data were verified using the Levene and Shapiro-Wilk's test, respectively. The data were expressed as mean ± standard deviation (SD) of three replicates, and differences were considered statistically significant when $P < 0.05$. The analysis was performed using the Statistical Package for the Social Sciences (SPSS).

4.2 Case study II: Carciofo di Malegno

Article 4

Plant material

Thirty capitula of "Carciofo di Malegno" was collected from a local farmer, Felice Pezzoni, growing it in the municipality of Malegno (Camonica Valley; Latitude 45°57′06″N; Longitude 10°16′30″E), who has cultivated this landrace since the 1940s. In addition, four commercial globe artichoke varieties (30 capitula each) were obtained from a local shop selling different Italian cultivars. All capitula were collected with approximately 10 cm of stem.

The samples were divided into three parts: non-edible flower head parts, stem, and receptacle with the inner bracts.

Each part was frozen at -80 °C using a freezer (Haier biomedical, Qingdao, P.R. China) and then milled with a kitchen mixer (Moulinex) to obtain a coarse powder. The powder was stored in sealed container (50mL, centrifuge tube) at -80 °C before the extraction.

Geometric morphometric analysis

The morphometric analysis of each artichoke's bracts and capitula was performed following the same procedure described for bean seeds.

Images were processed into black-and-white silhouettes and analyzed using elliptical Fourier descriptors (EFDs) in Momocs (R). Outlines were standardized by orientation and a common landmark. Fourier coefficients were calculated using 9 harmonics for bracts and 15 for capitula (≥99% harmonic power). PCA, DAPC, and MANOVA (with pairwise comparisons) were used to assess shape variation among cultivars, and mean shapes were reconstructed with the MSHAPES function.

Phytochemical analysis

All the solvents, reagents, and the analytical standards were purchased from Merck (Milan, Italy).

The extracts for the HPLC assays were obtained by a two steps solvent extraction procedure adapting literature procedure (Fratianni et al., 2014). The resulting phenolic extract was stored at -20 °C overnight and then centrifuged at 4000 rpm for 10 min. A 1 mL aliquot of the extract was filtered through a nylon 0.2 µm Millex®-GN syringe filter prior to the chromatographic analysis.

Acidic and alkaline hydrolyses of phenolic extracts were performed according to Nuutila et al (2022) and Lin et al (2008), respectively, with minor modifications. For acidic hydrolysis, 480 µL of extract were mixed with 120 µL of 6 N HCl and heated at 80 °C for 2 h in a screw-cap tube. After cooling, the mixture was diluted with 400 µL of methanol, sonicated, and filtered (0.2 µm nylon filter) prior to chromatographic analysis.

For alkaline hydrolysis, 1 mL of extract was evaporated to dryness under air, and the residue was dissolved in 300 µL of 4 N NaOH and incubated at room temperature for 18 h. The solution was then acidified with 150 µL of 12 N HCl, diluted with 550 µL of methanol, and filtered (0.2 µm nylon filter) before analysis.

The High-Performance Liquid Chromatography (HPLC) analysis was performed using a LC Agilent series 1200 apparatus (Waldbronn, Germany) consisting of a degasser, a quaternary gradient pump, an auto-sampler and a MWD detector (Waldbronn, Germany). A Luna® 5 µm C18 (150 × 4.6 mm) column (Phenomenex, Santa Clara, USA) at 40 °C was used for this analysis. Sample injections were made at 10 µL for all samples and standards; the run time was 40 mins. A binary gradient comprising of 0.1% aqueous formic acid (v/v) (A) and acetonitrile (B) at a flow rate of 0.8 mL min−1 was used as the mobile phase. The gradient profile was as follows: 0 min, 5% B; 20 mins, 25% B; and 30 mins, 95% B; 40 mins, 5%B.

Absorbance wavelength was 330 ± 10 and 370 ± 10 nm, for the caffeoylquinic acids and for flavonoids respectively.
Phytochemical data was analyzed using a one-way ANOVA test using R software to highlight the significant differences ($P < 0.05$) attributable to each genotype. All the results were expressed to dried weight of plant material.

Prediction of the potential distribution of "carciofo di Malegno"

The fields where the "carciofo di Malegno" is cultivated (and where was cultivated in the latest decades) were identified by consulting the local farmers and were georeferenced using a GPS device (Garmin Etrex 32x). Twenty-five georeferenced points (occurrence points) were collected to assess on the spatial distribution of "carciofo di Malegno" in Malegno whose coordinates were imported into R in CSV format.
Nineteen bioclimatic variables (Table 1) were retrieved as predictors to model the potential environmental niche of "carciofo di Malegno" based on its occurrence dataset. In particular, the bioclimatic layers were obtained from World Climate Database (WorldClim 2.1, http://worldclim.org) at a spatial resolution of 0.5 arc-second. All the bioclimatic variables were used to establish the distribution model of "Carciofo di Malegno" under the current conditions and future global warming scenarios (2021-2040 and 2041-2060).
One global climate model (CNRM-CM6-1) was obtained from WorldClim database for the future scenarios of the periods 2021-2040 and 2041–2060.
In this research, all models were run using the MaxEnt (Maximum Entropy) algorithm in R environment. The MaxEnt algorithm, instead, is a modelling technique used for making predictions of species distribution, particularly well-suited for applications involving presence-only data (occurrence data). It allows you to infer a probability distribution that has maximum entropy while still satisfying the constraints imposed by the available environmental information (Phillips et al., 2006).
The relative importance/weight of each bioclimatic preditor for the distribution model was assessed using the Jackknife test.

Table 1. Environmental variables used for modelling the potential distribution of *"Carciofo di Malegno"landrace and Italian saffron.*

Code (unit)	Bioclimatic variable
BIO1 (°C)	Annual Mean Temperature
BIO2 (°C)	Mean Diurnal Range (Mean of monthly (max temp - min temp)
BIO3 (-)	Isothermality (BIO2/BIO7 × 100)
BIO4 (°C)	Temperature Seasonality (standard deviation × 100)
BIO5 (°C)	Max Temperature of Warmest Month

BIO6 (°C)	Min Temperature of Coldest Month
BIO7 (°C)	Temperature Annual Range (BIO5-BIO6)
BIO8 (°C)	Mean Temperature of Wettest Quarter
BIO9 (°C)	Mean Temperature of Driest Quarter
BIO10 (°C)	Mean Temperature of Warmest Quarter
BIO11 (°C)	Mean Temperature of Coldest Quarter
BIO12 (mm)	Annual Precipitation
BIO13 (mm)	Precipitation of Wettest Month
BIO14 (mm)	Precipitation of Driest Month
BIO15 (-)	Precipitation Seasonality (Coefficient of Variation)
BIO16 (mm)	Precipitation of Wettest Quarter
BIO17 (mm)	Precipitation of Driest Quarter
BIO18 (mm)	Precipitation of Warmest Quarter
BIO19 (mm)	Precipitation of Coldest Quarter

Participants of Consumer study- valorisation activity

35 subjects (40% men; mean age: 39 ± 15 years) were recruited among students and employees of the Centre of Applied Studies in the Sustainable Management of the Mountain Environment (Ge.S.Di.Mont.) of the University of Milan and among the population of the neighboring municipalities. This study was approved by the Ethics Committee of the University of Milan ad was conducted in compliance with the principles laid down in the Declaration of Helsinki. All participants signed a written informed consent to be involved in the study.

Samples

The Tevini distillery developed a hydro-alcoholic extract from the artichoke leaves, which was then used to produce three prototypes of spirits: a liqueur, a bitter, and a gin.

Liking assessment and sensory descriptive evaluation

Subjects were asked to taste the biscuits and to express their liking using a 10 cm visual analogue scale (VAS) anchored by the extremes “extremely disliked” (rated 0) and “extremely liked” (rated 10).

In order to obtain a sensory description of the samples, a focus group was preliminary performed to define and select the appropriate attributes (Ares et al., 2010; Jaeger et al., 2015). For each sample, subjects had to evaluate their overall liking and perform a sensory descriptive analysis by means of the check-all-that-apply (CATA) methodology.

For further methodological details, see Article 4 (page 141.)

4.3 Case study III: “Grano Siberiano Valtellinese’’

Original Data

Plant materials

Whole ‘‘Grano Siberiano Valtellinese’’ and its by-product (straw) sample from the productive season 2024 was collected directly from a custodian farmer (Patrizio Mazzucchelli, Raetia Biodiversità Alpine) at Teglio municipality (Sondrio, Valtellina, latitude: 46°1002500N, longitude: 10°0204800E) located at an altitude of about 900 m a.s.l. Approximately 500g of seed and dried plant collected from the field were milled into flour for nutritional and phytochemical analysis. Flours were obtained by finely grinding the plant materials with a commercial blender (IKA A10 basic, Werke GmbH & Co.KG, Staufen, Germany). All plant material was collected in compliance with national and international mandatory regulations.

Nutritional analysis

For the characterization of “Grano Saraceno Siberiano Valtellinese” and its straw, the contents of moisture, ash, protein, lipids, available carbohydrates, and dietary fibre were determined. The analyses were carried out following the specific methodologies previously described (see section Valorization 2), including standard AOAC protocols, enzymatic–gravimetric procedures for fibre fractions, and colorimetric determination of carbohydrates.

Polyphenol and water-soluble carbohydrates extraction

Polyphenols and water-soluble carbohydrates from “Grano Saraceno Siberiano Valtellinese” by-products were extracted using the same protocols reported above (chapter 4.1, original data 2), based on the procedures of Saura-Calixto (1998).

Total phenolic content and antioxidant assessment

Total phenolic content was quantified using the Folin–Ciocalteu method, while antioxidant activity was assessed through FRAP and ORAC assays, following the procedures previously described (Valorization 2). Measurements were carried out using a Synergy™ HTX Multi-Mode microplate reader, and results were expressed as mg GAE/g DW for TPC, and as mmol or µmol Trolox equivalents per gram of dry weight (TE/g DW) for FRAP and ORAC, respectively.

Data were analyzed using one-way ANOVA with Tukey post-hoc test. The assumptions of homogeneity of variances and normality of group data were verified using the Levene and

Shapiro-Wilk's test, respectively. The data were expressed as mean ± standard deviation (SD) of three replicates, and differences were considered statistically significant when $P < 0.05$.

HHP treatment assisted by Ultimase® L or Ultraflo® L

"Grano Siberiano Valtellinese" by-product (10% w/v) was treated with high hydrostatic pressure (HHP; 100, 200, 400 MPa), with or without the addition of Ultimase® L or Ultraflo® L enzymes (1:40, v/w; Novozymes Spain, S.A.), following the protocol previously described for "Copafam" bean pod (chapter 4.1, original data 2). Control samples were kept at atmospheric pressure (0.1 MPa). After treatment, samples were centrifuged (6000 rpm, 20 min), filtered (0.22 µm), and stored at −20 °C.

Water soluble carbohydrates analysis

Water-soluble carbohydrates, including polysaccharides (100–5.94 kDa), oligosaccharides (0.83–0.50 kDa), and simple sugars (0.34–0.18 kDa), were quantified by HPLC-RID, following the procedure previously described. Separation was performed using ultrapure water as the mobile phase, and identification was based on retention time comparison with commercial standards.

Characterization of phenolic compounds by Chromatography and Mass Spectrometry analysis

The phenolic profile of the extracts was characterized using HPLC-ESI-QTOF, as previously detailed. Compounds were identified based on retention times, UV spectra, and mass fragmentation patterns, using both positive and negative ionization modes. Quantification was carried out with calibration curves of commercial standards, and tentative compounds were also annotated based on accurate mass and fragmentation data.

Statistical analysis

Descriptive statistics of the data was determined, and the differences within groups were studied by one-way analysis of variance (ANOVA) and post-hoc Ryan-Einot-Gabriel-Welsch F. The analysis was performed using the Statistical Package for the Social Sciences (SPSS).

4.4 Case study IV: Italian Saffron

Article 5

Quality of Italian saffron by ISO 3632 1,2:2010-2011 standard procedure

To evaluate the quality of Italian saffron, the samples were pulverized using an MM400 vibrational mill (frequency: 30 Hz; duration: 1 minute).

Moisture content was determined by weighing 500 mg of dried powder saffron and incubating it in an oven for 16 hours at 103 ± 2 °C. Each sample was later weighed and moisture content (wMV) was calculated by the following formula:

$$wMV \ = \ m0 - m \times 100m0\%$$

where m0 is the mass, in grams, of the test portion before incubation and *m* is the mass, in grams, of the dry residue after incubation. Amounts of picrocrocin, safranal and crocin were determined by UV-Vis spectrophotometric analyses. the amount of picrocrocin, crocin and safranal expressed as the absorbance of a 1% aqueous solution of dried saffron at 257, 330 and 440 nm respectively, using a 1 cm pathway quartz cell. Picrocrocin, safranal and crocins determination [A1%1cm (λ max)] of each sample was calculated using the following formula:

$$A1\%1cm\,(\lambda\,max) \ = \ D \ \times \ 10000/m \ \times \ (100 \ - \ wMV)$$

where *D* is the specific absorbance; *m* is the mass, in grams, of the test portion; *wMV* is the moisture expressed as percentage mass fraction of the sample.

All analytical steps were conducted in the dark to keep the saffron solution away from all light.

NIR analysis

The MPA FT-NIR spectrophotometer (Bruker Optics, Milano, Italy), managed by the software Opus™ (v. 6.5, Bruker Optics, Milano, Italy), was used for saffron analysis. Measurements were performed in reflection, using an integrating sphere, and spectra collection was in the range of 12500-3800 cm-1, with a resolution of 8 cm-1, and with each measurement consisting of 32 scans for both samples and background. To carry out the analyses, special vials with 0.5 ± 0.01 g of saffron powder were used; two vials were filled for each sample and each of them was analyzed in duplicate.

Principal component analysis

Principal component analysis (PCA) was applied to explore sample distribution based on the data collected according to ISO analytical procedures and FT-NIR spectra.

PCA was applied to ISO results to identify sample grouping, which were further classified into (i.e., premium, superior, high-quality) for use as a priori information in classification model development.

For FT-NIR data, PCA was applied after spectral range reduction and pre-treatments. Specifically, the NIR spectral range was reduced to the region between 9000 – 3800 cm-1, and different spectral pretreatments, such as Standard Normal Variate (SNV) and first derivate alone or in combination, were applied. Samples were colour-coded based on the quality subcategories identified by the ISO analysis.

Statistical data analysis

Data exploration was performed in the MATLAB environment (v. 2017b, Mathworks, Inc., Natick, MA, USA) using the PLS toolbox (v. 8.5, Eigenvector Research, Inc., Aeattle, WA, USA). Classification models and variable selection were performed by the V-Parvus package (Forina et al., 1988).

Classification model development

The development of classification models for saffron quality prediction involved the associating the FT-NIR spectra with an a priori information. Subcategories, previously identified through analysis of saffron characterization using ISO methods, served as the basis for class definition (premium, superior, high-quality).

The classification models were developed by Linear Discriminant Analysis (LDA) and Soft Independent Modelling of Class Analogy (SIMCA).

As LDA, a supervised pattern recognition method, utilizes discriminant canonicals to calculate the center of matrix covariance, it requires a number of samples exceeding the number of variables. Thus, a feature selection approach (fifteen wavenumbers) was implemented using the SELECT algorithm (Forina et al., 1988, Kowalski and Bender, 1976).

SIMCA, a supervised classification method developed by Wold and Sjostrom (1977), followed the same feature selection approach as LDA. SIMCA begins with an independent PCA on the spectral variables of calibration sets for the considered subcategories (premium, superior, high-quality). Then, in the PCA-reduced space, SIMCA constructs a multidimensional space for classifying external test set samples based on the distance between each sample and the models. SIMCA creates models equal to the number of classes, leading to sample assignments as follows: (1) exclusive assignment to one class; (2) no assignment to any class; (3) fitting two or more classes.

Finally, supervised classification models were validated through both internal cross-validation, by 5 cancellation groups, and prediction, by an independent random external test set containing around 30% of the total data.

Model performance was evaluated in terms of sensitivity (TP/(TP + FN)), specificity (TN/(TN + FP)), and correct classification percentage, a.k.a. the proportion of samples correctly accepted in the external test set belonging to the modelled class.

For further methodological details, see Article 5 (page 182).

Article 6

Occurrence data source

The geographical coordinates of 721 areas (occurrence points) where Italian saffron is produced were collected in March 2023 from the database of the "Val.Te.Mo." association (https://www.valtemo.it). This association, which aims to enhance the resources of mountain territories of Italy, has been conducting qualitative analyses (according to the ISO 3632 1,2:2010-2011 standard) of saffron produced in Italy in collaboration with the University of Milan since 2015. The database of the Val.Te.Mo. association is the most comprehensive and up-to-date database regarding saffron produced in Italy (both by farmers and hobbyists), and it was possible to access it following an agreement. This research only considered the geographical coordinates of areas where top-quality saffron (first-category saffron as defined by ISO 3632 standards) has been produced for more than one year. In detail, top-quality saffron must have the following characteristics: flavor strength (picrocrocin) ≥70; aroma strength (safranal): 20-50; coloring strength (crocin) ≥200; moisture content ≤12% (ISO 3632).

Spatial distribution prediction

Species distribution models (SDMs) were adopted to predict the spatial distribution (current and future) of C. sativus in Italy based on bioclimatic data considering 19 bioclimatic variables (Table 1) in addition to the 721occurrence data (presence only) of this species. All the bioclimatic variables were used to establish the distribution model of *C. sativus* in Italy under current climatic conditions (2016-2020) and for two future periods: 2021-2040 and 2041-2060. In this research, the distribution models of *C. sativus* were generated in the R environment using the "dismo" package (Hijmans et al., 2023) and two different algorithms: Bioclim (Busby, 1991) and MaxEnt (Phillips et al., 2006). The Bioclim algorithm is a classic method widely used in species distribution modelling. It assesses location suitability by comparing environmental variables to a percentile distribution of known occurrence sites, with locations closer to the median considered more suitable (Busby, 1991). The MaxEnt algorithm, instead, was performed as previous described.

The final product of the distribution models of C. sativus is a georeferenced map indicating the probability of the species occurrence (0 for low probability; 1 for high probability). In this research, nine model-generated habitat suitability maps of *C. sativus* in Italy were created: 3 maps produced by applying the Bioclim algorithm, considering the climatic conditions of three time periods (2016-2020, 2021-2040, and 2041-2060); 3 maps produced by applying the MaxEnt algorithm, considering the climatic conditions of the same periods; and 3 maps resulting from the combination of the prediction models produced with the two algorithms for the three periods.

For further methodological details, see Article 6 (page 194).

Valorization activities

To investigate farmers' perspectives on the proposed quality subclassification and to collect information on cultivation practices, a survey was conducted using Google Forms. The questionnaire was distributed online to Italian saffron growers, and responses were collected over a two-year period. The survey included both closed and open questions, focusing on production experience, perception of quality parameters, and evaluation of the usefulness of the new classification system.

4.5 *Bibliography*

Abdel-Aal, E.-S.M.; Hucl, P. A Rapid Method for Quantifying Total Anthocyanins in Blue Aleurone and Purple Pericarp Wheats.Cereal Chem. 1999, 76, 350–354.

American Association of Cereal Chemists (AACC). ApprovedMethods of Analysis; Method n_ 08-01.01. 11th ed.; AACC International: St. Paul, MN, USA, 1999.

Alcázar-Valle, M., Lugo-Cervantes, E., Mojica, L., Morales-Hernández, N., Reyes-Ramírez, H., Enríquez-Vara, J. N., & García-Morales, S. (2020). Bioactive Compounds, Antioxidant Activity, and Antinutritional Content of Legumes: A Comparison between Four Phaseolus Species. Molecules, 25(15), 3528. https://doi.org/10.3390/molecules25153528

AOAC International. AOAC Method n_9991.36; 945.18; 993.40. In Official Methods of Analysis of AOAC International, 18th ed.; Horwitz,W., Latimer, G.W., Eds.; AOAC International: Gaithersburg, ML, USA, 2005.

Ares, G., Deliza, R., Barreiro, C., Giménez, A., & Gámbaro, A. (2010). Comparison of two sensory profiling techniques based on consumer perception. Food Quality and Preference, 21(4), 417–426. https://doi.org/10.1016/j.foodqual.2009.10.006

Benzie, I. F. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of “Antioxidant Power”: The FRAP Assay. Analytical Biochemistry, 239(1), 70–76. https://doi.org/10.1006/abio.1996.0292

Bonhomme, V., Picq, S., Gaucherel, C., & Claude, J. (2014). Momocs: Outline Analysis Using R. Journal of Statistical Software, 56(13). https://doi.org/10.18637/jss.v056.i13

Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci. Technol.1995, 28, 25–30

Busby, J.R., 1991: Bioclim, a Bioclimatic Analysis and Prediction System. In: Margules, C.R., Austin, M.P. (eds.), Nature Conservation: Cost Effective Biological Surveys and Data Analysis. Canberra: CSIRO, pp. 64-68.

Claude, J. (2008). Morphometrics with R. Springer.

De La Peña-Armada, R., Villanueva-Suárez, M. J., Rupérez, P., & Mateos-Aparicio, I. (2020). High Hydrostatic Pressure Assisted by Celluclast® Releases Oligosaccharides from Apple By-Product. Foods, 9(8), 1058. https://doi.org/10.3390/foods9081058

Development Core Team. R: A Language and Environment or Statistical Computing. R Foundation for Statistical Computing. Available online: http://www.r-project.org/ (accessed on 30 September 2023).

Forina, M. R., Leardi; C., Armanino; S., Lanteri; Conti, Paolo; P., Princi. PARVUS: An extendable package of programs for data exploration, classification and correlation. *J. Chemometr.* **4** (2), 191–193 (1988). https://hdl.handle.net/11581/11010

Fratianni, F., Pepe, R., & Nazzaro, F. (2014). Polyphenol Composition, Antioxidant, Antimicrobial and Quorum Quenching Activity of the “Carciofo di Montoro” (<i>Cynara cardunculus</i> var. <i>scolymus</i>) Global Artichoke of the Campania Region, Southern Italy. Food and Nutrition Sciences, 05(21), 2053–2062. https://doi.org/10.4236/fns.2014.521217

Ghidoli, M., Geuna, F., De Benedetti, S., Frazzini, S., Landoni, M., Cassani, E., Scarafoni, A., Rossi, L., & Pilu, S. R. (2024). Genetic study of Camelina sativa oilseed crop and selection of a new variety by the bulk method. Frontiers in Plant Science, 15, 1385332. https://doi.org/10.3389/fpls.2024.1385332

Giupponi, L., Pilu, R., Scarafoni, A., & Giorgi, A. (2020). Plant agro-biodiversity needs protection, study and promotion: Results of research conducted in Lombardy region (Northern Italy). Biodiversity and Conservation, 29(2), 409–430. https://doi.org/10.1007/s10531-019-01889-3

Grime, J. P. (2001). Plant strategies, vegetation processes and eco- system properties. Wiley, Chichester. The American Naturalist, 111(982), 1169–1194. https://doi.org/10.1086/283244

Hijmans, R.J., Phillips, S., Leathwick, J., Elith, J., 2023: Dismo: Species Distribution Modeling. R Package Version 1.3-14. http://CRAN.R-project.org/package=dismo

ISO 14902:2001; Animal Feeding Stuffs—Determination of Trypsin Inhibitor Activity of Soya Products. International Standardization Organization: Geneva, Swizzerland, 2010.

ISO 3632-1:2011. Spices:Saffron (Crocus sativus L.)—Part 1: Specification. International Standardization Organization: Geneva, Swizzerland, 2010

ISO 3632-2.:2010. Spices: Saffron (Crocus sativus L.)–Part 2: Test methods. International Standardization Organization: Geneva, Swizzerland, 2010

Jaeger, S. R., Beresford, M. K., Paisley, A. G., Antúnez, L., Vidal, L., Cadena, R. S., Giménez, A., & Ares, G. (2015). Check-all-that-apply (CATA) questions for sensory product characterization by consumers: Investigations into the number of terms used in CATA questions. Food Quality and Preference, 42, 154–164. https://doi.org/10.1016/j.foodqual.2015.02.003

Jombart, T., Devillard, S., & Balloux, F. (2010). Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. BMC Genetics, 11(1), 94. https://doi.org/10.1186/1471-2156-11-94

Kowalski, B. R. & Bender, C. F. An orthogonal feature selection method. Patt. Recognit. 8 (1), 1–4. https://doi.org/10.1016/0031-3203(76)90023-6 (1976).

Lattanzio, V., Kroon, P. A., Linsalata, V., & Cardinali, A. (2009). Globe artichoke: A functional food and source of nutraceutical ingredients. Journal of Functional Foods, 1(2), 131–144. https://doi.org/10.1016/j.jff.2009.01.002

Laureati, M., Spinelli, S., Monteleone, E., Dinnella, C., Prescott, J., Cattaneo, C., Proserpio, C., De Toffoli, A., Gasperi, F., Endrizzi, I., Torri, L., Peparaio, M., Arena, E., Bonello, F., Condelli, N., Di Monaco, R., Gatti, E., Piasentier, E., Tesini, F., & Pagliarini, E. (2018). Associations between food neophobia and responsiveness to “warning” chemosensory sensations in food products in a large population sample. Food Quality and Preference, 68, 113–124. https://doi.org/10.1016/j.foodqual.2018.02.007

Lavelli, V., & Sri Harsha, P. S. C. (2019). Microencapsulation of grape skin phenolics for pH controlled release of antiglycation agents. Food Research International, 119, 822–828. https://doi.org/10.1016/j.foodres.2018.10.065

Lin, L.-Z., Harnly, J. M., Pastor-Corrales, M. S., & Luthria, D. L. (2008). The polyphenolic profiles of common bean (Phaseolus vulgaris L.). Food Chemistry, 107(1), 399–410. https://doi.org/10.1016/j.foodchem.2007.08.038

Luca, G., & Annamaria, G. (2019). Effectiveness of modern leaf analysis tools for the morpho-ecological study of plants: The case of Primula albenensis. Nordic Journal of Botany, 37(8), njb.02386. https://doi.org/10.1111/njb.02386

Nuutila, A. M., Kammiovirta, K., & Oksman-Caldentey, K.-M. (2002). Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. Food Chemistry, 76(4), 519–525. https://doi.org/10.1016/S0308-8146(01)00305-3

Phillips, S. J., Anderson, R. P., & Schapire, R. E. (2006). Maximum entropy modeling of species geographic distributions. Ecological Modelling, 190(3–4), 231–259. https://doi.org/10.1016/j.ecolmodel.2005.03.026

Pierce, S., Negreiros, D., Cerabolini, B. E. L., Kattge, J., Díaz, S., Kleyer, M., Shipley, B., Wright, S. J., Soudzilovskaia, N. A., Onipchenko, V. G., Van Bodegom, P. M., Frenette-Dussault, C., Weiher, E., Pinho, B. X., Cornelissen, J. H. C., Grime, J. P., Thompson, K., Hunt, R., Wilson, P. J., … Tampucci, D. (2017). A global method for calculating plant CSR ecological strategies applied across biomes world-wide. Functional Ecology, 31(2), 444–457. https://doi.org/10.1111/1365-2435.12722

Pignatti, S.; Guarino, R.; La Rosa, M. Flora d'Italia, 2nd ed.; Edagricole: Bologna, Italy, 2017.

Rodríguez Madrera, R., Campa Negrillo, A., Suárez Valles, B., & Ferreira Fernández, J. J. (2021). Phenolic Content and Antioxidant Activity in Seeds of Common Bean (Phaseolus vulgaris L.). Foods, 10(4), 864. https://doi.org/10.3390/foods10040864

Saura-Calixto, F. (1998). Antioxidant Dietary Fiber Product: A New Concept and a Potential Food Ingredient. Journal of Agricultural and Food Chemistry, 46(10), 4303–4306. https://doi.org/10.1021/jf9803841

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods, 9(7), 671–675. https://doi.org/10.1038/nmeth.2089

Serra, A. T., Duarte, R. O., Bronze, M. R., & Duarte, C. M. M. (2011). Identification of bioactive response in traditional cherries from Portugal. Food Chemistry, 125(2), 318–325. https://doi.org/10.1016/j.foodchem.2010.07.088

Spataro, G., & Negri, V. (2013). The European seed legislation on conservation varieties: Focus, implementation, present and future impact on landrace on farm conservation. Genetic Resources and Crop Evolution, 60(8), 2421–2430. https://doi.org/10.1007/s10722-013-0009-x

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology, 299, 152–178.

Southgate, D. A. T. (1969). Determination of carbohydrates in foods. I.—Available carbohydrate. Journal of the Science of Food and Agriculture, 20(6), 326–330. https://doi.org/10.1002/jsfa.2740200602

Villa, T. C. C., Maxted, N., Scholten, M., & Ford-Lloyd, B. (2005). Defining and identifying crop landraces. Plant Genetic Resources, 3(3), 373–384. https://doi.org/10.1079/PGR200591

Wold, S. & Sjöström, M. SIMCA: a method for analyzing chemical data in terms of similarity and analogy. (1977).

5. Case study I: Copafam Bean

5.1 *Introduction*

Despite national efforts to safeguard agrobiodiversity, Lombardy, one of the most industrialized and horticulturally productive Italian regions, still faces major challenges in identifying and protecting its landraces (Giupponi et al., 2020). The first Italian inventory of *in situ* maintained cultivars included only eight herbaceous landraces for this region (Giupponi et al., 2020), highlighting the need for updated censuses, agronomic and nutritional characterization, and stronger legislative tools to ensure both varietal and territorial protection.

Among crop families, Fabaceae (legumes) are particularly relevant for their agricultural importance, role in traditional diets, and contribution to food security in marginal and mountain areas (Alcázar-Valle et al., 2020; Ceccarelli et al., 2012; Giupponi et al., 2021). Beans (*Phaseolus* spp.), with their high protein content (\~20 g/100 g) and cultural value, have historically served as a staple and meat substitute for rural communities, including those in Lombardy (Alicandri et al., 2024). Yet, most Lombard bean landraces remain poorly documented in terms of nutritional, genetic, morphological, and ecological traits, limiting their potential for valorization (Losa et al., 2025). Systematic characterization is therefore essential to safeguard these genetic resources and to promote their role in sustainable agri-food systems (Rossi et al., 2019).

Within this framework, the “Copafam” bean (*Phaseolus coccineus* L.) emerges as a particularly emblematic case. Traditionally cultivated in the pre-Alpine areas of Lombardy, especially the Brescia Pre-Alps, it originated from long-standing farmer selection that led to its adaptation to local pedoclimatic conditions (Giupponi et al., 2018). Over time, it became strongly tied to the agricultural and gastronomic heritage of the region. However, as with many traditional landraces, “Copafam” is currently at risk of extinction due to rural depopulation and the abandonment of mountain farming. Today, its cultivation is limited to a few hobby farmers, who mainly preserve it for self-consumption and the preparation of traditional dishes such as “Copafam” soup or pasta with beans (Giupponi et al., 2018).

In this context, the first study was carried out to provide a comprehensive characterization of some traditional beans cultivars still present in Lombardy, including the “Copafam” bean. Sixteen previously undocumented landraces were collected from alpine and hilly areas and genetically, morphologically, ecologically, and nutritionally characterized. The aim was to assess their diversity and distinctive traits to deepen knowledge of plant agrobiodiversity in Northern Italy, producing data supporting their possible inclusion in national and European registers dedicated to agrobiodiversity protection, and promote their use in crop improvement programs and in sustainable, high-value agri-food systems.

Building on the results of the first investigation, the second study focused exclusively on the "Copafam" bean to explore its nutritional and phytochemicals profile and its potential for wider valorization compared to more common and commercial bean varieties (*P. vulgaris* and *P. coccineus*). Additionally, the research examined the suitability of "Copafam" flour for innovative product development, by assessing its impact on sensory properties and consumer acceptance in a model food formulation (biscuits). The working hypothesis was that "Copafam" could represent a valuable resource for initiating unique, high-quality agri-food chains with low environmental impact, thereby supporting sustainable and inclusive economic growth in mountain areas.

5.2 *Characterization*

The combined results of the two studies demonstrated that Lombardy's bean landraces, encompassing both *P. vulgaris* and *P. coccineus*, exhibited unique and distinctive traits. These characteristics differentiated them not only from landraces of other species, but also from those belonging to the same species. Molecular analyses confirmed the genetic separation between *P. coccineus* and *P. vulgaris*, in line with their phenotypic, ecological, and reproductive differences. The former is predominantly allogamous and adapted to cooler mountain environments, while the latter is mainly self-pollinating and suited to lowlands (Ciaffi et al. 2024). Commercial cultivars were clearly distinguishable from traditional landraces confirming previous studies on the genetic differences between these two species (Bosmali et al. 2024), an important result for germplasm conservation and the identification of modern varieties derived from breeding programs. In contrast, seed morphology, functional strategies, and nutritional traits did not clearly separate species or commercial respect of traditional varieties, as each genotype exhibited unique and specific features. Morphometric analyses revealed high intra- and inter-specific variability within *Phaseolus*, identifying three main seed shapes (reniform, elliptic, and roundish). This variability likely reflects the absence of strong selection pressure in landraces compared to commercial beans, highlighting their value as diverse genetic resources.

Given the agri-food relevance of beans, the nutritional data obtained in the first study were particularly important for promoting the cultivation and, consequently, the on-farm conservation of these landraces in developed countries such as Italy, where mountain and marginal agricultural systems are increasingly oriented toward producing quality and niche products rather than quantity. The Multidimensional Scaling (MDS) analysis (Figure 1) revealed that the commercial varieties lie in the centre of the principal component plot, reflecting their uniformity and lack of extreme traits, while the landraces had a broader dispersion highlighting their diversity. The *P. coccineus* landraces are concentrated in the upper left quadrant (first quadrant) an area associated with elevated levels of RSO, flavonoids and strong antioxidant activity. Conversely, several *P. vulgaris* landraces were found in the lower region of the plot. These genotypes exhibit the highest levels of both protein and polyphenols among all accessions analyzed.

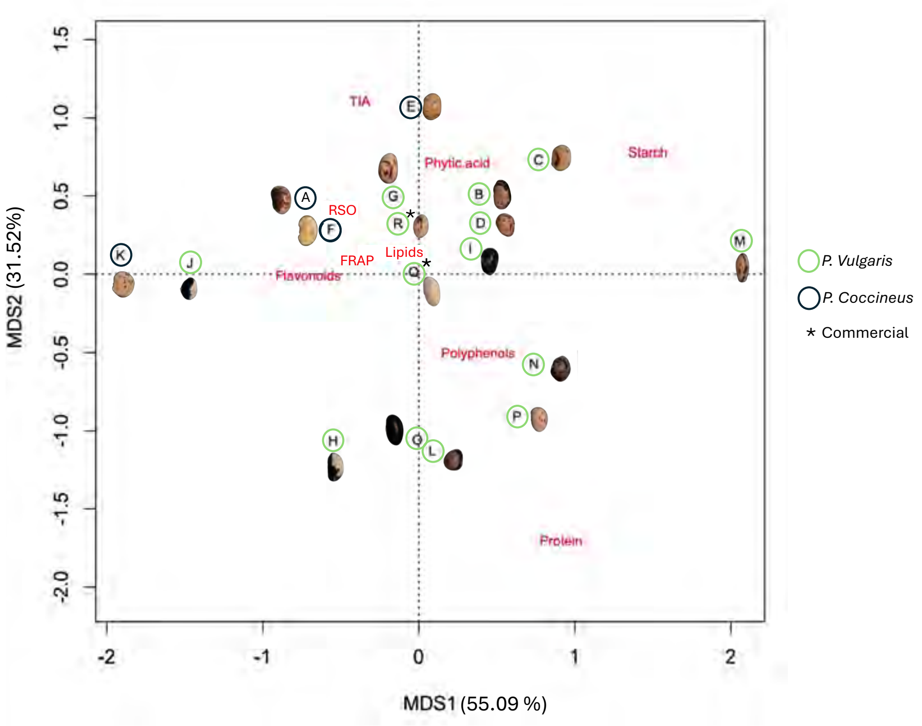


Figure 1. MDS (multidimensional scaling) biplot of beans samples associated with nutritional and phytochemical parameters. The identification codes of bean samples (capital letter) are referred to in figure 1. Key: TIA (trypsin inhibitor activity); FRAP (ferric reducing antioxidant power); RSO (raffinose series oligosaccharides)

Nutritional assessments revealed that several landraces had high levels of bioactive compounds and antioxidant activity, along with excellent protein content, suggesting their potential for functional food development. For these reasons, the studied landraces should be regarded as plant genetic resources with untapped potential for innovation in food production and the creation of new value chains. Their protection and valorization were considered essential, especially since they were still cultivated by only a few farmers, mainly hobbyists, for self-consumption.

The second study, which focused specifically on “Copafam,” revealed that it had the lowest protein content among the four cultivars analyzed (21.93 ± 0.41 g/100 g dw), but the highest dietary fiber content (34.83 ± 2.48 g/100 g dw) and low lipid and sucrose levels, aligning with nutritional recommendations for reduced fat and sugar intake.

From a phytochemical perspective, “Copafam” stood out as the richest source of total phenols (121.36 ± 5.31 mg GAE/g dw) and anthocyanins (28.11 ± 0.16 mg Cy3 G/kg dw), with high flavonoid content and strong antioxidant activity. Hydroxycinnamic acids were the main phenolic compounds across all beans, but *P. coccineus* varieties, including “Copafam,” contained more synapic acid and less ferulic and p-coumaric acids than *P. vulgaris*. Moreover,

“Copafam” showed the lowest levels of phytic acid and trypsin inhibitor activity, suggesting better protein digestibility and mineral bioavailability.
Multivariate analyses (heatmap and PCA) confirmed its distinctive profile, characterized by high antioxidant-related traits and low antinutrient content, which clearly separated it from both commercial and traditional counterparts.
Overall, the integrated findings from both studies emphasized that each landrace exhibited unique and distinctive traits that differentiated it from other landraces, even within the same species. The combination of genetic, morphological, ecological, and nutritional uniqueness underscored the untapped potential of these beans for innovation in agri-food systems. In the case of “Copafam,” its environmental adaptation, cultural value, and favorable nutritional and phytochemical profile positioned it as an excellent candidate for inclusion in sustainable, niche agri-food chains. To prevent the loss of such genetic resources, further research efforts should be promoted, as scientific characterization is the fundamental starting point for their conservation and valorization.

5.3 Valorization

Today, only few farmers in the Brescia Pre-Alps and surrounding areas continue to cultive “Copafam” bean mainly for home consumption. Conversely, “Copafam” is not commercially available and its cultivation remains limited to small-scale, non-commercial production by hobby farmers, with no initiatives to bring it to the market.
The second study evaluated the potential of “Copafam” flour for developing innovative and unique food products by assessing its effect on sensory properties and consumer acceptance in a model formulation (biscuits). The underlying goal was to explore its suitability for initiating high-quality agri-food chains with low environmental impact, supporting sustainable and inclusive growth in mountain areas.
The results showed that the incorporation of “Copafam” flour into biscuit formulations produced sensory results comparable to control samples, despite the common challenges of integrating legume flours into baked goods. The positive hedonic responses indicated that “Copafam” could be used to develop innovative, functional food products without compromising consumer acceptance.
In parallel with the development of new foods with “Copafam” bean, the valorization of its byproducts, such as the cooking water represents a strategy to exploit the full potential of this underutilized landrace.
The growing production and demand for pulses, driven by health, sustainability, and plant-based market trends, has also increased food chain byproducts, including cooking water (Nartea et al., 2023). Known as aquafaba (AQ), this liquid is rich in soluble proteins,

oligosaccharides, polyphenols, and other bioactive compounds, and is valued as a plant-based egg substitute in vegan and gluten-free applications due to its ability to form stable foams and emulsions (Serventi et al. 2020).

However, AQ has low solid content, making drying energy-intensive; thus, entrapment in alginate hydrogels is proposed as an alternative to retain proteins and bioactives (Molino et al. 2022). Alginate, a pH-sensitive hydrocolloid that gels in the presence of cations (H^+ or Ca^{2+}), is widely used for controlled release and has shown effectiveness in encapsulating anthocyanins, polyphenols, and proteins, improving stability and functionality (Lavelli and Sri Harsha 2019; Da Silva Carvalho et al. 2019; Norkaew et al. 2019)).

In this study, AQ from "Copafam" and a commercial bean variety was entrapped in Ca^{2+}-gelled alginate microbeads to evaluate retention of proteins and phenolics at different pH level (3-6). Additionally, rutin was encapsulated as a model bioactive compound to assess the potential of the alginate–AQ system.

Cooking water from both "Copafam" and Borlotto (*Phaseolus vulgaris*) beans proved to be a relevant source of bioactive compounds, including proteins and phenolics, that can be valorized through alginate encapsulation. However, despite the higher initial protein content in Borlotto beans, the percentage of proteins recovered from their cooking water was lower compared to "Copafam" landrace.

Table 2. Protein and polyphenols content of Hardening solution and “Copafam” bean beads

	Hardening		Beads		Hardening		Beads	
sample	Protein concentration (g/100 g)	Protein Hardening (recovery %)	Protein concentration (g/100 g)	Protein Beads (recovery %)	Polyphenols concentration (mg/ g)	Polyphenols Hardening (recovery %)	Polyphenols concentration (mg/ g)	Polyphenols Beads (recovery %)
"Copafam Bean" _pH3	0.10 ± 0.00	68.71 ± 2.33^{c}	0.43 ± 0.01	31.07 ± 0.63^{a}	0.28 ± 0.01	37.79 ± 1.79^{b}	4.87 ± 0.11	62.21 ± 1.79^{d}
"Copafam Bean"_pH4	0.10 ± 0.00	68.25 ± 3.14^{bc}	0.59 ± 0.02	31.93 ± 1.27^{ab}	0.35 ± 0.01	47.64 ± 1.60^{d}	5.66 ± 0.15	52.36 ± 1.60^{b}
"Copafam Bean"_pH5	0.09 ± 0.00	58.34 ± 1.24^{ab}	0.50 ± 0.02	39.12 ± 1.5^{abc}	0.32 ± 0.01	41.96 ± 0.85^{c}	4.17 ± 0.05	58.04 ± 0.85^{c}
"Copafam Bean"_pH6	0.09 ± 0.00	54.62 ± 4.64^{a}	0.59 ± 0.04	47.17 ± 1.78^{c}	0.40 ± 0.00	52.15 ± 0.52^{e}	3.65 ± 0.06	48.85 ± 0.52^{a}
"Copafam Bean"_pH6*	0.51 ± 0.02	61.40 ± 0.03^{abc}	0.63 ± 0.01	40.78 ± 0.05^{bc}	0.42 ± 0.00	15.29 ± 0.12^{a}	1.05 ± 0.01	84.71 ± 0.12^{e}

Key: (*): enriched

In Borlotto bean cooking water, protein encapsulation was highly efficient at pH 5 with 39.5 ± 0.61% of proteins retained in the beads and ~60% lost to the hardening solution.
In contrast, "Copafam" bean proteins showed stronger pH sensitivity. The highest protein recovery in the beads was achieved at pH 6 (47. 17 ± 1.78%), followed by pH 5 (39.12 ± 1.5%). When considering phenolic compounds, encapsulation efficiency was markedly higher than for proteins. In "Copafam" cooking water, polyphenol retention reached 62. 21 ± 1.79% at pH 3, but decreased to 48.85 ± 0.52% at pH 6, which was the optimal condition for protein recovery (47%). By contrast, in Borlotto bean cooking water, protein encapsulation was more efficient and consistent across all tested pH values, with 62–64% of proteins retained in the beads and 36–38% lost to the hardening solution.
Since the primary objective was to maximize protein recovery, encapsulation trials were carried out under the optimal pH (6) using an enriched system, where alginate was directly dissolved in the cooking water.
In the enriched system significantly increased polyphenol retention within beads. Notably, while protein recovery did not improve with this approach and even slightly decreased (40.78 ± 0.05% in beads), the amount of phenolics retained in the gel increased considerably reaching 84.71 ± 0.12%, confirming a stronger interaction between alginate and polyphenolic molecules compared to proteins.
To further assess the system's capacity, an external flavonoid, rutin, was introduced into the enriched cooking water. Remarkably, 97% of rutin was successfully encapsulated, confirming the robustness of alginate hydrogels in retaining flavonoid-type compounds. Rutin content was quantified by both HPLC and spectrophotometric methods, ensuring accuracy and reproducibility of the results.
These findings illustrate the feasibility of transforming bean processing by-products into functional hydrogels enriched in proteins and antioxidants compounds, contributing to circular economy strategies and the sustainable valorization of mountain agrobiodiversity.
This thesis was focused also on a second "Copafam" bean byproduct (Figure 2), its pod, to valorize this landrace in line with circular economy principles.
Beans are usually harvested dry, and their pods are often discarded despite being rich in dietary fiber, polyphenols, and antioxidants with potential health benefits (Bitocchi et al., 2017) (Tassoni et al., 2020). Current recovery methods focus mainly on proteins, with fewer studies targeting fibers or phenolics (Sun et al., 2020). Conventional solvent extractions are limited by low selectivity, degradation of thermolabile compounds, and environmental concerns, and many antioxidants are bound to plant cell walls, reducing extractability (Gligor et al. 2019; Lemes et al. 2022). Emerging approaches such as enzyme-assisted extraction and high hydrostatic pressure (HHP) can overcome these issues by improving the release of bioactives. (Gligor et al., 2019; Mateos-Aparicio et al., 2020).Their combined use has proven effective in

recovering fiber and phenolics from various agro-industrial by-products (De la Peña-Armada et al.2020; Mateos-Aparicio et al., 2020).

Therefore, the third valorization part presented in this study aimed to evaluate the nutritional and phytochemical composition of the "Copafam" bean pod to understand if it can be used as sources of bioactive compounds in food industry. Finally, the synergistic effect of high hydrostatic pressure (100, 200, and 400 MPa) and food-grade enzymes (Ultimase® and Pectinex Yieldmax®) on the recovery of soluble carbohydrates, dietary fiber, and antioxidant compounds was investigated.

Figure 2. "Copafam" bean pod

The "Copafam" bean pods revealed a nutritional profile dominated by dietary fiber (76.88 ± 2.55 g/100 g dw), of which the vast majority was insoluble (65.88 ± 1.39 g/100 g dw) and only a minor fraction was soluble (11.0 ± 1.21 g/100 g dw). Proteins (5.37± 0.71g/100 g dw), lipids (1.32± 0.01g/100 g dw), and available carbohydrates (2.2 ± g/100 g dw) were present in low amounts, confirming that pods are not an energy-rich fraction but a fiber-dense matrix. This composition is consistent with literature on legume pod residues, where lignocellulosic polysaccharides such as cellulose, hemicellulose, and lignin account for the majority of biomass (Tassoni et al., 2020; Nartea et al., 2023).

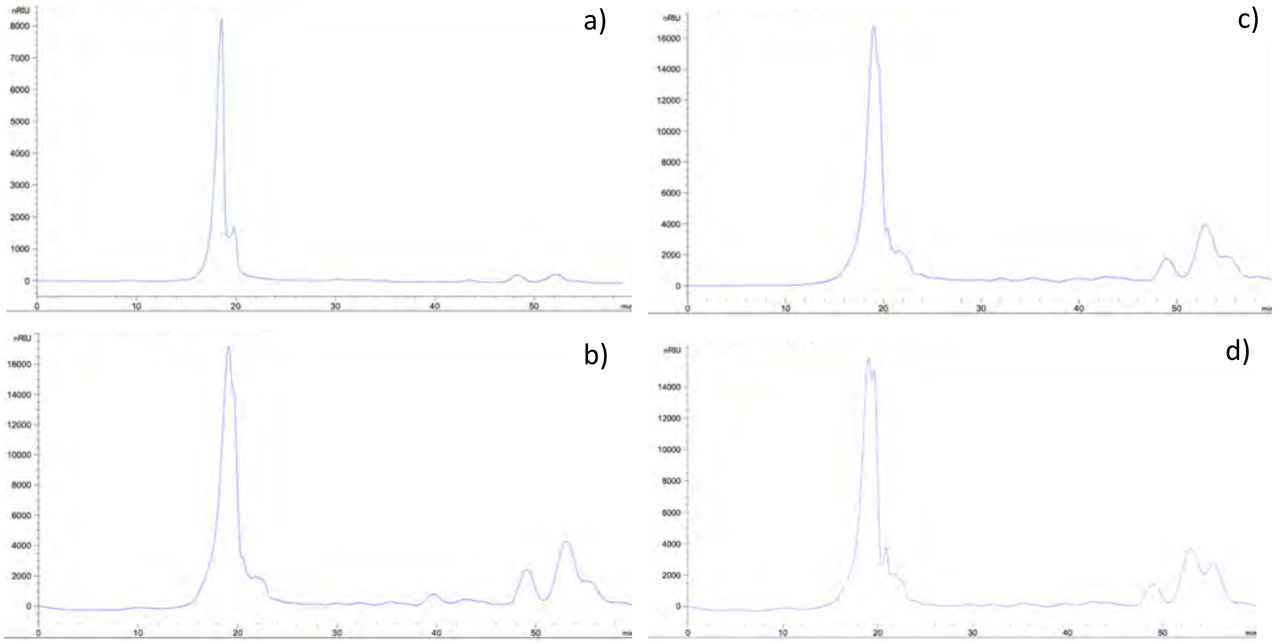


Figure 3. HPLC-RID analysis of water-soluble carbohydrates released from "Copafam" pod after HHP treatment assisted by Ultiase ® and Pectinex Yieldmax®. Key: (a) control; (b) 100Mpa; (c) 100Mpa+ Ultimase ®; (d) 100mPa + Pectinex Yieldmax®.

Application of high hydrostatic pressure combined with enzymatic hydrolysis substantially modified the carbohydrate profile of the pods (Figure 3). Untreated sample (control) was dominated by high-molecular-weight carbohydrates (HMWC), whereas after treatments at 100–200 MPa, a shift towards low-molecular-weight carbohydrates (LMWC) and oligosaccharides was evident. Raffinose family oligosaccharides (raffinose and verbascose), cellobiose, sucrose, glucose, fructose, and arabinose were detected in high quantity, particularly in samples with the presence of enzymes. In table 3, Ultimase® promoted the release of cellobiose and glucose through its cellulase/hemicellulase activity, while Pectinex® Yieldmash favored the liberation of fructose, arabinose, and RFOs due to its pectinolytic/xylanolytic action. The most effective condition was 200 MPa combined with enzymatic treatment, which maximized the release of soluble fiber and prebiotic oligosaccharides. At 400 MPa, however, excessive degradation led to the loss of oligosaccharides and monosaccharides, suggesting that over-treatment compromises functionality.

Table 3. Phenolic acid composition (µg/g) of "Copafam" bean pod after HHP treatment

Samples	Protocatequic acid	Trans p cumaric acid	Ferulic acid	Trans ferulic acid	Caffeic acid	Galic acid	Neochlorogenic acid	Quinic acid	Quercetin	Apigening	Luteolin
Control	21.49 ± 2.59^{c}	5.69 ± 0.37^{d}	15.88 ± 0.66^{f}	0.60 ± 0.08^{b}	0.26 ± 0.09^{abc}	-	-	-	30.87 ± 4.67^{b}	23.99 ± 0.48^{d}	7.00 ± 0.01^{f}
V100	3.53 ± 0.17^{a}	3.76 ± 0.04^{c}	2.30 ± 0.38^{c}	-	0.50 ± 0.02^{c}	0.33 ± 0.01^{ab}	-	-	3.54 ± 0.60^{a}	7.97 ± 0.31^{bc}	3.09 ± 0.05^{b}
V100 UT1	3.70 ± 0.03^{a}	3.77 ± 0.05^{c}	1.98 ± 0.05^{c}	-	0.36 ± 0.07^{bc}	0.41 ± 0.06^{bc}	-	-	2.04 ± 0.22^{a}	8.38 ± 0.22^{c}	4.32 ± 0.06^{d}
V100 Y	4.84 ± 0.03^{ab}	0.82 ± 0.01^{a}	1.83 ± 0.07^{bc}	-	0.12 ± 0.01^{ab}	0.31 ± 0.04^{a}	-	-	1.80 ± 0.45^{a}	8.05 ± 0.06^{c}	3.81 ± 0.06^{c}
V200	6.38 ± 0.06^{ab}	-	-	-	-	0.50 ± 0.03^{cd}	-	-	1.67 ± 0.06^{a}	7.19 ± 0.09^{ab}	3.99 ± 0.02^{c}
V200 UT	3.63 ± 0.05^{a}	3.52 ± 0.03^{c}	2.38 ± 0.22^{c}	-	0.48 ± 0.03^{c}	0.46 ± 0.01^{c}	-	-	1.09 ± 0.43^{a}	7.68 ± 0.09^{bc}	2.60 ± 0.02^{a}
V200 Y	7.27 ± 0.04^{b}	-	0.70 ± 0.15^{ab}	-	-	0.58 ± 0.01^{d}	-	-	0.90 ± 0.14^{a}	8.00 ± 0.01^{bc}	6.34 ± 0.08^{e}
V400	4.09 ± 0.03^{ab}	1.62 ± 0.02^{b}	13.25 ± 0.02^{e}	0.34 ± 0.00^{a}	0.52 ± 0.01^{c}	-	0.32 ± 0.07^{b}	8.72 ± 0.28^{b}	-	7.77 ± 0.12^{bc}	12.07 ± 0.01^{h}
V400 UT	4.87 ± 0.03^{ab}	1.26 ± 0.01^{ab}	14.30 ± 0.44^{e}	0.37 ± 0.01^{a}	0.49 ± 0.05^{abc}	-	0.30 ± 0.07^{ab}	8.59 ± 0.44^{b}	-	6.49 ± 0.04^{a}	13.51 ± 0.10^{i}
V400 Y	3.37 ± 0.01^{a}	1.22 ± 0.01^{ab}	11.33 ± 0.24^{d}	0.36 ± 0.03a	0.41 ± 0.01^{bc}	-	0.27 ± 0.02^{b}	5.56 ± 1.99^{a}	-	7.22 ± 0.08^{ab}	10.76 ± 0.20^{g}

Key: (V) Pod; (UT) Ultimase ®; (Y) Pectinex Yieldmax®.

Table 4. HPLC-RID analysis of water-soluble carbohydrates released from “Copafam” pod after HHP treatment. HMWC: high molecular weight carbohydrates; LMWC: low molecular weight carbohydrates

				100 MPa			200 MPa			400 MPa		
Peak n°	Standard		Control	No enzyme	Ultimase®	Pectinex® Yieldmash	No enzyme	Ultimase®	Pectinex® Yieldmash	No enzyme	Ultimase®	Pectinex® Yieldmash
1	Pullullan 100	HMWC a	3.05 ± 0.26^{c}	3.19 ± 0.33^{cd}	4.00 ± 0.23^{d}	3.29 ± 0.15^{cd}	3.03 ± 0.44^{c}	2.99 ± 0.10^{c}	3.05 ± 0.56^{c}	2.80 ± 0.20^{bc}	1.96 ± 0.38^{ab}	1.67 ± 0.23^{a}
2	Pullullan 100	HMWC b	166.98 ± 8.15^{a}	-	-	-	-	-	-	-	-	-
3	Pullullan 5	LMWC a	-	98.31 ± 2.93^{abcd}	99.11 ± 5.57^{bcd}	93.56 ± 1.06^{abc}	93.36 ± 5.35^{ab}	89.54 ± 4.12^{a}	95.81 ± 1.07^{abc}	102.48 ± 2.68^{cd}	100.53 ± 2.05^{bcd}	106.79 ± 0.72^{d}
4	Pullullan 5	LMWC b	-	31.84 ± 1.29^{b}	33.55 ± 1.00^{bc}	41.64 ± 0.98^{d}	34.74 ± 1.54^{bc}	33.90 ± 1.05^{bc}	45.26 ± 0.00^{d}	24.84 ± 2.31^{a}	25.32 ± 2.49^{a}	36.92 ± 0.53^{c}
5	Pullullan 5	LMWC c	19.18 ± 1.38^{a}	-	-	-	-	-	-	-	-	-
6	Pullullan 5	LMWC d	8.60 ± 0.44^{a}	-	-	-	-	-	-	-	-	-
7	Pullullan 5	LMWC e	-	11.65 ± 0.73^{b}	6.77 ± 0.23^{a}	12.55 ± 0.73^{b}	7.38 ± 0.17^{a}	6.55 ± 0.29^{a}	12.73 ± 1.29^{b}	-	-	-
8	Pullullan 5	LMWC f	-	-	5.55 ± 0.20^{b}	-	4.40 ± 0.40^{a}	6.34 ± 0.55^{b}	-	-	-	-
9	Pullullan 5	LMWC g	-	9.24 ± 0.47^{b}	7.30 ± 0.26^{a}	7.14 ± 0.16^{a}	9.95 ± 0.53^{b}	6.04 ± 0.51^{a}	6.44 ± 0.62^{a}	19.28 ± 1.06^{c}	19.02 ± 0.53^{c}	21.15 ± 0.11^{d}
10	Pullullan 5	LMWC h	-	5.53 ± 0.06^{b}	7.41 ± 0.13^{c}	7.18 ± 0.33^{c}	4.27 ± 0.23^{a}	7.35 ± 0.73^{c}	7.77 ± 0.25^{c}	4.36 ± 0.19^{a}	4.73 ± 0.28^{ab}	4.83 ± 0.38^{ab}
11	Verbascose	O-5a	-	3.64 ± 0.18^{c}	2.90 ± 0.11^{b}	2.76 ± 0.13^{b}	2.70 ± 0.26^{b}	1.87 ± 0.21^{a}	2.54 ± 0.12^{b}	-	-	-
12	Verbascose	O-5b	-	2.69 ± 0.19^{d}	2.26 ± 0.06^{c}	1.67 ± 0.07^{ab}	1.97 ± 0.25^{bc}	2.07 ± 0.07^{bc}	1.55 ± 0.02^{a}	-	-	-
13	Estaquiose	Estaquiose	0.82 ± 0.03^{a}	2.65 ± 0.08^{e}	1.61 ± 0.23^{bcd}	1.85 ± 0.09^{cd}	0.80 ± 0.03^{a}	1.00 ± 0.11^{a}	1.13 ± 0.13^{ab}	1.97 ± 0.32^{d}	2.02 ± 0.20^{d}	1.29 ± 0.10^{abc}
14	Raffinose	Raffinose	0.35 ± 0.00^{a}	2.50 ± 0.52^{b}	1.80 ± 0.33^{b}	2.08 ±0.22^{b}	0.33 ± 0.10^{a}	0.62 ± 0.13^{a}	0.80 ± 0.17^{a}	-	-	-
15	Maltotriose	O-3	5.51 ± 0.13^{f}	3.16 ± 0.23^{de}	2.58 ± 0.14^{bc}	2.61 ± 0.15^{bc}	2.80 ± 0.22^{cd}	2.11 ± 0.02^{a}	2.98 ± 0.12^{cd}	2.30 ± 0.07^{ab}	3.53 ± 0.19^{e}	2.60 ± 0.12^{bc}
16	Maltotriose	O-3	-	2.11 ± 0.42^{a}	2.04 ± 0.15^{a}	2.06 ± 0.07^{a}	2.35 ± 0.15^{a}	1.80 ± 0.05a	-	1.97 ± 0.21^{a}	-	-
17	Cellobiose	Cellobiose	-	3.14 ± 0.09^{a}	4.84 ± 0.44^{c}	3.02 ± 0.12^{a}	3.18 ± 0.08^{ab}	3.96 ± 0.05^{b}	2.71 ± 0.02^{a}	-	-	-
18	Sucrose	Disaccarides	-	3.29 ± 0.47^{bc}	4.63 ± 0.06^{e}	3.86 ± 0.21^{cd}	2.77 ± 0.04^{b}	4.34 ± 0.37^{de}	3.68 ± 0.18^{cd}	1.20 ± 0.22^{a}	-	1.16 ± 0.06^{a}
19	Sucrose		2.01 ± 0.13^{ab}	2.87 ± 0.51^{cd}	2.10 ± 0.19 ^{ab}	2.65 ± 0.26^{bcd}	2.97 ± 0.04^{d}	2.19 ± 0.27^{abc}	2.60 ± 0.07^{bcd}	2.45 ± 0.15^{abcd}	4.59 ± 0.32^{e}	1.75 ± 0.10^{a}
20	Glucose	Glucose	9.24 ± 0.26^{c}	7.95 ± 0.64^{b}	11.53 ± 0.64^{d}	7.88 ± 0.13^{b}	7.99 ± 0.51^{b}	12.70 ± 0.34^{e}	7.89 ± 0.01^{b}	5.42 ± 0.36^{a}	11.16 ± 0.50^{d}	5.85 ± 0.10^{a}
21	Fructose	Fructose	9.23 ± 0.47^{a}	35.92 ± 1.18^{c}	41.52 ± 0.99^{d}	35.45 ± 0.51^{c}	36.44 ± 2.19^{c}	42.51 ± 0.77^{d}	35.34 ± 0.51^{c}	9.87 ± 0.52^{a}	17.16 ± 0.61^{b}	10.72 ± 0.17^{a}
22	Fructose	Fructose	1.99 ± 0.21^{a}	12.52 ± 1.10^{bc}	11.3 0 ± 0.60^{b}	22.87 ± 0.51^{d}	12.48 ± 0.42^{bc}	11.47 ± 0.85^{b}	22.40 ± 0.10^{d}	1.70 ± 0.26^{a}	2.74 ± 0.08^{a}	13.51 ± 1.03^{c}
23	Arabinose	Arabinose	-	4.02 ± 0.75^{c}	2.38 ± 0.15^{a}	2.70 ± 0.32^{ab}	3.54 ± 0.01^{bc}	1.86 ± 0.10^{a}	2.56 ± 0.21^{ab}	-	-	-

Parallel trends were observed in the polyphenol and antioxidant profile. The total polyphenols recovered in the control were 105.78 µg/g, the highest among all samples. However, the untreated byproduct had an Folin value of 0.35± 0.03 g GAE/100g and relatively low antioxidant capacity (236.96 8.58 µmol TE/g).

The phenolic composition of “Copafam” pods was dominated by quercetin (30.87 ± 4.67), apigenin (23.99 ± 0.48 µg/g), and protocatechuic acid (21.49 ± 2.59 µg/g), with only minor amounts of and other phenolic acids.

HHP treatments markedly altered the balance between phenolic content and antioxidant activity. At 100 MPa, total phenolics recovered dropped to 25 µg/g, yet antioxidant performance increased, with ORAC reaching 1096.56 ± 88.95 µmol TE/g and peaking at 1751.36 ± 58.22 µmol TE/g under 100 MPa + Ultimase®, despite FRAP values remaining moderate (1.45 ± 0.19g TE/100 g). At 200 MPa, phenolic content remained low (19–24 µg/g), while ORAC values ranged between 505.90 ± 23.50 and 788.61± 116.11 µmol TE/g, depending on the enzyme treatment. This is in line with the literature because many antioxidant compounds exist in insoluble-bound forms, covalently linked to plant cell wall components, limiting their extractability (Acosta-Estrada et al., 2014).

At 400 MPa, phenolic recovery was partially restored (48.69 to 50.17 µg/g), although still lower than the control. However, FRAP values dropped to the lowest levels (from 0.76 ± 0.14 to 0.86 ± 0.11 g TE/100 g). Interestingly, the combination of 400 MPa + Pectinex® (V400_Y) generated an exceptionally high ORAC value (4549.49 ± 327.88 µmol TE/g), this could reflect the formation of minor highly reactive antioxidant compounds released under these conditions

Table 5. Polyphenols content and antioxindant activity of "Copafam" bean pod after HHP treatment

Samples	Total polyphenols (µg/g)	Total phenolic content (g GAE/100g)	FRAP (g TE/100g)	ORAC (µmol TE/g)
Control	105.78	0.35 ± 0.03^{c}	1.75 ± 0.09^{c}	236.96 ± 8.58^{a}
V100	24.58	0.54 ± 0.03^{d}	1.45 ± 0.13^{bc}	1096.56 ± 88.95^{i}
V100_UT	24.95	0.50 ± 0.08^{c}	1.45 ± 0.19^{c}	1751.36 ± 58.22^{j}
V100_Y	21.58	0.59 ± 0.05^{d}	1.48 ± 0.22^{bc}	595.39 ± 43.78^{e}
V200	19.73	0.58 ± 0.03^{d}	1.54 ± 0.2^{c}	639.64 ± 20.67^{f}
V200_UT	21.84	0.57 ± 0.03^{d}	1.51 ± 0.14^{c}	505.90 ± 23.5^{d}
V200_Y	23.79	0.54 ± 0.01^{d}	1.10 ± 0.3^{a}	788.61 ± 116.11^{h}
V400	48.69	0.24 ± 0.02^{ab}	0.79 ± 0.15^{a}	650.68 ± 51.01^{g}
V400_UT	50.17	0.24 ± 0.02^{b}	0.86 ± 0.11^{ab}	385.66 ± 44.72^{b}
V400_Y	40.50	0.20 ± 0.03^{a}	0.76 ± 0.14^{a}	4549.49 ± 327.88^{k}

These results demonstrate that "Copafam" bean pods can be successfully valorized through moderate HHP and enzymatic hydrolysis. This approach both enhances soluble fiber release, enriching the matrix in oligosaccharides with prebiotic potential, and improves antioxidant activity, linked to the increased availability of reactive phenolic compounds.

Additionally, their incorporation into food products can enhance nutritional, sensory, and technological properties (Serventi et al., 2020).

Among the tested conditions, 200 MPa combined with enzymes emerged as the most consistent and effective strategy, while excessively high pressure (400 MPa) promoted over-degradation. The dual improvement in fiber quality and antioxidant potential underlines the opportunity to transform Copafam pods into a source of functional ingredient, supporting circular economy models and contributing to the sustainable valorization of mountain agrobiodiversity.

Finally, the "Copafam" bean was officially included in the Slow Food Ark of Taste, and a formal application was submitted for its registration in the National Register of Conservation Varieties, thus ensuring both recognition and future protection.

5.4 *Conclusion and future activities*

This study demonstrated that Lombardy's bean landraces represent a valuable reservoir of agrobiodiversity, offering distinctive nutritional, phytochemical, and functional traits that can support the development of innovative agri-food products. Their diversity enables the selection of varieties adapted to different dietary needs and health goals, aligning with the Farm to Fork strategy of the European Green Deal, which promotes sustainable food systems and greater use of legumes as alternative protein sources. The multidisciplinary characterization presented here provides a foundation for their valorization, offering new opportunities to integrate these landraces into niche markets and functional food formulations while supporting on-farm conservation. From a broader perspective, the genetic and ecological analyses revealed clear distinctions between *P. vulgaris* and *P. coccineus* landraces and confirmed substantial intra-specific variability across mountain areas. Morphometric and nutritional assessments further demonstrated that each landrace possesses unique and distinctive traits, which may largely reflect the influence of the environments in which they evolved. These specific pedoclimatic conditions have shaped their morphological, nutritional, and phytochemical characteristics, reinforcing their potential for innovation in food production and for building strong, territorially linked value chains.

The "Copafam bean" emerged as a particularly relevant case, both for its unique nutritional profile, rich in dietary fiber, phenolics, and anthocyanins, and for the valorization potential of its by-products. Pods, typically discarded, were successfully transformed through high hydrostatic pressure combined with enzymatic treatments, enhancing the release of soluble fiber, prebiotic oligosaccharides, and phenolic compounds with antioxidant activity. Similarly, cooking water proved to be a complementary source of proteins and polyphenols, with encapsulation experiments confirming high polyphenol retention (up to 85%) and exceptional rutin encapsulation efficiency (97%), supporting its use as a basis for functional hydrogels. Together, these results highlight how by-products can be reimagined as resources, contributing to circular bioeconomy strategies and the sustainable valorization of mountain agrobiodiversity.

Future activities should therefore prioritize:

- Expanding scientific characterization of neglected landraces to provide the data required for their registration in national and European agrobiodiversity registers.
- Strengthening participatory approaches with custodian farmers, local associations, and small-scale enterprises to embed landraces into viable production systems.
- Promoting product innovation, including functional ingredients derived from by-products, to generate added value and stimulate market demand.
- Integrating conservation and valorisation strategies into regional development policies, ensuring that agrobiodiversity acts as both a cultural heritage and an economic driver for mountain areas.

5.5 *Bibliography*

Alcázar-Valle, M., Lugo-Cervantes, E., Mojica, L., Morales-Hernández, N., Reyes-Ramírez, H., Enríquez-Vara, J. N., & García-Morales, S. (2020). Bioactive Compounds, Antioxidant Activity, and Antinutritional Content of Legumes: A Comparison between Four Phaseolus Species. Molecules, 25(15), 3528. https://doi.org/10.3390/molecules25153528

Alicandri E, Paolacci AR, Coluccia L, et al (2024) Exploring molecular, morphological, and biochemical diversity of Phaeolus vulgaris landraces cultivated in the Aniene Valley (Lazio region, Italy). Curr Plant Biol 39:100374. https://doi.org/10.1016/j.cpb.2024.100374

Belščak-Cvitanović, A., Đorđević, V., Karlović, S., Pavlović, V., Komes, D., Ježek, D., Bugarski, B., & Nedović, V. (2015). Protein-reinforced and chitosan-pectin coated alginate microparticles for delivery of flavan-3-ol antioxidants and caffeine from green tea extract. Food Hydrocolloids, 51, 361–374. https://doi.org/10.1016/j.foodhyd.2015.05.039

Bitocchi, E., Rau, D., Bellucci, E., Rodriguez, M., Murgia, M. L., Gioia, T., Santo, D., Nanni, L., Attene, G., & Papa, R. (2017). Beans (Phaseolus ssp.) as a Model for Understanding Crop Evolution. Frontiers in Plant Science, 8, 722. https://doi.org/10.3389/fpls.2017.00722

Bosmali, I., Lagiotis, G., Ganopoulos, I., Stefanidou, E., Madesis, P., & Biliaderis, C. G. (2024). Phaseolus coccineus L. Landraces in Greece: Microsatellite Genotyping and Molecular Characterization for Landrace Authenticity and Discrimination. BioTech, 13(2), 18. https://doi.org/10.3390/biotech13020018

Ceccarelli S (2012) Landraces: importance and use in breeding and environmentally friendly agronomic systems. In: Maxted N, Ehsan Dulloo M, Ford-Lloyd BV, et al. (eds) Agrobiodiversity conservation: securing the diversity of crop wild relatives and landraces, 1st edn. CABI, UK, pp 103–117

Ciaffi, M., Paolacci, A. R., Marcomeni, M., Coluccia, L., Taviani, P., & Alicandri, E. (2024). The Characterization of the Morphological and Molecular Traits of Phaseolus coccineus in the Aniene Valley: Insights into Genetic Diversity and Adaptation. Plants, 13(23), 3320. https://doi.org/10.3390/plants13233320

Comunian, T. A., Silva, M. P., & Souza, C. J. F. (2021). The use of food by-products as a novel for functional foods: Their use as ingredients and for the encapsulation process. Trends in Food Science & Technology, 108, 269–280. https://doi.org/10.1016/j.tifs.2021.01.003

Da Silva Carvalho, A. G., Da Costa Machado, M. T., De Freitas Queiroz Barros, H. D., Cazarin, C. B. B., Maróstica Junior, M. R., & Hubinger, M. D. (2019). Anthocyanins from jussara (Euterpe edulis Martius) extract carried by calcium alginate beads pre-prepared using ionic gelation. Powder Technology, 345, 283–291. https://doi.org/10.1016/j.powtec.2019.01.016

De La Peña-Armada, R., Rupérez, P., Villanueva-Suarez, M. J., & Mateos-Aparicio, I. (2022). High hydrostatic pressure assisted by food-grade enzymes as a sustainable approach for the development of an antioxidant ingredient. LWT, 169, 113968. https://doi.org/10.1016/j.lwt.2022.113968

De La Peña-Armada, R., Villanueva-Suárez, M. J., Molina-García, A. D., Rupérez, P., & Mateos-Aparicio, I. (2021). Novel rich-in-soluble dietary fiber apple ingredient obtained from the synergistic effect of high hydrostatic pressure aided by Celluclast®. LWT, 146, 111421. https://doi.org/10.1016/j.lwt.2021.111421

De La Peña-Armada, R., Villanueva-Suárez, M. J., Rupérez, P., & Mateos-Aparicio, I. (2020). High Hydrostatic Pressure Assisted by Celluclast® Releases Oligosaccharides from Apple By-Product. Foods, 9(8), 1058. https://doi.org/10.3390/foods9081058

Echeverria-Jaramillo, E., Kim, Y., Nam, Y., Zheng, Y., Cho, J. Y., Hong, W. S., Kang, S. J., Kim, J. H., Shim, Y. Y., & Shin, W.-S. (2021). Revalorization of the Cooking Water (Aquafaba) from Soybean Varieties Generated as a By-Product of Food Manufacturing in Korea. Foods, 10(10), 2287. https://doi.org/10.3390/foods10102287

Giupponi L, Tamburini A, Giorgi A (2018) Prospects for Broader Cultivation and Commercialization of Copafam, a Local Variety of Phaseolus coccineus L., in the Brescia Pre-Alps. Mt Res Dev 38:24–34. https://doi.org/10.1659/MRD-JOURNAL-D-17-00013.1

Giupponi L, Pilu R, Scarafoni A, Giorgi A (2020) Plant agro-biodiversity needs protection, study and promotion: results of research conducted in Lombardy region (Northern Italy). Biodivers Conserv 29:409–430. https://doi.org/10.1007/s10531-019-01889-3

Giupponi L, Pedrali D, Leoni V, et al (2021) The Analysis of Italian Plant Agrobiodiversity Databases Reveals That Hilly and Sub-Mountain Areas Are Hotspots of Herbaceous Landraces. Diversity 13:70. https://doi.org/10.3390/d13020070

Gligor, O., Mocan, A., Moldovan, C., Locatelli, M., Crişan, G., & Ferreira, I. C. F. R. (2019). Enzyme-assisted extractions of polyphenols – A comprehensive review. Trends in Food Science & Technology, 88, 302–315. https://doi.org/10.1016/j.tifs.2019.03.029

He, Y., Purdy, S. K., Tse, T. J., Tar'an, B., Meda, V., Reaney, M. J. T., & Mustafa, R. (2021). Standardization of Aquafaba Production and Application in Vegan Mayonnaise Analogs. Foods, 10(9), 1978. https://doi.org/10.3390/foods10091978

Lavelli, V., & Sri Harsha, P. S. C. (2019). Microencapsulation of grape skin phenolics for pH controlled release of antiglycation agents. Food Research International, 119, 822–828. https://doi.org/10.1016/j.foodres.2018.10.065

Lemes, A. C., Egea, M. B., Oliveira Filho, J. G. D., Gautério, G. V., Ribeiro, B. D., & Coelho, M. A. Z. (2022). Biological Approaches for Extraction of Bioactive Compounds From Agro-industrial By-products: A Review. Frontiers in Bioengineering and Biotechnology, 9, 802543. https://doi.org/10.3389/fbioe.2021.802543

Losa, A., Sala, T., Toppino, L., Fricano, A., Rossi, G., Gipli, V., & Landoni, M. (2025). Genetic Diversity and Distinctiveness of Common Beans (Phaseolus vulgaris L.) Between Landraces and Formal Cultivars Supporting Ex Situ Conservation Policy: The Borlotti Case Study in Northern Italy. Agronomy, 15(4), 786. https://doi.org/10.3390/agronomy15040786

Mateos-Aparicio, I., De La Peña Armada, R., Pérez-Cózar, M. L., Rupérez, P., Redondo-Cuenca, A., & Villanueva-Suárez, M. J. (2020). Apple by-product dietary fibre exhibits potential prebiotic and hypolipidemic effectsin high-fat fed Wistar rats. Bioactive Carbohydrates and Dietary Fibre, 23, 100219. https://doi.org/10.1016/j.bcdf.2020.100219

Molino, S., Rufián Henares, J. Á., & Gómez-Mascaraque, L. G. (2022). Tannin-rich extracts improve the performance of amidated pectin as an alternative microencapsulation matrix to alginate. Current Research in Food Science, 5, 243–250. https://doi.org/10.1016/j.crfs.2022.01.014

Nartea, A., Kuhalskaya, A., Fanesi, B., Orhotohwo, O. L., Susek, K., Rocchetti, L., Di Vittori, V., Bitocchi, E., Pacetti, D., & Papa, R. (2023). Legume byproducts as ingredients for food applications: Preparation, nutrition, bioactivity, and techno-functional properties. Comprehensive Reviews in Food Science and Food Safety, 22(3), 1953–1985. https://doi.org/10.1111/1541-4337.13137

Norkaew, O., Thitisut, P., Mahatheeranont, S., Pawin, B., Sookwong, P., Yodpitak, S., & Lungkaphin, A. (2019). Effect of wall materials on some physicochemical properties and release characteristics of encapsulated black rice anthocyanin microcapsules. Food Chemistry, 294, 493–502. https://doi.org/10.1016/j.foodchem.2019.05.086

Pedrali, D., Scarafoni, A., Giorgi, A., & Lavelli, V. (2023). Binary Alginate-Whey Protein Hydrogels for Antioxidant Encapsulation. Antioxidants, 12(6), 1192. https://doi.org/10.3390/antiox12061192

Rossi G, Guzzon F, Canella M, et al (2019) Le varietà agronomiche lombarde tradizionali a rischio di estinzione o di erosione genetica. Ortive e cerealicole: uno sguardo d'insieme, first ed. Pavia, Lombardia, Italy.

Serventi, L. (2020). Upcycling Legume Water: From wastewater to food ingredients. Springer International Publishing. https://doi.org/10.1007/978-3-030-42468-8

Shim, Y. Y., He, Y., Kim, J. H., Cho, J. Y., Meda, V., Hong, W. S., Shin, W.-S., Kang, S. J., & Reaney, M. J. T. (2021). Aquafaba from Korean Soybean I: A Functional Vegan Food Additive. Foods, 10(10), 2433. https://doi.org/10.3390/foods10102433

Tassoni, A., Tedeschi, T., Zurlini, C., Cigognini, I. M., Petrusan, J.-I., Rodríguez, Ó., Neri, S., Celli, A., Sisti, L., Cinelli, P., Signori, F., Tsatsos, G., Bondi, M., Verstringe, S., Bruggerman, G., & Corvini, P. F. X. (2020). State-of-the-Art Production Chains for Peas, Beans and Chickpeas—Valorization of Agro-Industrial Residues and Applications of Derived Extracts. Molecules, 25(6), 1383. https://doi.org/10.3390/molecules25061383

Article 1: *under review in plant genetic and crop evolution* 2025

Characterization and prospects for the valorization of plant agro-biodiversity: the bean (*Phaseolus* spp.) landraces of the Lombardy Alps (Northern Italy)

Davide Pedrali[1,2], Martina Ghidoli[2], Sara Margherita Borgonovi[3], Rachele Stentella[4], Luca Giupponi [1,2], Alex Alberto[1,2], Elena Cassani[2], Stefano Sangiorgio[2], Bettina Bussi[4], Giuseppe De Santis[4], Roberto Pilu[2], Alessio Scarafoni[3], Annamaria Giorgi[1,2]*

1Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy

2Department of Agricultural and Environmental Sciences, Production, Landscape and Agroenergy-(DISAA), University of Milan, 20133 Milan, Italy

3 Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy

4 Rete Semi Rurali ETS, Scandicci, Italy

* Corresponding author: Luca Giupponi; luca.giupponi@unimi.it

Abstract

The loss of agrobiodiversity is a world damage both in biological and cultural aspects. Although mountainous areas in Italy still preserve many traditional cultivars (landraces), they face increasing risk from genetic erosion and disappearance. This study focused on the identification and multidisciplinary characterization of 16 previously undocumented bean landraces (*Phaseolus vulgaris* and *P. coccineus*) from the Lombardy Alps (Italy) using genetic, morphological, nutritional and ecological approaches. Genetic analyses revealed clear differentiation between landraces and commercial controls, as well as high intra-specific variability among landraces, likely due to their geographic origin and lack of breeding programs. In contrast, morphological outline analysis and nutritional profiling showed that each landrace possesses unique and distinctive traits without major intra-specific variation. All beans showed high levels of proteins (20-29%) and some accessions (pigmented seeds) had a good quantity of antioxidants and other functional compounds, reinforcing their value as nutritious and health-promoting products. Furthermore, the ecological assessment indicated a shared competitive/competitive-ruderal strategy while outline analyses showed three main beans forms: reniform, elliptic, roundish. The results underscore the remarkable intra-genus and intra-specific diversity within *Phaseolus* landraces suggesting that this variability is genetic, nutritional and

phenotypic. Their cultivation (on farm conservation) and integration into local value chains could support sustainable development in marginal areas and create new opportunities for mountain economies. Moreover, the data collected provide a scientific basis for the registration of these landraces in Italian and European agrobiodiversity registers, a key step for their protection and promotion.

Keywords: plant diversity, conservation, traditional cultivars, mountain resources, nutritional composition, biodiversity loss.

Introduction

In the last few decades, the principles and methods of biodiversity conservation have become some of the most crucial and debated issues, due to the alarming fact that over a third of known species are now at risk of extinction, including those of agri-food interest (agro-biodiversity). The Food and Agriculture Organization of the United Nations (FAO) reports that 75% of global agro-biodiversity has been lost in just a century, and today, three-quarters of the world's food supply comes from just 12 plant species and five animal species (Hammer et al. 1996; Esquinas-Alcázar 2005; FAO 1999, 2004, 2007, 2010).

This decline poses a serious threat not only to the planet's biodiversity but also to the variety of foods available to humans and other species (Spataro and Negri 2013). Plant genetic resources for food and agriculture include modern varieties, crop wild relatives, and landraces (Villa et al. 2005; Spataro and Negri 2013). Landraces, also called local varieties or traditional cultivars, are defined as dynamic populations of cultivated plants with a historical origin, a distinct identity, and no formal crop improvement (Spataro and Negri 2013). They are locally adapted and are closely tied to traditional farming systems (Villa et al. 2005; Piergiovanni and Lioli 2010; Almeida et al. 2023; Romero-Astudillo et al. 2024).

Additionally, the erosion of these resources undermines the cultural heritage linked to agri-food traditions, which play a crucial role in shaping the identity of specific communities and regions (Losa et al. 2025). As genetic erosion intensifies, the sustainable farming practices are at risk of disappearing, taking with them centuries-old knowledge that could hold the key to tackling upcoming agricultural issues (Frison et al. 2011; Giupponi et al. 2020b, 2021b; Raggi et al. 2022).

In response to these challenges, the European Union has developed a series of strategic initiatives aimed at protecting agrobiodiversity, including the EU Biodiversity Strategy 2020 (EU Commission 2010), the National Recovery and Resilience Plan (PNRR), the 2030 Agenda for Sustainable Development (United Nations 2015) and the European Register of Conservation Varieties (EU Commission 2023). The latter, represents one of the most modern instruments for in situ conservation of landraces adopted by the EU (Spataro and Negri 2013).

Italy is rich in plant agrobiodiversity and it boasts a significant number of landraces, particularly in mountainous and hilly regions (Giupponi et al. 2020b, 2021b). However, it also faces the progressive loss of traditional cultivars (Hammer et al. 1996; Pacicco et al. 2018; Negri 2003) and the Italian government has established the National Register of Agrobiodiversity (Ministerial Decree 2019/39407; https://rica.crea.gov.it/APP/anb/ accessed on 1 September 2023) with the objective of protecting and promoting agricultural and food resources from the risk of genetic erosion and extinction. This register was established in December 2019 in accordance with Law No. 194/2015 (“Provisions for the conservation and enhancement of biodiversity of agricultural and food interest”) and has collected all information on landraces submitted by the regions to the Italian Ministry for Agriculture and Forestry (MiPAAF) in recent years. The register indicated that 12 out of 20 Italian regions had no landraces, including Lombardy (Giupponi et al. 2021b), one of the most industrialized areas in the Alpine macro-region (EU Commission, 2017) and among the most productive Italian regions for horticulture (ISTAT, 2020; ISMEA, 2014). Despite these efforts, regions like Lombardy still face significant challenges in identifying and protecting their unique landraces, which are essential not only for conserving biodiversity but also for fostering sustainable agricultural practices.

A previous study which assessed the presence of landraces currently cultivated in Lombardy (Giupponi et al. 2020b), mainly in alpine and hilly zones, revealed that only eight herbaceous landraces were reported in the first Italian inventory of *in situ* maintained landraces. Based on the outcomes of this study, several actions were recommended to strengthen Lombardy’s and Italy's efforts to protect plant agrobiodiversity. These actions include performing a comprehensive census and regular updates of both cultivated and conserved landraces, characterizing the agronomic and nutritional properties of landraces to promote the development of sustainable agri-food chains and implementing legislative measures to protect both the landraces and the territories from which they originate.

Among these, the *Fabaceae* family (legumes) stands out, both for its agricultural importance and for its role in traditional diets (Ceccarelli 2012; Giupponi et al. 2021b). In fact, they might represent one of the main food and income sources in marginal and mountain areas (Alcázar-Valle et al. 2020; Rodríguez Madrera et al. 2021; Aquino-Bolaños et al. 2021). In these areas, legumes such as beans (*Phaseolus* spp.) have historically been a primary source of food and income, offering high-protein content (around 20g/100g) and serving as a meat substitute for rural communities (Alicandri et al. 2024), including that of Lombardy.

In developed countries like Italy, the nutritional properties of bean landraces are important for promoting their integration into local agri-food chains and supporting their cultivation and *on-farm* (*in situ*) conservation (Pedrali et al. 2022). Nevertheless, most Italian and Lombardy bean landraces remain largely undocumented in terms of distinctness traits, such as nutritional composition, genetic structure, morphological variability, and ecological characteristics, limiting their potential for valorization (Losa et al. 2025). Exploring and disseminating this information is essential for safeguarding these resources and enhancing their role in sustainable agri-food systems (Alicandri et al. 2024).

Landraces are indeed genetic resources of particular interest for both the technical and scientific sectors (for example, for the development of products or food ingredients with high added value and in plans for the genetic improvement of crops) in socio-economic areas, especially marginal areas, since an intensive farming model is not applicable for several physical, social, cultural or economic limitations (Giupponi and Leoni 2020; Raggi et al. 2022; Fenzi and Couix 2022).

In recent years, a renewed interest in local bean varieties has emerged in Northern Italy, driven by consumers seeking traditional and high-quality products. However, despite their nutritional and agronomic value, many traditional Lombardy bean varieties remain undocumented and at risk of genetic erosion (Giupponi et al. 2020b). Most are not preserved in germplasm banks but are still cultivated by a handful of small-scale farmers and hobbyists, making them particularly vulnerable to extinction (Giupponi et al. 2020b; Rossi et al. 2020).

For these reasons, this research aims to genetically, morphologically, ecologically, and nutritionally characterization of 16 previously undocumented bean landraces cultivars (*P. vulgaris* and *P. coccineus*) cultivated and preserved for decades in the Lombardy region. The objective is to assess their diversity and distinctive traits in order to deepen knowledge of plant agrobiodiversity in Northern Italy, support the inclusion of these landraces in national and European registers dedicated to agrobiodiversity protection, and promote their use in crop improvement programs and in high-value, sustainable agri-food systems.

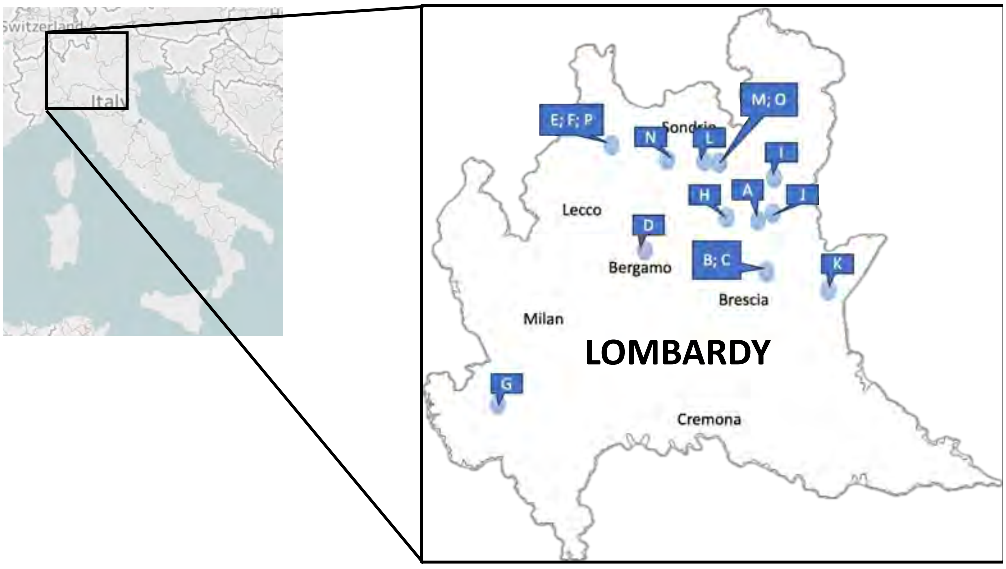
Materials and Methods

Plant material

Based on the census of Lombardy landraces by Giupponi et al. (2020), 16 bean landraces were identified in Lombardy (Northern Italy; Latitude N: 47°1.122'; Longitude E: 12°20.553') between 2020 and 2022 (Figure 1).

These landraces have been cultivated in mountainous areas for over 30 years by local farmers, mainly hobbyists, primarily for self-consumption. Twelve landraces are *P. vulgaris* L. while only four are *P. coccineous* L. Plant identification was conducted using the dichotomous keys of Pignatti (2017) and the landrace data collected was verified to ensure it matched the characteristics outlined by Camacho Vila et al. (2005).

In 2023, the seeds of the 16 landraces were cultivated again in their original fields, following traditional farming practices under the supervision of researchers. This approach aimed to prevent potential hybridizations or phytopathological issues and allowed for the collection of morphological and agronomic data for each landrace. Approximately 500 grams of each landrace were allocated for genetic analysis, and for morphometric and nutritional assessments of the seeds. For comparison, two commercial *P. vulgaris* varieties (Cannellino and Borlotto), purchased at a local market, were cultivated in two experimental fields.



COD	Name	Species	Origin	Lat (°)	Long (°)	Altitude m.a.s.l.
A	Copafam	*P. coccineus*	Val Camonica (BS)	45.9547731	10.2687306	400
B	Tavela longa	*P. vulgaris*	Pertica Bassa (BS)	45.7659132	10.3028845	1070
C	Nosenta	*P. vulgaris*	Pertica Bassa (BS)	45.7659132	10.3028845	1070
D	Sussia	*P. vulgaris*	San Pellegrino Terme (BG)	45.8353335	9.6461925	470
E	Borlotto Camoscio	*P. coccineus*	Val Codera (SO)	46.245352	9.4573499	890
F	Bianco di Codera	*P. coccineus*	Val Codera (SO)	46.245352	9.4573499	890
G	Borlotto di Gambolò	*P. vulgaris*	Gambolò (PV)	45.2576289	8.8379816	100
H	Dihiplì	*P. vulgaris*	Ossimo (BS)	45.946293	10.236272	870
I	Zio Doro	*P. vulgaris*	Garda di Sonico (BS)	46.1275053	10.3440663	790
J	Convento di Vogel	*P. vulgaris*	Niardo (BS)	45.9760913	10.3221444	315
K	Val Vestino	*P. coccineus*	Navazzo di Gargnano (BS)	45.6842632	10.6282474	500
L	Togn	*P. vulgaris*	Ponte in Valtellina (SO)	46.1739215	9.9699667	540
M	Clelia Mangiatutto	*P. vulgaris*	Teglio (SO)	46.1725475	10.0258667	745
N	Emma	*P. vulgaris*	Castione Andevenno (SO)	46.1879228	9.7647217	470
O	Maria Sciuccetti	*P. vulgaris*	Teglio (SO)	46.1725475	10.0258667	745
P	Borlotto di Codera	*P. vulgaris*	Val Codera (SO)	46.245352	9.4573499	890
Q	Cannellino (commerciale)	*P. vulgaris*	Local market	-	-	
R	Borlotto (commerciale)	*P. vulgaris*	Local market	-	-	

163

Fig. 1 Bean samples and their cultivation areas. Capital letters are the identification code (COD) of each genotype

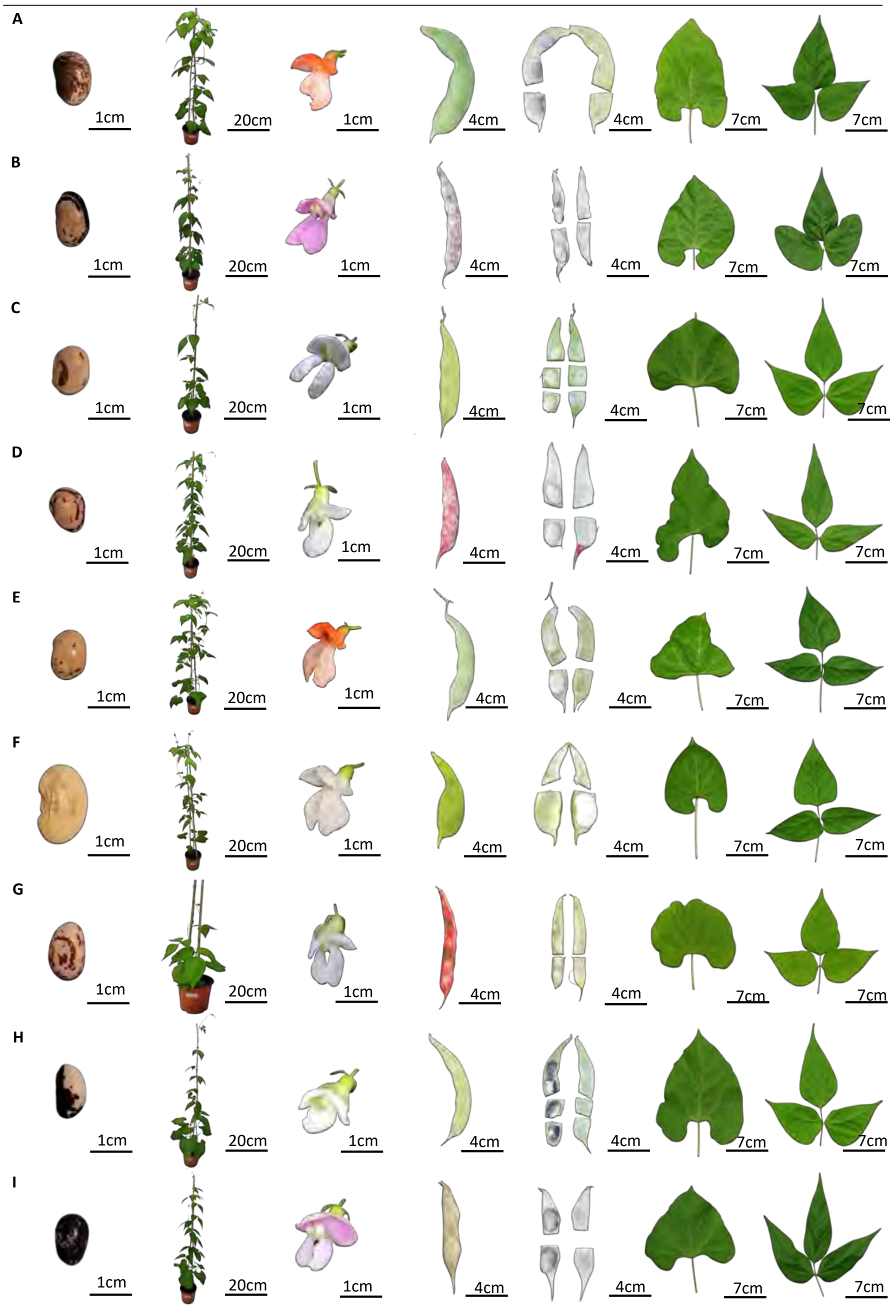


Fig. 2 Representative mature dry seeds, plants after one month, flowers, entire pods, sectioned pods, entire basal leaf and trifoliate leaf from each accession of *Phaseolus* spp. studied. The identification codes of bean samples (capital letter) are referred to in Figure 1.

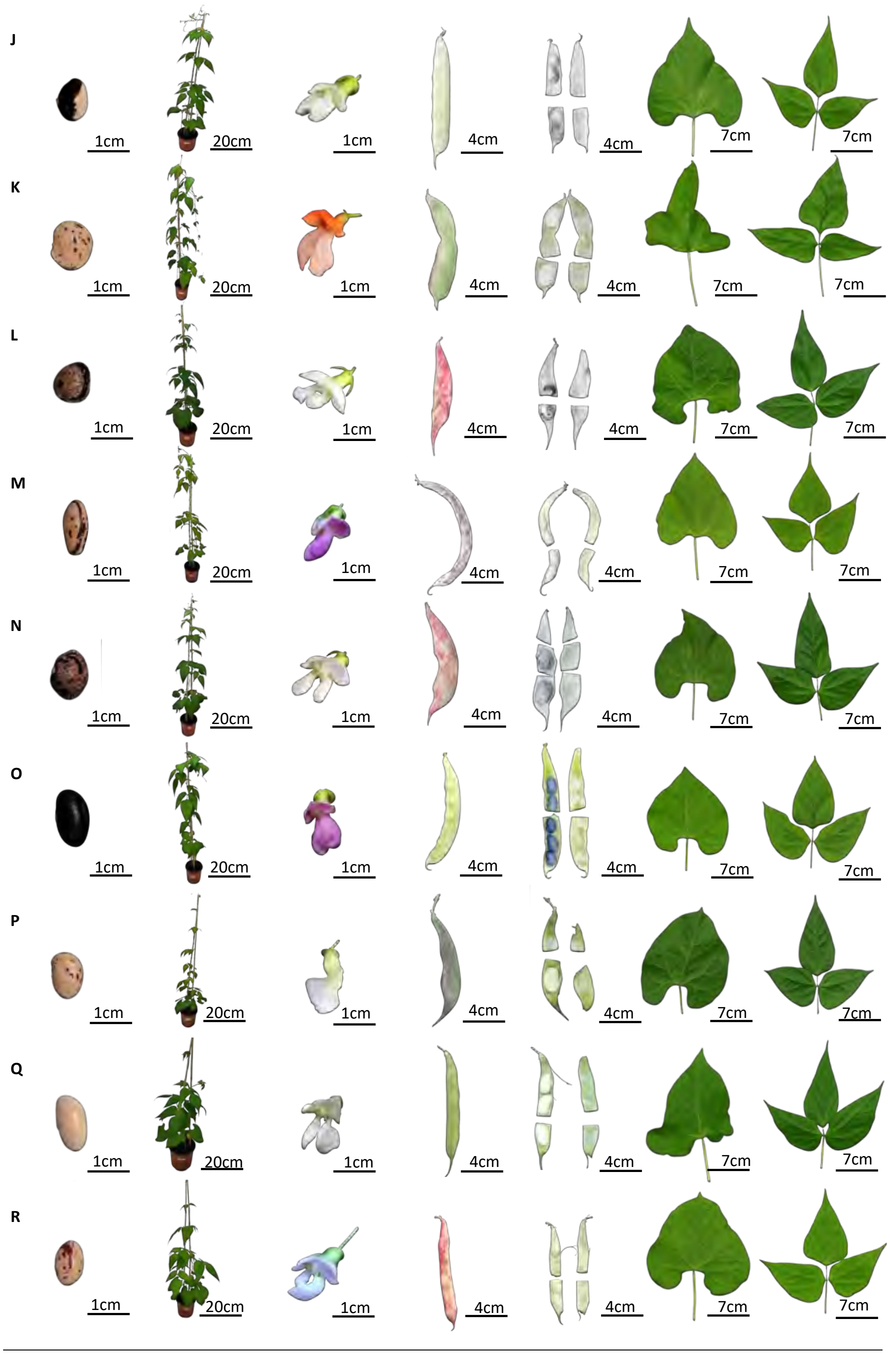


Fig.2 (continued)

Genetic analysis

The genetic analysis was performed on the DNA extracted from the leaves of the 18 varieties (16 landraces and the two commercials' beans) (Figure 2) using 20 molecular markers SSRs (Table 1).

For each variety, two seeds were germinated in the laboratory under controlled conditions and their leaves were sampled and crushed and then used for DNA extraction following the Steve1 method modified (Ghidoli et al. 2024). The extracted DNA was diluted 5 times, and 1µL was utilized for Polymerase Chain Reactions (PCR). PCR were carried out as described by Yu (2000) by using labelled primers with 6-FAM. Sizing was performed in outsourcing by BMR genomics (Padova, Italy).

Out of 20 SSRs we used 15 markers (PV128, PV131, PV167, BM153, and BM187 were discarded) that showed the most robust amplification throughout all the work.

PCR reactions were performed in a thermocycler, where the amplification conditions consisted of a first denaturation cycle at 94°C for 2' followed by 35 denaturation cycles for 1' at 94°C, primer annealing at the reported annealing temperature in Table 1 (Tm) for 1' and extension for 1' at 72°C, with a final extension cycle for 5' at 72°C.

By electrophoresis, the amplified fragments were fractionated using 4% agarose gel and coloured with ethidium bromide. The amplifiers were outsourced to BMR Genomics for sizing. The results obtained were used to compile the binary data matrix used to generate the Principal Coordinate Analysis (PCoA) using the GenAlEx 6.5 program. Finally, a cluster analysis using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm was performed to evaluate the similarity among landraces and commercial varieties.

Table 1. 20 SSRs identified in bean and used for the molecular analysis (modified from Grisi et al., 2007)

SSRs	SEQ PRIMER FORWARD	SEQ PRIMER REVERSE	Tm (°C)
PV31	AATGGCAGGTCAGTGAAACA	ATGACCACGCAGTGACAGAG	56
PV87	CTCATTGCGTCTACCAGTGC	CCTAGGTTCCGCAGCATGT	56
PV93	TGGGGTGAGAGAGAAAGGTG	TACCATAGCAGGCGTTGTTG	56
PV94	ACGACGGAGAGAGAGGTTGA	CCGTGTTCCTTCTGTCTGTG	56
PV106	CAACAAACAAGGCTGAAAAACA	AAAAAGAGAGGAGAGAGAAGAGAGC	56
PV112	AACAACACCACCCTGGAGAC	ACAAAAAGCGAGGAATCACG	56
PV113	TGCATTCTTCCTCCCATCTT	TTGATTTGATTTGAGCAGTGGTG	56
PV128	GAAGAGCTCTCATCGCAACG	CTAGCTCCCTCCCCTCGTAA	56
PV131	GCGTCTGAGGAGAAGGAGGT	CTCCCAATCTCACCAAAACC	56
PV167	GGCAAAAACAAAACCATTTCA	GCCATTTCTCCACTGTCTGG	56
PV185	TGGTAAAGCAAAAACGATGG	GACAGAAGAGTGAGGGTGTGAA	56
PV191	AATTTTGAACTGAAAGATAACAAAGC	CGCTTCATTCAACTCCGAAA	56
PV218	TGTAAATGGCAGGCAGTGAA	ATGACCACGCAGTGACAGAG	54
PV233	AGAGAGGGTTGTGGTTGGTG	TTAATCCCGCTTTACGCAAC	56
PV269	TCGCCCCATATTCACTTTTC	TGGTGTGCAGAAAGTCTGTGA	56
BM141	TGAGGAGGAACAATGGTGGC	CTCACAAACCACAACGCACC	62
BM153	CCGTTAGGGAGTTGTTGAGG	TGACAAACCATGAATATGCTAAGA	63
BM154	TCTTGCGACCGAGCTTCTCC	CTGAATCTGAGGAACGATGACCAG	68
BM160	CGTGCTTGGCGAATAGCTTTG	CGCGGTTCTGATCGTGACTTC	65
BM187	TTTCTCCAACTCACTCCTTTCC	TGTGTTTGTGTTCCGAATTATGA	63

Ecological and agronomic analysis

The analysis of the competitor, stress-tolerator, ruderal (CSR) functional strategy of Grime (Grime 1974, 1977, 2001) of all 18 genotypes was performed according to the method proposed by Pierce et al. (2017). In detail, 10 fully expanded leaves of each genotype were collected in the experimental fields during July 2023. The leaf samples were collected from different plants considering those without disease. All the 10 leaf samples collected were wrapped in moist paper and stored at 4 °C overnight. Leaf fresh weight was measured using an analytical weight scale (Precisa XB 220A, 0.0001 g). The beans leaves were digitized with a digital scanner (Samsung X3280NR) and ImageJ software (Schneider et al. 2012) was used to calculate their leaf area. Beans dry weight was measured after oven drying 105 °C overnight. CSR values and functional strategy of beans samples were determined using ‘StrateFy’ spreadsheet (Pierce et al. 2017) and were plotted in the CSR ternary graph using the ‘ggplot2’ package of R software (R Core Team 2024).

In field, phenotypic data were collected for all landraces through the completion of UPOV morphological descriptors. These forms provide essential information for the potential registration of these varieties in official biodiversity or conservation registers.

Geometric morphometric analysis

All landraces seed samples and those of the two commercial beans collected from local markets were used for the outline analysis (elliptical Fourier descriptors analysis) (Giupponi and Giorgi 2019). For each genotype, 30 seeds were randomly selected. Each seed sample was positioned on a white table and photographed using a Canon EOS 2000D digital camera installed perpendicularly to the surface. The images of the beans were modified using Adobe Photoshop software removing the shadows and the images were transformed into black and white. The outline coordinates were extracted with Momocs 1.4.0 (Claude 2008; Bonhomme et al. 2014) in an R environment (R Core Team 2024) and converted into Fourier coefficients considering 8 harmonics that accumulated at least 99% of the total harmonic power (Bonhomme et al. 2014). To control the asymmetry, the samples were flipped in the same direction, and a landmark was defined as a starting point for importing outline coordinates. The contours were centered, and the outline analysis was performed without numerical normalization. Principal Component Analysis (PCA) was performed on the matrix of coefficients to reduce dimensionality, and the samples were plotted on the first two principal components. Linear discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was carried out. The mean shape of the beans of each genotype was reconstructed using the MSHAPES function. The average bean shape was reconstructed using the MSHAPES function in Momocs, and a multivariate analysis of variance (MANOVA) was conducted to assess differences among beans' shapes. Subsequently, pairwise MANOVA was applied to identify specific differences between samples

Proximate composition of the seeds

Approximately 300g of seeds collected from the field were milled into flour for nutritional analysis. A blender (IKA A10 basic, Werke GmbH & Co.KG, Staufen, Germany) was used to obtain a bean whole flours. All plant material was collected in compliance with national and international mandatory regulations.

The measurement of the total starch content was done according to the AACC 76-13.01 method, following the manufacturer’s instructions. Values were expressed as g/100 g of flour.

The Kjeldahl method was utilized to the protein content determination, in according to International Standard ISO 20483:2013. Nitrogen was converted into protein using a conversion factor of 6.25 and the results were expressed as g/100g.

To determine the total lipid content, 0.5 grams of flour were extracted with 5 mL of pentane under agitation at 37°C for 2 hours and 30 minutes. This process was repeated twice. After extraction, the samples were placed in an oven at 40°C overnight to ensure complete evaporation of the pentane. The lipid content was then calculated using the following calculation:

$$\%\, lipids = \frac{mi - mf}{mi} \times 100 \tag{1}$$

Where *mi* is the initial mass, and *mf* is the final mass.

Folin-Ciocalteau spectrophotometric method (Singleton et al. 1999) was used to analyze the total polyphenol content. Quantification was expressed as gallic acid equivalents (mg GAE/g bean flour) after measuring the absorbance at 765 nm.

The determination of total flavonoids was performed by aluminum chloride test (Alcázar-Valle et al. 2020). The absorbance was measured at 415 nm in a spectrophotometer, and the results were expressed as mg of catechin/g of flour.

The antioxidant activity of beans was evaluated using the Fe (III)/tripyridyltriazine complex (FRAP), following the procedure of Benzie and Strain (1996 Ferric sulfate (FeSO4) concentrations in the range 100-1000 µmol/l were used for calibration. Results were expressed in µmolFe2+/g based on the calibration curve.

Trypsin inhibitor activity (TIA) was performed following the ISO 14902:2001 assay.

The phytic acid and Raffinose Series Oligosaccharides content were determined using K-PHYT and K-RAFGL Megazyme assay kit, respectively, (Megazyme International Ireland Ltd, Wicklow, Ireland) following the manufacturer's instructions. The results were measured by milligrams of phytic acid per gram of bean flour and g/100 g

One-way ANOVA and a Tukey post-hoc test were used to analyze the data for proximate analysis. The Levene and Shapiro-Wilk's tests were used to verify the assumption that variances were homogeneous, and group data was normal. The data were expressed as mean ± standard deviation (SD) of three replicates, and differences were considered statistically significant when $P < 0.05$.

Results

The molecular analysis conducted using 15 SSRs, as reported in Figure 3, allowed for the unique discrimination of all 18 genotypes. Furthermore, the PCoA (Percentage of variation explained by the two axes = 66.01%) and UPGMA analyses clearly demonstrated the presence of three main clusters. It was possible to highlight the differences not only between the two different species (*P. vulgaris* vs. *P. coccineus*) but also within *P. vulgaris*, distinguishing the two commercial benchmarks (“Cannellino” commercial (Q); “Borlotto” commercial (R)) from the traditional varieties/landraces.

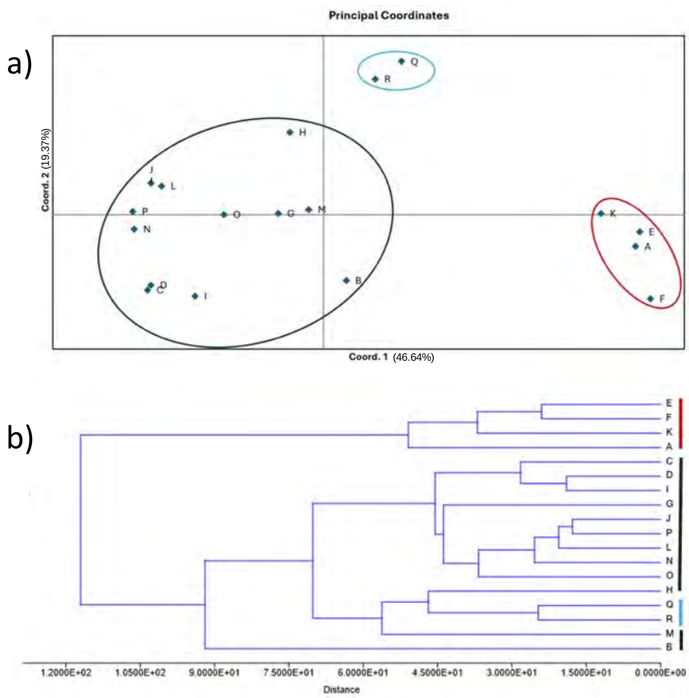


Fig. 3. Molecular analysis. (a) PCoA (Eigenvalue scaling) and (b) UPGMA clustering using 15 SSR markers across 18 different varieties. *P. coccineus* is shown in red, *P. vulgaris* commercial varieties in light blue, and *P. vulgaris* landraces in black. The identification codes of bean samples (capital letter) are referred to in Figure 1.

In Table 2 were represented the results of the ecological analysis (functional strategy). CSR functional strategy revealed that most samples had a competitor/competitive-ruderal strategy (C/CR). The genotypes do show less o no adaptation to stress, understood as phenomena which restrict photosynthetic production such as shortages of light, water, and mineral nutrients, or suboptimal temperatures (Grime 2001).

Also from the results of the functional strategy, no differences are observed between the two species considered (*P. vulgaris* and *P. coccineus*), nor between landraces and commercial varieties. The only two genotypes that exhibit a slightly different functional strategy (CR) from the others are "Tavela longa" (B) and "Zio Doro" (I) (Table 3). Both are, in fact, more ruderal (R >39%) and less competitive than the others (C <61%), which suggests that these landraces may be more resistant to disturbances (understood as the set of biotic and/or abiotic phenomena capable of destroying plant biomass), and therefore more resilient to potential mechanical damage (such as attacks by phytophagous insects or herbivores and/or hailstorms and wind damage, etc.) compared to the other genotypes.

Table 2. Results of Grime's CSR functional strategy. Key: C, competitor; S, stress-tolerator; R, ruderal. The identification codes of bean samples (capital letter) are referred to in Figure 1.

COD	C (%)	S (%)	R (%)	Strategy
A	67.98	0.00	32.02	C/CR
B	60.33	0.00	39.67	CR
C	73.41	0.00	26.59	C/CR
D	70.99	0.28	28.73	C/CR
E	70.12	0.00	29.88	C/CR
F	61.27	6.68	32.05	C/CR
G	72.05	0.58	27.37	C/CR
H	69.99	8.64	21.37	C/CR
I	56.09	0.00	43.91	CR
J	65.42	0.37	34.21	C/CR
K	69.19	0.00	30.81	C/CR
L	68.96	0.00	31.04	C/CR
M	64.66	5.47	29.87	C/CR
N	70.63	3.18	26.19	C/CR
O	63.91	0.00	36.09	C/CR
P	69.34	0.00	30.66	C/CR
Q	72.12	4.90	22.98	C/CR
R	68.16	1.82	30.02	C/CR

Drawing on the data collected during fieldwork, a UPOV morphological descriptor form was filled out for each bean landrace, providing a standardized description of their phenotypic traits. The results of UPOV forms were reported in supplementary material (Supplementary Information SI 1).

The mean shapes of the eighteen bean samples returned by the outline analysis are shown in Figure 4a, while Figure 4b presents the Linear Discriminant Analysis of principal components (LDA) results. Along the horizontal axis (LD1 = 60.5%), the seed shapes transition from more elliptic to more rounded, while along the vertical axis (LD2 = 30.4%), moving from top to bottom, the seed shapes shift from less reniform to more reniform. Figure 4 illustrates the considerable morphological variation in seed shape among the analyzed genotypes, with each accession displaying a distinct and specific shape profile. However, in contrast to the genetic analysis, the results of the morphometric analysis do not allow for any clear clustering of accessions, neither according to species (*P. vulgaris* and *P. coccineus*) nor between the 16 traditional landraces and the two commercial varieties.

The landraces “Bianco di Codera” (F), “Borlotto Camoscio” (E) and “Fagiolo della Val Vestino” (K), placed in the left part of the graph LDA, had the most elliptic seeds, the landraces “Togn” (L), “Convento di Vogel” (J) and “Zio Doro” (I), placed on the right side of the graph, presented the roundish seeds, while the “Clelia mangiatutto” (M) and “Borlotto di Codera” (F), placed at the bottom of the graph, have the most rhino-shaped seeds. The other varieties have morphometric characteristics intermediate between those mentioned above. These results were based on the data reported in supplementary material (SI 2). Multivariate analysis of variance (MANOVA) showed that the only varieties among which there are no statistically significant differences in seed shape are “Dihiplì” (H), commercial “Borlotto” (R), “Sussia” (D) and “Fagiolo Emma” (N).

Finally, based on LDA and PCA results (Figure 4c), three main shapes of bean seeds emerged: reniform, elliptic, roundish.

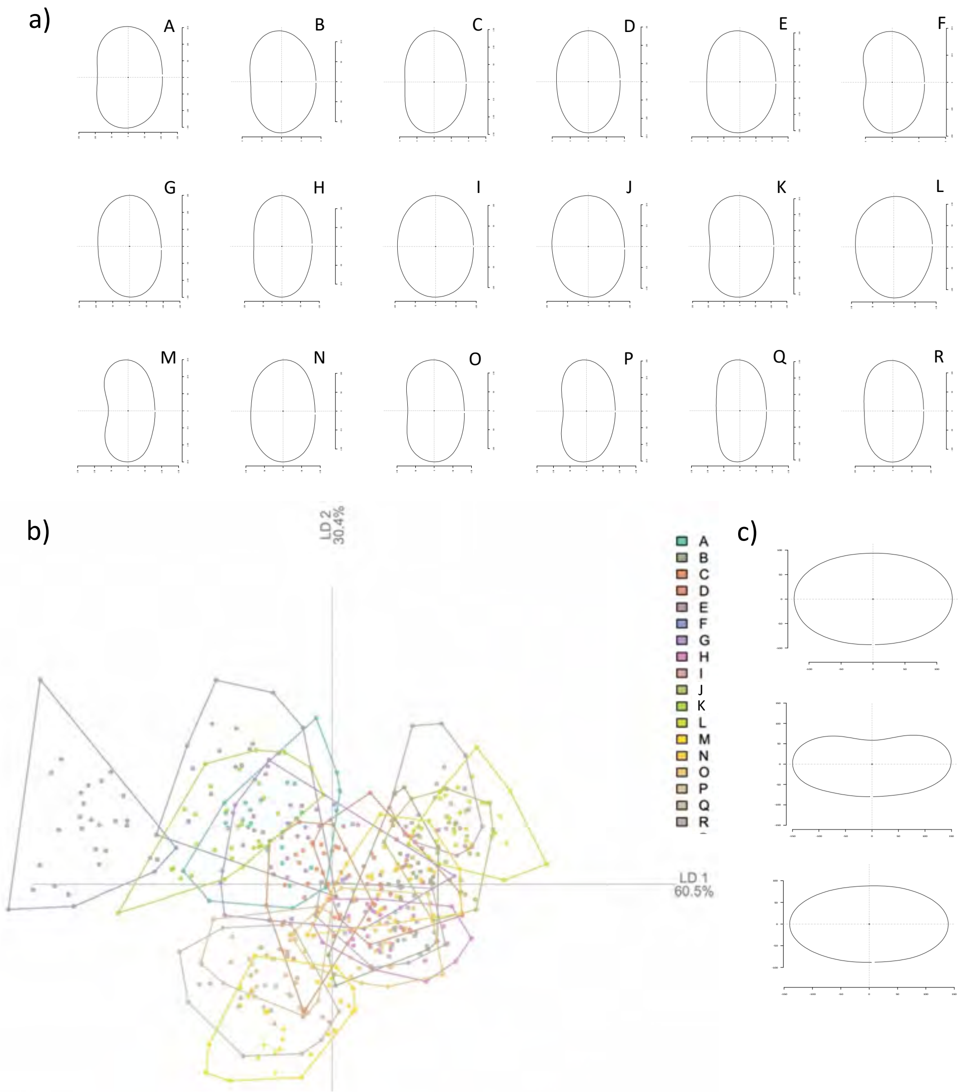


Fig. 4. Mean shapes of the eighteen bean samples (a), LDA analysis results (b) and the three main morphologies of bean seeds (c). The identification codes of bean samples (capital letter) are referred to in Figure 1.

Table 3 shows the nutritional and phytochemical characteristics of bean samples. The analysis of bean samples reveals significant variability in their nutritional and phytochemical composition, making it challenging to establish clear groupings or identify consistent patterns of similarity among genotypes.

Regarding the proximate composition, “Clelia Mangiatutto” (M) sample has the highest starch content (46.55 g/100g), while “Val Vestino “bean (K) has the lowest (29.39 g/100g).

All beans had a protein content above 20%, with sample P reaching 29.68 g/100g and the lowest amount was found in K (20.01 g/100g) and in the other *P. coccineus* beans.

Lipid content varies among samples, with “Val Vestino bean”sample (K) (6.00 g/100g), (M) “Clelia Mangiatutto” and “Borlotto di Codera” (P) (5.20 g/100g) having the highest and “Togn” bean (L) the lowest (1.20 g/100g).

The highest polyphenol content was found in *P. Vulgaris* samples, particularly “Emma” (N), “Borlotto di Codera” (P), “Togn” (L), and “Maria Sciuccetti” (O). The flavonoid level was higher in K and N samples (1.33 and 0.61 mg/g, respectively), suggesting a strong presence of functional and bioactive compounds. G has the lowest flavonoid content (0.16 mg/g), indicating a limited antioxidant potential.

Sample K has the highest antioxidant capacity (3.42 µmol Fe^{2+}/g), making it potentially beneficial for oxidative stress protection, while N, Q and F beans had the lowest mean value (less than 0.4 µmol Fe^{2+}/g).

“Zio Doro” bean (I) has the highest TIA value (3.94 mg/g), which may reduce protein digestibility, while “Emma” (N) has the lowest activity (0.08 mg/g), making it a more digestible variety. Generally, *P. coccineus* samples showed a higher value of trypsin inhibitor activity than the other beans. The same trend was shown in phytic acid content, where sample I scored a mean value of 1.32 g/100g in contrast to “Emma” bean (N) which had the lowest content (0.51 g/100g), making it a better choice for those with mineral deficiencies. This antinutrient was high in commercial beans too.

In the dataset, *P. coccineus* samples displayed the highest oligosaccharide (α-Galactosides) content, with “Borlotto Camoscio” (E) reaching 5.79 g/100g. In contrast, samples C and F exhibit the lowest value (1.13 and 2.06 g/100g, respectively), potentially making them more digestible for individuals with intestinal issues related to oligosaccharides.

The Multidimensional Scaling (MDS) analysis (Fig. 5) showed distinct patterns in the distribution of the analyzed bean accessions, as far as nutritional and phytochemical parameters concerns. The commercial varieties lie in the center of the principal component plot, reflecting their uniformity and lack of extreme traits, while the landraces had a broader dispersion highlighting their diversity. The *P* *coccineus* landraces are concentrated in the upper left quadrant (first quadrant) an area associated with elevated levels of RSO, flavonoids and strong antioxidant activity. Conversely, several *P. vulgaris* landraces were found in the lower region of the plot. These genotypes exhibit the highest levels of both protein and polyphenols among all accessions analyzed.

Table 3. Nutritional and phytochemical composition of samples beans. The identification codes of bean samples (capital letter) are referred to in Figure 1. Key: TIA (Trypsin inhibitor activity); FRAP (Ferric Reducing Antioxidant Power); RSO (Raffinose Series Oligosaccharides)

COD	Starch (g/100g)	Protein (g/100g)	Lipids (g/100g)	Polyphenols (mg GAE/g)	Flavonoids (mg/g)	FRAP (µmol Fe2+/g)	TIA (mg/g)	Phytic acid (g/100g)	RSO (g/100g)
A	35.84 ±1.5^{defg}	21.76 ±1.58^{abc}	2.98 ±0.00^{def}	1.16 ±0.05^{fghi}	0.31 ±0.01^{ab}	0.74 ±0.1^{cde}	2.40 ±0.02^{ef}	1.01 ±0.02^{efg}	4.82 ±0.07^{f}
B	40.08 ±0.18^{jk}	23.28 ±0.99^{cde}	3.22 ±0.11^{def}	1.08 ±0.04^{efgh}	0.49 ±0.04^{bc}	1.43 ±0.04^{g}	2.35 ±0.15^{ef}	0.90 ±0.00^{cde}	4.54 ±0.31^{f}
C	42.03 ±1.41^{k}	23.02 ±0.61^{cd}	2.41 ±0.02^{bcd}	1.06 ±0.07^{efgh}	0.33 ±0.16^{ab}	0.72 ±0.16^{cde}	3.24 ±0.04^{g}	1.06 ±0.01^{gh}	1.13 ±0.10^{a}
D	39.70 ±0.18^{ijk}	23.57 ±0.89^{cde}	2.72 ±0.11^{cde}	0.91 ±0.02^{cdefg}	0.31 ±0.04^{ab}	0.67 ±0.04^{cde}	0.55 ±0.02^{b}	1.17 ±0.01^{hi}	3.32 ±0.05^{cde}
E	39.51 ±0.82^{hij}	20.18 ±0.72^{ab}	3.45 ±0.12^{ef}	1.20 ±0.01^{ghij}	0.33 ±0.01^{ab}	1.13 ±0.01^{f}	3.61 ±0.02^{h}	0.89 ±0.03^{cd}	5.79 ±0.02^{g}
F	35.36 ±0.27^{def}	21.96 ±0.50^{bc}	3.15 ±0.11^{def}	0.60 ±0.00^{ab}	0.41 ±0.36^{abc}	0.39 ±0.36^{a}	3.13 ±0.08^{g}	1.19 ±0.03^{i}	2.06 ±0.00^{b}
G	37.65 ±1.58^{fghij}	21.90 ±0.09^{bc}	3.78 ±0.22^{fg}	1.43 ±0.12^{ij}	0.16 ±0.05^{a}	0.79 ±0.05^{de}	2.22 ±0.03^{e}	1.20 ±0.00^{ij}	3.52 ±0.12^{cde}
H	32.42 ±0.49^{bc}	27.78 ±0.05^{f}	4.50 ±0.18^{gh}	1.31 ±0.09^{hij}	0.27 ±0.11^{ab}	0.58 ±0.11^{bc}	1.81 ±0.06^{d}	0.93 ±0.03^{cdef}	3.15 ±0.19^{cd}
I	38.94 ±0.74^{hij}	24.94 ±0.58^{e}	3.52 ±0.04^{ef}	0.73 ±0.07^{bc}	0.20 ±0.10^{a}	0.47 ±0.10^{ab}	3.94 ±0.09^{j}	1.32 ±0.03^{j}	3.74 ±0.20^{e}
J	31.17 ±0.47^{ab}	20.23 ±0.23^{ab}	2.80 ±0.40^{cde}	0.85 ±0.01^{bcde}	0.47 ±0.22^{bc}	0.64 ±0.22^{cd}	0.11 ±0.00^{a}	0.71 ±0.01^{b}	3.33 ±0.01^{cde}
K	29.39 ±0.32^{a}	20.01 ±0.41^{a}	6.00 ±0.40^{i}	1.02 ±0.01^{defgh}	1.33 ±0.12^{d}	3.42 ±0.00^{j}	3.38 ±0.01^{gh}	0.85 ±0.02^{c}	4.90 ±0.02^{f}
L	35.05 ±0.30^{de}	29.28 ±0.10^{fg}	1.20 ±0.40^{a}	1.28 ±0.05^{hij}	0.41 ±0.09^{abc}	0.81 ±0.09^{e}	0.69 ±0.03^{bc}	0.83 ±0.00^{bc}	3.78 ±0.03^{e}
M	46.55 ±0.56^{l}	28.11 ±0.14^{fg}	5.20 ±0.4^{hi}	1.20 ±0.09^{hij}	0.42 ±0.09^{abc}	2.02 ±0.09^{i}	0.84 ±0.01^{c}	1.04 ±0.01^{fg}	3.03 ±0.03^{c}
N	39.12 ±11^{hij}	28.20 ±0.45^{fg}	2.80 ±0.40^{cde}	1.40 ±0.08^{ij}	0.61 ±0.06^{c}	0.35 ±0.06^{a}	0.08 ±0.03^{a}	0.51 ±0.00^{a}	3.25 ±0.03^{cde}
O	34.74 ±0.3^{cd}	28.97 ±0.22^{fg}	2.00 ±0.40^{bc}	0.89 ±0.05^{bcdef}	0.31 ±0.01^{ab}	0.62 ±0.01^{cd}	1.83 ±0.21^{d}	0.91 ±0.01^{cde}	3.24 ±0.06^{cde}
P	37.87 ±0.4^{ghij}	29.68 ±0.01^{g}	5.20 ±0.40^{hi}	1.46 ±0.33^{j}	0.30 ±0.03^{ab}	1.90 ±0.03^{h}	0.78 ±0.09^{bc}	1.00 ±0.00^{defg}	3.04 ±0.03^{c}
Q	37.08 ±0.47^{defgh}	24.36 ±0.09^{de}	1.85 ±0.11^{ab}	0.32 ±0.02^{a}	0.27 ±0.24^{ab}	0.39 ±0.24^{a}	2.35 ±0.13^{ef}	1.31 ±0.02^{j}	3.59 ±0.58^{cde}
R	37.37 ±1.35^{efghi}	22.83 ±0.45^{cd}	2.50 ±0.11b^{cd}	0.76 ±0.04^{bcd}	0.32 ±0.06^{ab}	0.73 ±0.06^{cde}	2.63 ±0.20^{f}	1.07 ±0.14^{gh}	3.66 ±0.27^{de}

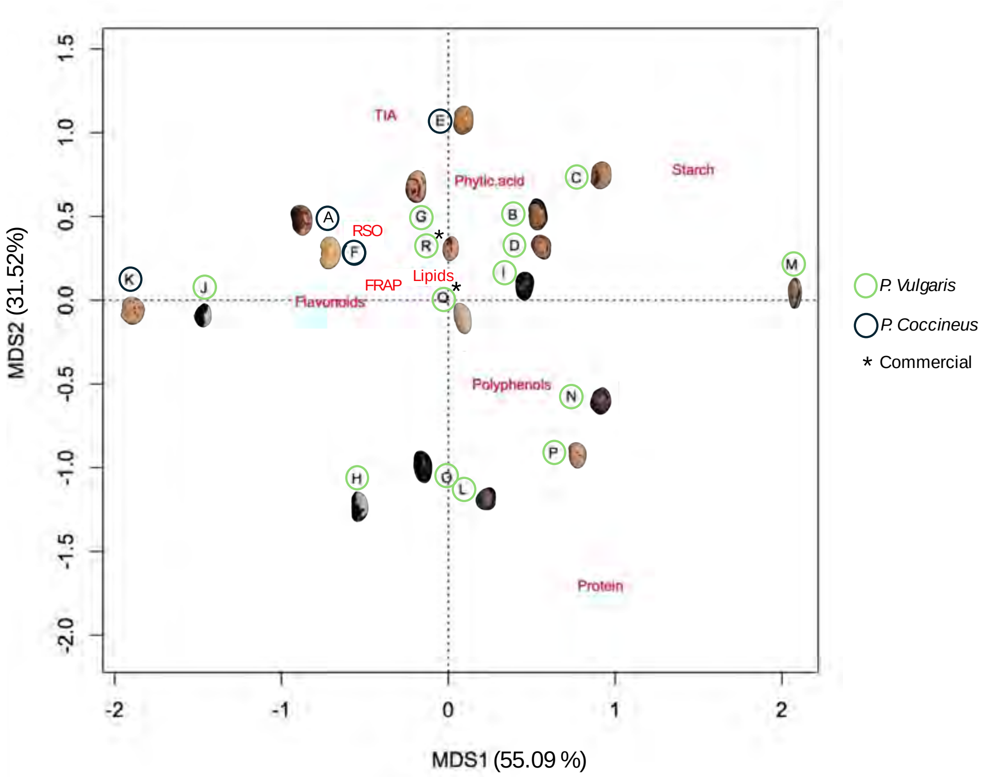


Fig.5 MDS (Multidimensional Scaling) biplot of beans samples associated with nutritional and phytochemical parameters. The identification codes of bean samples (capital letter) are referred to in Figure 1. Key: TIA (Trypsin inhibitor activity); FRAP (Ferric Reducing Antioxidant Power); RSO (Raffinose Series Oligosaccharides)

Discussion

This research contributed to knowledge on characterizing several previously undocumented Lombardy bean landraces, bridging the lack of scientific data.

The molecular analysis shown in Fig.3 highlighted the differences between the species *P. coccineus* and *P. vulgaris*. Considering the phenotypic differences, growth environments, and reproductive cycles of the two species, these results confirm previous studies on the genetic differences between these two species (Bosmali et al. 2024). It is well known that *P. coccineus* is predominantly an allogamous species that requires pollinating insects for fruit set and seed production, whereas *P. vulgaris* is capable of self-pollination (Ciaffi et al. 2024). Additionally, *P. coccineus* thrives in environments with lower average temperatures compared to *P. vulgaris*, favoring hilly or mountainous regions (Aquino-Bolaños et al. 2021). Within the *P. vulgaris* species, it is possible to distinguish the two commercial varieties used (Q: "Cannellino" commercial; R: "Borlotto" commercial), employed as benchmarks, from the traditional varieties studied. This result is particularly important for future work on the characterization and conservation of the germplasm of this species, as it allows for the rapid identification of newly synthesized commercial cultivars that might be mistaken for traditional varieties or landraces (Landoni et al. 2024). Modern newly synthesized varieties, having been subjected to improvement programs, exhibit a modified genetic structure with alleles conferring superior traits related to resistance to biotic and abiotic stresses, enabling their distinction from traditional genetic materials (Mercati et al. 2015).

Unlike the results of the genetic analysis, those concerning seed morphology, functional strategy, and nutritional traits did not reveal clear distinctions between the two species (*P. vulgaris* and *P. coccineus*) or between the landraces and the commercial bean varieties. Each genotype exhibited unique and specific characteristics, which, particularly in terms of seed shape and nutritional composition, clearly differentiated it from the others. These findings highlight the high intra-genus and intra-specific diversity within *Phaseolus* and suggest that this variability is both intrinsic (genetic, eco-physiological, and phytochemical) and phenotypic (seed shape and morphological traits, see Fig. 2).

In this research, the outline analyses demonstrated differences in the beans assessed, which present more variable shapes. This might be because landraces are not commercial varieties having not been subjected to strong morphological selection (and genetic depletion) to produce standardized products. The morphometric analysis has revealed three main shapes of bean seeds: reniform, elliptic and roundish (Fig 4c).

Modern geometric morphometric methods, such as outline analysis, provide a powerful approach for analyzing and comparing shapes in biological studies. These techniques evaluate the spatial relationships of landmarks and outlines to measure form and size and have been successfully applied in plant biology, for example, to distinguish leaf shapes (Chitwood and Otoni 2017; Giupponi and Giorgi 2019b) or to characterize landraces (Giupponi et al. 2019a, 2020a; Pedrali et al. 2024). These analyses offer an objective (based of statistical analysis), cheap and modern method for the characterization and identification of landraces, reducing subjectivity and human error. They could support or even replace traditional morphological descriptors used in Distinctness, Uniformity, and Stability (DUS) testing, as defined by the International Union for the Protection of New Varieties of Plants (UPOV) and the Community Plant Variety Office (CPVO), which are mandatory for the registration of cultivars in both the register of conservation varieties and the plant agrobiodiversity register (https://www.upov.int/resource/en/dus_guidance.html).

The CSR functional classification of the studied beans revealed a prevailing C/CR strategy, with no particular differences among the genotypes considered. The observed C/CR profile suggests that bean plants combine the ability to efficiently exploit available resources with a certain degree of tolerance to disturbances. In particular, the presence of competitive traits in the analysed genotypes indicates that the different bean varieties share the ability to thrive in environments where plants do not experience stress from factors such as insufficient light, nutrients, or water in the soil. This occurs in cultivated or managed fields where humans, even in mountainous regions, have contributed to the development of agro-ecosystems with more favorable soil characteristics compared to natural soils, for example, through regular fertilization and stone removal (Sicard et al. 2005).

Moreover, the ruderal traits highlighted in the various genotypes studied indicate that beans, in general, are fairly tolerant to disturbances, meaning they are capable of good vegetative recovery following mechanical damage caused, for instance, by insect attacks or adverse weather events. This characteristic explains the presence of many bean landraces in Italian mountain environments (Giupponi et al. 2021), where there is a higher likelihood of adverse weather conditions (storms, strong winds, tempests) capable of destroying plant biomass. In fact, the landrace “Copafam” (sample A) in the Italian Alps is cultivated even above 1,000 m a.s.l. (Giupponi et al. 2018) and can produce shoots, flowers, and fruits late in the season.

Although the analysis of CSR functional strategy has been mainly applied to wild plants (Pierce et al. 2017), it has also been assessed for a few Italian landraces (Giupponi et al. 2019; Zuccolo et al. 2023), one of which, “Ciuenlai” (*Cyclanthera pedata* (L.) Schrad.) (Zuccolo et al. 2023), exhibits a CSR strategy similar (C/CR) to that of the beans considered in this research and is cultivated in Val Camonica (Lombardy Alps) in the same fields where some bean landraces, including “Copafam”, are also grown.

In the future, it would be advisable to apply this methodology to other plants of agricultural and food interest, if only to investigate intra-specific ecological variability and gather information on the functional/adaptive traits of varieties, useful for those who cultivate and/or conserve them *in situ* (*on-farm*).

Moreover, the nutritional composition was similar in all landraces analyzed but they exhibited equal or superior phytochemical profiles compared to commercial varieties especially those with pigmented seeds that demonstrated high levels of functional molecules and antioxidant activity. Starch is the primary energy source in beans but it hadn't showed strong variation among traditional cultivars as well as compared with the commercial samples. Its content ranged from 46.55 g/100g to 31.17 g/100g (sample M and K, respectively), indicating that certain landraces may be more suitable for energy-dense diets, while others, the *P. coccineus* beans, may cater to low-glycemic nutritional needs. A similar carbohydrate content (45.66 ± 0.3 - 55.24 ± 0.4 g/100g) was found by Nor Azmah (2023) that studied four underutilized legumes in Malaysia and confirmed literature data (Maphosa and Jideani 2017; Serventi 2020).

The protein content is a key nutritional feature in legume seeds. With protein levels exceeding 20 g per 100 g across all samples, these legumes are particularly well-suited for vegetarian and vegan diets. "Borlotto di Codera" sample (P) displayed the highest protein concentration at 29.28 ± 0.01 g/100g. The MDS analysis results showed a group of traditional cultivars (all belonging to *P. vulgaris*) that are particularly rich in protein content, more than the commercial ones.

These results are congruent with previous findings by Pedrali (2022), which compared a mountain bean landrace ("Copafam bean", which is included in this research too) to three other commercial beans. All these samples had a protein content ranging from 21.9 to 26.48 g/100g dw and the *P. coccineus* beans had a lower value than the *P. vulgaris* ones.

As expected, lipid content was low in all samples, with limited variability. Each sample exhibited a lipid level below 10%, confirming that these beans represent a healthy food choice aligned with World Health Organization (WHO) dietary recommendations. The WHO advises limiting the intake of high-fat foods to help combat the rising prevalence of overweight and obesity worldwide. This result is according to Maphosa (2017) and Pedrali (2022), who found a total lipid content ranging from 1-5% in beans (Maphosa and Jideani 2017).

Anti-nutritional compounds such as trypsin inhibitor activity (TIA) and phytic acid, which can impair protein digestion and mineral absorption, were lower in the traditional landraces compared to the commercial variety analyzed (Alcázar-Valle et al. 2020; Cominelli et al. 2022). This suggests that landraces may offer improved digestibility and be more appropriate for individuals with mineral deficiencies or sensitive digestive systems.

In the dataset, *P. coccineus* samples exhibited the highest oligosaccharide content, with "Borlotto Camoscio" (E) reaching a peak value of 5.79 g/100 g. This suggests a greater presence of these sugars, which are often associated with the anti-nutritional effects of legumes. Due to the lack of α-galactosidase, these oligosaccharides remain undigested until they reach the lower intestine, where they are fermented by gut microbiota producing gases such as methane, which can result in flatulence. Consequently, the presence of galactosyl-sucrose oligosaccharides could represent a factor limiting these beans use in human diets (Han and Baik 2006). In contrast, "Nosenta" (C) and "Bianco di Codera" (F) samples showed the lowest oligosaccharide levels (1.13 and 2.06 g/100g, respectively), potentially making them more suitable for individuals with digestive sensitivities to these compounds.

Phytochemical analysis highlighted significant differences among samples. Some samples belonging to the *P. vulgaris* genus recorded the highest polyphenol (1.46 ± 0.33 mgGAE/g) suggesting a strong antioxidant profile. In contrast, *P. coccineus* samples had a high flavonoid level and antioxidant capacity, measured by the FRAP assay. This trend was confirmed by MDS biplot graph (Figure 5). In fact, the MDS analysis revealed the *P. coccineus* landraces cluster in the upper left quadrant (first quadrant), an area associated with higher levels of flavonoids and antioxidant activity indicating an ability to counter oxidative stress and potentially lower chronic disease risk. However, these samples also show elevated concentrations of trypsin inhibition activity and oligosaccharides.

In contrast, several *P. vulgaris* landraces are found in the lower portion of the plot. These genotypes exhibit the highest levels of both protein and polyphenols among all accessions analyzed. Their nutritional profiles suggest a strong potential for valorization within the context of functional food development, especially in developed countries where plant-based protein sources and bioactive compounds are increasingly in demand (King et al. 2024). These landraces could therefore serve as a valuable basis for promoting high-quality, locally adapted bean varieties that support both agrobiodiversity conservation and the growing interest in health-oriented diets.

Meanwhile, the commercial varieties are positioned near the center of the principal component plot, indicating an absence of distinctive biochemical traits. This distribution likely reflects the effects of breeding programs aimed at standardization, which typically select for “average” characteristics to ensure uniformity and consumer acceptance.

The presence of numerous traditional landraces in Italian mountain areas provides valuable opportunities to enhance food choices while preserving agricultural biodiversity. This diversity enables the selection of bean varieties best suited to individual dietary needs and health goals, offering a wide range of nutritional and functional profiles. Promoting the cultivation and use of plant-based products, such as legumes as alternative protein sources, is one of the main objectives of the Farm to Fork strategy within the European Green Deal (NRP 2021–2027). This strategy supports the transition toward sustainable food systems by encouraging innovative and environmentally friendly solutions, including increased legume consumption (NRP 2021–2027; Falcione et al. 2022).

In light of this, the preliminary results of the multidisciplinary characterization of Lombardy bean landraces presented in this study could represent a first step toward the valorization of legumes in line with the goals of the Green Deal. Furthermore, as consumer awareness shifts towards the health-promoting properties of food, the demand for products rich in bioactive compounds continues to increase (Firoozzare et al. 2024). In this study we have shown that Lombardy bean landraces offer significant potential for the agri-food sector, particularly in the development of functional foods designed to increase protein, fiber, and antioxidant intake.

These landraces, therefore, have the potential to not only be consumed in their traditional form but also integrated as functional ingredients into processed foods, enhancing both their nutritional quality and health-promoting properties (Lin et al. 2008, Pedrali et al. 2022; King et al. 2024; Bosmali et al. 2025).

The production and consumption of food products from underutilized traditional cultivars strongly linked to specific territories and traditions offers a unique opportunity to create employment and revitalize rural and mountain communities (Giupponi et al. 2018, 2020b; Zuccolo et al. 2023; Pedrali et al. 2022, 2024).

Lombardy features a highly diverse landscape that has long supported the cultivation and conservation of numerous plant species (Giupponi et al. 2020b; Colombo et al. 2022; Rossi et al. 2020). All the landraces included in this study were found in mountainous and hilly areas, as the lowland zones, particularly the Po Valley, have undergone intense urbanization and land degradation over the past century (Giupponi et al. 2013, 2015). Today, large-scale farms in these lowland areas mainly grow commercial varieties, while the mountain regions remain hotspots of agro-biodiversity (Alicandri et al. 2024; Giupponi et al. 2020b, 2021b). These beans should be viewed as strategic resources for the sustainable development of rural and marginal areas (Giorgi and Scheurer 2015; Falcione et al. 2022). In this context, mountain territories could play a dual role, not only as economically and culturally significant areas, but also as key sites for the *in situ* conservation of bean landraces (Alicandri et al. 2024;).

However, the abandonment of agricultural activities, partly caused by a generational turnover in the Alps, remains an ongoing concern, leading to the loss of traditional practices and local genetic resources, including landraces (Nordregio 2004; Keenleisyde and Tucker 2010; Terres et al. 2013, Cislaghi et al. 2019, Didonna et al. 2024). To reverse this trend, greater support is needed to involve new farmers and/or young people in modern, sustainable agricultural systems, particularly those that include the cultivation of landraces (Puneeth et al. 2024).

Rural Development Programmes (RDPs) represent a valuable opportunity to engage and encourage the involvement of young and new farmers, offering financial support and training pathways that can help them start and sustain agricultural activities (https://agriculture.ec.europa.eu/common-agricultural-policy/rural-development/country_en). The RDPs were established by Regulation (EU) No. 1305/2013 and entered into force since 1 January 2014. The RDPs are a key instrument of the European Common Agricultural Policy (CAP), and their objectives include supporting farmers and encouraging environmentally sustainable agricultural practices (https://agriculture.ec.europa.eu/common-agricultural-policy_en). The RDPs operate on a seven-year cycle and are financed through the European Agricultural Fund for Rural Development (EAFRD), which has a total budget of €95.5 billion for the 2021–2027 programming period (https://agriculture.ec.europa.eu/common-agricultural-policy/financing-cap/cap-funds_en). Part of these funds is specifically allocated to research and innovation activities, such as the project presented in this study, which contributes to the conservation and valorization of agrobiodiversity and supports more resilient and diversified agri-food systems.

In line with European directives aimed at the protection of genetic resources, Italy has also established a National Register of Agrobiodiversity, an innovative tool designed to safeguard traditional genetic resources (https://rica.crea.gov.it/APP/anb/anagrafe-nazionale-35.php). Article 3 of Law No. 194/2015 created this Register within the Ministry of Agricultural, Food and Forestry Policies. The Register includes all local genetic resources of food and agricultural interest, of plant, animal, or microbial origin, that are at risk of extinction or undergoing genetic erosion. Its purpose is to formally recognize and protect these resources, which are crucial for preserving agrobiodiversity and ensuring the resilience and sustainability of national agri-food systems (https://rica.crea.gov.it/APP/anb/anagrafe-nazionale-35.php).

A notable example of successful registration on the European register of conservation variety is the “Nero Spinoso” maize (Cassani et al. 2017), while “Grano Siberiano Valtellinese” (Giupponi et al. 2019b), “Ciuenlai” (Zuccoolo et al. 2023) and “Mais delle Fiorine di Clusone” (Giupponi et al.2021a) landraces which are now protected under the National agrobiodiversity Registry. Their inclusion has led to an expansion of the cultivated area, thus enhancing their *in situ* conservation, and could promote their valorization through the development of new agri-food products (Alicandri et al. 2024).

Recently, some farmers have formed associations or consortia to conserve and promote landraces (Negri 2003; Piergiovanni and Lioli 2010; Montesano et al. 2012; Raggi et al. 2022; Fenzi and Couix 2022; Colombo et al. 2022), though most of the varieties identified in this study are maintained by individual, often elderly, hobby farmers who are not affiliated with any formal network generations (Negri 2003; Colombo et al. 2022).

Although Italy has a lot of traditional cultivars (more than 1,650 only considering herbaceous plants), mainly discovered in mountain and sub-mountain, the majority of them remains unknown to most farmers and only a small fraction of them has been registered in the European Register of Conservation Varieties and/ or in the National Register of Agrobiodiversity (Giupponi et al. 2020b). So far, the use of these registers has been and still is limited. In fact, out of the twenty regions in Italy, only eleven, Calabria, Emilia Romagna, Lombardy, Piedmont, Umbria, Liguria, Marche, Tuscany, Apulia, Trentino Alto Adige and Veneto, have recorded conservation varieties. Combined, these registrations (1,148 varieties in total, considering both herbaceous and tree landraces, even if 80% of the landraces listed are tree species). Of these, Tuscany stands out with 738 registrations (64.28%), while Lombardy has only 13 (1.13%) (https://rica.crea.gov.it/APP/anb/anagrafe-nazionale-35.php). This highlights the need for a better evaluation of the effectiveness and accessibility of such regulatory tools in the coming years and increase the awareness of citizens on biodiversity conservation connecting researchers, population, seed savers and farmers (Didonna et al. 2024; Bocci et al. 2025).

Gene banks and seedbanks are examples of tools has been born after the introduction of the concept of “dynamic conservation” (as the “preservation of genetic diversity within and between cultivated plant species, including both *in situ* conservation and *ex situ* conservation, with the aim of a sustainable use of plant genetic resources and agrobiodiversity”) and they are involved in seed saving, on-farm conservation and agro-biodiversity management (Didonna et al. 2024). To protect and conserve the seeds of the landraces studied in this research they have been entrusted to “Rete Semi Rurali” (RSR), the Italian seed network (https://rsr.bio/), which safeguards them in its seedbanks and it could distribute these traditional cultivars to farmers committed to cultivating them in purity promoting both *ex situ* and on-farm conservation (Alicandri et al. 2024; Bocci et al. 2025).

Following scientific characterization, the next crucial step for the conservation and protection of these bean landraces is to support farmers in registering their traditional cultivars in the Italian and European agrobiodiversity registers, thereby granting them the recognition and legal protection they deserve at both national and international levels. The data collected in this study are essential for this purpose, as the registration process requires a detailed description of the distinctive traits of each landrace (https://www.upov.int/resource/en/dus_guidance.html). The characterization of these Lombardy bean landraces and the RSR involvement contributes to their on farm conservation, recognizing them as foundational resources for resilient and sustainable agri-food systems (Losa et al. 2025).

This status not only safeguards the landrace itself but also supports the cultural and environmental value of the territory in which it has historically been cultivated (Pedrali et al. 2022; Zuccolo et al. 2023).

Without concrete support for the preservation and valorization of Lombardy beans landraces, and the others traditional cultivars generally, there is a tangible risk of losing valuable agro-biodiversity. This not only undermines conservation efforts but also limits the development of high-quality, low-impact agri-food chains that could play a pivotal role in promoting smart, inclusive, and sustainable growth in their origin areas (Cleveland et al. 1994; Fideghelli and Engel 2009; EU Commission 2010; Newton et al. 2010). Simultaneously, greater emphasis must be placed on deepening the understanding and promoting the role of agro-biodiversity in food security, cultural identity, and climate change resilience (Falcione et al. 2022; Pedrali et al. 2024; Losa et al. 2025). Raising awareness and educating younger generations on these issues will facilitate future efforts toward the conservation and valorization of agro-biodiversity, especially in mountain areas (Puneeth et al. 2024).

Conclusion

This research enabled the genetic, morphological, ecological, and nutritional characterization of 16 bean landraces from the Lombardy Alps, previously undocumented. The results highlighted that, from a genetic perspective, these landraces were clearly distinguishable not only by species (*P. vulgaris* and *P. coccineus*), but also from the two commercial varieties for a comparison. Furthermore, a high level of genetic variability was observed within the landraces themselves, likely due to their origin from distinct mountain areas across Lombardy (often adjacent or overlapping) and to the absence of breeding or selection programs While the ecological analysis revealed that the Lombardy beans follow a C/CR functional strategy without substantial intra-genus or intra-specific differentiation, the outline and nutritional analyses demonstrated that each landrace exhibits unique and distinctive traits. These characteristics differentiate them not only from landraces of other species but also from those of the same species. Given the agrifood relevance of beans, the nutritional data obtained in this study are particularly valuable for promoting the cultivation, and thus the *on-farm* conservation, of these landraces in developed countries such as Italy, where mountain and marginal agricultural systems are increasingly oriented toward producing quality and niche products rather than quantity.

Nutritional assessments of the 16 landraces revealed that some possess high levels of bioactive compounds and antioxidant activity, along with excellent protein content, suggesting potential use in the development of functional foods. For these reasons, the studied landraces should be regarded as plant genetic resources with untapped potential for innovation in food production and new value chains. Their protection and valorization are essential, especially considering they are still grown by only a few farmers (mainly hobbyists) for self-consumption. To prevent the loss of agrobiodiversity, more research projects must be supported in the future to characterize plant agrobiodiversity, since scientific knowledge represents the fundamental starting point for promoting/valorizing, and conserving landraces.

References

American Association of Cereal Chemists (AACC). Approved Methods of Analysis; Method n_ 08-01.01. 11th ed.; AACC International: St. Paul, MN, USA, 1999.

Alcázar-Valle M, Lugo-Cervantes E, Mojica L, et al (2020) Bioactive Compounds, Antioxidant Activity, and Antinutritional Content of Legumes: A Comparison between Four Phaseolus Species. Molecules 25:3528. https://doi.org/10.3390/molecules25153528

Alicandri E, Paolacci AR, Coluccia L, et al (2024) Exploring molecular, morphological, and biochemical diversity of Phaeolus vulgaris landraces cultivated in the Aniene Valley (Lazio region, Italy). Curr Plant Biol 39:100374. https://doi.org/10.1016/j.cpb.2024.100374

Almeida MJ, Pinheiro De Carvalho MÂA, Barata AM, et al (2023) Crop landraces inventory for Portugal. Genet Resour Crop Evol 70:1151–1161. https://doi.org/10.1007/s10722-022-01492-6

AOAC International. AOAC Method n_9991.36; 945.18; 993.40. In Official Methods of Analysis of AOAC International, 18th ed.; Horwitz, W., Latimer, G.W., Eds.; AOAC International: Gaithersburg, ML, USA, 2005.

Aquino-Bolaños EN, Garzón-García AK, Alba-Jiménez JE, et al (2021) Physicochemical Characterization and Functional Potential of Phaseolus vulgaris L. and Phaseolus coccineus L. Landrace Green Beans. Agronomy 11:803.

https://doi.org/10.3390/agronomy11040803

Benzie, I.F.F., Strain, J.J (1996). The Ferric Reducing Ability of Plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry, 239, 70-76

Bocci R, Bartha B, Maierhofer H, et al (2025) Community seedbanks in Europe: their role between ex situ and on-farm conservation. Genet Resour 147–161. https://doi.org/10.46265/genresj.OHNK3179

Bonhomme V, Picq S, Gaucherel C, Claude J (2014) Momocs : Outline Analysis Using R. J Stat Softw 56:. https://doi.org/10.18637/jss.v056.i13

Bosmali I, Lagiotis G, Ganopoulos I, et al (2024) Phaseolus coccineus L. Landraces in Greece: Microsatellite Genotyping and Molecular Characterization for Landrace Authenticity and Discrimination. BioTech 13:18. https://doi.org/10.3390/biotech13020018

Bosmali I, Kotsiou K, Matsakidou A, et al (2025) Fortification of wheat bread with an alternative source of bean proteins using raw and roasted Phaseolus coccineus flours: Impact on physicochemical, nutritional and quality attributes. Food Hydrocoll 158:110527. https://doi.org/10.1016/j.foodhyd.2024.110527

Canella M, Ardenghi NMG, Müller JV, et al (2022) An updated checklist of plant agrobiodiversity of northern Italy. Genet Resour Crop Evol 69:2159–2178. https://doi.org/10.1007/s10722-022-01365-y

Cassani E, Puglisi D, Cantaluppi E, et al (2017) Genetic studies regarding the control of seed pigmentation of an ancient European pointed maize (Zea mays L.) rich in phlobaphenes: the "Nero Spinoso" from the Camonica valley. Genet Resour Crop Evol 64:761–773. https://doi.org/10.1007/s10722-016-0399-7

Ceccarelli S (2012) Landraces: importance and use in breeding and environmentally friendly agronomic systems. In: Maxted N, Ehsan Dulloo M, Ford-Lloyd BV, et al.

(eds) Agrobiodiversity conservation: securing the diversity of crop wild relatives and landraces, 1st edn. CABI, UK, pp 103–117

Chitwood DH, Otoni WC (2017) Morphometric analysis of Passiflora leaves: The relationship between landmarks of the vasculature and elliptical Fourier descriptors of the blade. GigaScience, 6, giw008.

Ciaffi M, Paolacci AR, Marcomeni M, et al (2024) The Characterization of the Morphological and Molecular Traits of Phaseolus coccineus in the Aniene Valley: Insights into Genetic Diversity and Adaptation. Plants 13:3320. https://doi.org/10.3390/plants13233320

Cislaghi A, Giupponi L, Tamburini A, Giorgi A, Bischetti GB (2019) The effects of mountain grazing abandonment on plant community, forage value and soil properties: observations and field measurements in an alpine area. CATENA 181:104086. https://doi.org/10.1016/j. catena.2019.104086

Claude J (2008) Morphometrics with R. Springer, New York

Cleveland DA, Soleri D, Smith SE (1994) Do folk crop varieties have a role in sustainable agriculture? Bioscience 11:740–751

Colombo F, Franguelli N, Licheri G, et al (2022) Agriculture in Marginal Areas: Reintroduction of Rye and Wheat Varieties for Breadmaking in the Antrona Valley. Agronomy 12:1695. https://doi.org/10.3390/agronomy12071695

Cominelli E, Sparvoli F, Lisciani S, et al (2022) Antinutritional factors, nutritional improvement, and future food use of common beans: A perspective. Front Plant Sci 13:992169. https://doi.org/10.3389/fpls.2022.992169

Didonna A, Bocci R, Renna M, Santamaria P (2024) The Conservation Varieties Regime: Its Past, Present and Future in the Protection and Commercialisation of Vegetable Landraces in Europe. Horticulturae 10:877. https://doi.org/10.3390/horticulturae10080877

EU Commission (2017) Region Innovation Monitor Plus. https://ec.europa.eu/growth/tools-databases/regional-innovation-monitor/base-profile/lombardy. Accessed 11 April 2024

EU Commission. EUROPE 2020 a Strategy for Smart, Sustainable and Inclusive Growth. 2010. Available online: https: //eurlex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52010DC2020 (accessed on 11 September 2024).

Esquinas-Alcázar J (2005) Protecting crop genetic diversity for food security: political, ethical and technical challenges. Nat Rev Genet 6:946–953. https://doi.org/10.1038/nrg1729

Falcione M, Simiele M, Renella A, et al (2022) A Multi-Level Approach as a Powerful Tool to Identify and Characterize Some Italian Autochthonous Common Bean (Phaseolus vulgaris L.) Landraces under a Changing Environment. Plants 11:2790. https://doi.org/10.3390/plants11202790

FAO (2007) The state of the world's animal genetic resources for food and agriculture: in brief. Food and Agriculture Organization of the United Nations.

FAO (2010) Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture. Commission on Genetic Resources for Food and Agriculture; Food and Agriculture Organization of the United Nations: Rome, Italy, 2010.

Fenzi M, Couix N (2022) Growing maize landraces in industrialized countries: from the search for seeds to the emergence of new practices and values. Int J Agric Sustain 20:327–345. https://doi.org/10.1080/14735903.2021.1933360

Fideghelli C, Engel P (2009) Biodiversity and local genetic resources: from knowledge to exploitation. Acta Hortic 817:295–310

Firoozzare A, Boccia F, Yousefian N, Ghazanfari S, Pakook (2024) Understanding the role of awareness and trust in consumer purchase decisions for healthy food and products. Food Quality and Preference, 121: 105275. https://doi.org/10.1016/j.foodqual.2024.105275.

Frison EA, Cherfas J, Hodgkin T (2011) Agricultural Biodiversity Is Essential for a Sustainable Improvement in Food and Nutrition Security. Sustainability 3:238–253. https://doi.org/10.3390/su3010238

Ghidoli M, Geuna F, De Benedetti S, et al (2024) Genetic study of Camelina sativa oilseed crop and selection of a new variety by the bulk method. Front Plant Sci 15:1385332. https://doi.org/10.3389/fpls.2024.1385332

Giorgi A, Scheurer T (2015) Alpine Resources: Assets for a Promising Future—Conclusions from the ForumAlpinum 2014. Mt Res Dev 35:414–415. https://doi.org/10.1659/MRD-JOURNAL-D-15-00061

Giupponi L, Giorgi A (2019a) Effectiveness of modern leaf analysis tools for the morpho-ecological study of plants: the case of Primula albenensis. Nord J Bot 37:njb.02386. https://doi.org/10.1111/njb.02386

Giupponi L, Borgonovo G, Panseri S, Giorgi A (2019b) Multidisciplinary study of a little known landrace of Fagopyrum tataricum Gaertn. of Valtellina (Italian Alps). Genet Resour Crop Evol 66:783–796. https://doi.org/10.1007/s10722-019-00755-z

Giupponi L, Leoni V (2020) VegeT: An Easy Tool to Classify and Facilitate the Management of Seminatural Grasslands and Dynamically Connected Vegetation of the Alps. Land 9:473. https://doi.org/10.3390/land9120473

Giupponi L, Leoni V, Colombo F, et al (2021a) Characterization of "Mais delle Fiorine" (Zea mays L.) and nutritional, morphometric and genetic comparison with other maize landraces of Lombardy region (Northern Italy). Genet Resour Crop Evol 68:2075–2091. https://doi.org/10.1007/s10722-021-01118-3

Giupponi L, Leoni V, Pedrali D, et al (2020a) Morphometric and phytochemical characterization and elevation effect on yield of three potato landraces of the

Ligurian Apennines (Northern Italy). J Appl Bot Food Qual 234-243 Pages. https://doi.org/10.5073/JABFQ.2020.093.028

Giupponi L, Pedrali D, Leoni V, et al (2021b) The Analysis of Italian Plant Agrobiodiversity Databases Reveals That Hilly and Sub-Mountain Areas Are Hotspots of Herbaceous Landraces. Diversity 13:70. https://doi.org/10.3390/d13020070

Giupponi L, Pilu R, Scarafoni A, Giorgi A (2020b) Plant agro-biodiversity needs protection, study and promotion: results of research conducted in Lombardy region (Northern Italy). Biodivers Conserv 29:409–430. https://doi.org/10.1007/s10531-019-01889-3

Giupponi L, Tamburini A, Giorgi A (2018) Prospects for Broader Cultivation and Commercialization of Copafam, a Local Variety of Phaseolus coccineus L., in the Brescia Pre-Alps. Mt Res Dev 38:24–34. https://doi.org/10.1659/MRD-JOURNAL-D-17-00013.1

Grime JP (1977) Evidence for the Existence of Three Primary Strategies in Plants and Its Relevance to Ecological and Evolutionary Theory. Am Nat 111:1169–1194. https://doi.org/10.1086/283244

Grime JP (1974) Vegetation classification by reference to strategies. Nature 250:26–31

Grime JP (2001) Plant strategies, vegetation processes and eco- system properties. Wiley, Chichester

Hammer K, Knpffer H, Xhuveli L, Perrino P (1996) Estimating genetic erosion in landraces ? two case studies. Genet Resour Crop Evol 43:329–336. https://doi.org/10.1007/BF00132952

Han IH, Baik B (2006) Oligosaccharide Content and Composition of Legumes and Their Reduction by Soaking, Cooking, Ultrasound, and High Hydrostatic Pressure.

Cereal Chem 83:428–433. https://doi.org/10.1094/CC-83-0428

ISMEA (2014) Stime di produzione - Cereali autunnali, mais, semi oleosi. http://www.ismeamercati.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/4292. Accessed 11 April 2024

ISO 14902:2001; Animal Feeding Stuffs—Determination of Trypsin Inhibitor Activity of Soya Products. International Standardiza- tion Organization: Geneva, Swizzerland, 2010.

ISTAT (2020) Censimento Agricoltura 2020. https://esploradati.istat.it/databrowser/#/it/censimentoagricoltura/categories/AU. Accessed 11 April 2024

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet 11:94. https://doi.org/10.1186/1471-2156-11-94

Keenleyside C, Tucker GM (2010) Farmland Abandonment in the EU: an Assessment of Trends and Prospects Report Prepared for WWF. Institute for European Environmental Policy, London

King J, Leong SY, Alpos M, et al (2024) Role of food processing and incorporating legumes in food products to increase protein intake and enhance satiety. Trends Food Sci Technol 147:104466. https://doi.org/10.1016/j.tifs.2024.104466

Landoni M, Bertoncini A, Ghidoli M, et al (2024) PGRFA Management of Outcrossing Plants Propagated by Seed: From On-Farm to Ex Situ Conservation and Some Italian Maize Case Studies. Agronomy 14:1030. https://doi.org/10.3390/agronomy14051030

Losa A, Sala T, Toppino L, et al (2025) Genetic Diversity and Distinctiveness of Common Beans (Phaseolus vulgaris L.) Between Landraces and Formal Cultivars Supporting Ex Situ Conservation Policy: The Borlotti Case Study in Northern Italy.

Agronomy 15:786. https://doi.org/10.3390/agronomy15040786

Maphosa Y, Jideani VA (2017) The Role of Legumes in Human Nutrition. In: Hueda MC (ed) Functional Food - Improve Health through Adequate Food. InTech

Mercati F, Catarcione G, Paolacci AR, et al (2015) Genetic diversity and population structure of an Italian landrace of runner bean (Phaseolus coccineus L.): inferences for its safeguard and on-farm conservation. Genetica 143:473–485. https://doi.org/10.1007/s10709-015-9846-1

Montesano V, Negro D, Sarli G, et al (2012) Landraces in Inland areas of the Basilicata region, Italy: monitoring and perspectives for on farm conservation. Genet Resour Crop Evol 59:701–716. https://doi.org/10.1007/s10722-011-9712-7

Negri V Landraces in central Italy: where and why they are conserved and perspectives for their on-farm conservation

Nor Azmah U, Makeri MU, Bagirei SY, Shehu AB (2023) Compositional characterization of starch, proteins and lipids of long bean, dwarf long bean, mung bean and French bean seed flours. Meas Food 12:100111. https://doi.org/10.1016/j.meafoo.2023.100111

Nordregio (2004) Mountain Areas in Europe: Analysis of mountain areas in EU member states, acceding and other European countries. Commissioned report by the European Commission—DG Regional Policy, Brussels. https://ec.europa.eu/regional_policy/sources/docgener/studies/pdf/montagne/mount1.pdf. Accessed 11 April 2024

NRP 2021–2027. https://www.mur.gov.it/it/aree-tematiche/ricerca/programmazione/programma-nazionale-la-ricerca

Pacicco L, Bodesmo M, Torricelli R, Negri V (2018) A methodological approach to identify agro-biodiversity hotspots for priority in situ conservation of plant genetic resources. PLOS ONE 13:e0197709. https://doi.org/10.1371/journal.pone.0197709

Pedrali D, Proserpio C, Borgonovi SM, et al (2022) Nutritional Characterization and Novel Use of “Copafam” Bean (Phaseolus coccineus L.) for the Sustainable Development of Mountains Areas. Sustainability 14:13409. https://doi.org/10.3390/su142013409

Pedrali D, Zuccolo M, Giupponi L, et al (2024) Characterization and Future Distribution Prospects of “Carciofo di Malegno” Landrace for Its In Situ Conservation. Plants 13:680. https://doi.org/10.3390/plants13050680

Pierce S, Negreiros D, Cerabolini BEL, et al (2017) A global method for calculating plant CSR ecological strategies applied across biomes world-wide. Funct Ecol 31:444–457. https://doi.org/10.1111/1365-2435.12722

Piergiovanni AR, Lioi L (2010) Italian Common Bean Landraces: History, Genetic Diversity and Seed Quality. Diversity 2:837–862. https://doi.org/10.3390/d2060837

Puneeth GM, Gowthami R, Katral A, et al (2024) On-farm crop diversity, conservation, importance and value: a case study of landraces from Western Ghats of Karnataka, India. Sci Rep 14:10712. https://doi.org/10.1038/s41598-024-61428-1

R Development Core Team. R: A Language and Environment or Statistical Computing. R Foundation for Statistical Computing. Available online: http://www.r-project.org/ (accessed on June 2024).

Raggi L, Pacicco LC, Caproni L, et al (2022) Analysis of landrace cultivation in Europe: A means to support in situ conservation of crop diversity. Biol Conserv 267:109460. https://doi.org/10.1016/j.biocon.2022.109460

Rodríguez Madrera R, Campa Negrillo A, Suárez Valles B, Ferreira Fernández JJ (2021) Phenolic Content and Antioxidant Activity in Seeds of Common Bean (Phaseolus vulgaris L.). Foods 10:864. https://doi.org/10.3390/foods10040864

Romero-Astudillo MJ, Tapia C, Giménez De Azcárate J, Montalvo D (2024)

Diversity of Common Bean (Phaseolus vulgaris L.) and Runner Bean (Phaseolus coccineus L.) Landraces in Rural Communities in the Andes Highlands of Cotacachi—Ecuador. Agronomy 14:1666. https://doi.org/10.3390/agronomy14081666

Rossi G, Guzzon F, Canella M, et al (2019) Le varietà agronomiche lombarde tradizionali a rischio di estinzione o di erosione genetica. Ortive e cerealicole: uno sguardo d'insieme, first ed. Pavia, Lombardia, Italy.

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. https://doi.org/10.1038/nmeth.2089

Serventi L (2020) Upcycling Legume Water: from wastewater to food ingredients. Springer International Publishing, Cham

Sicard D, Nanni L, Porfiri O, et al (2005) Genetic diversity of Phaseolus vulgaris L. and P. coccineus L. landraces in central Italy. Plant Breed 124:464–472. https://doi.org/10.1111/j.1439-0523.2005.01137.x

Spataro G, Negri V (2013) The European seed legislation on conservation varieties: focus, implementation, present and future impact on landrace on farm conservation. Genet Resour Crop Evol 60:2421–2430. https://doi.org/10.1007/s10722-013-0009-x

Terres JM, Nisini L, Anguiano E. (2013) Assessing the risk of farmland abandonment in the EU. Final report EUR 25783EN, Joint Research Centre of the European Commission, Luxembourg

United Nations. Transforming Our World: The 2030 Agenda for Sustainable Development. 2015. Available online: https: //undocs.org/A/RES/70/1 (accessed on 11 September 2024).

Villa TCC, Maxted N, Scholten M, Ford-Lloyd B (2005) Defining and identifying crop landraces. Plant Genet Resour 3:373–384. https://doi.org/10.1079/PGR200591

Yu K, Park S J, Poysa V, Gepts P (2000) Integration of simple sequence repeat (SSR) markers into a molecular linkage map of common bean (Phaseolus vulgaris L.). The Journal of heredity. Vol.91 (6), p.429-434.

Zuccolo M, Pedrali D, Leoni V, et al (2023) Characterization of an Italian landrace of Cyclanthera pedata (L.) Schrad. of herbal and horticultural interest. Genet Resour Crop Evol 70:1455–1469. https://doi.org/10.1007/s10722-022-01514-3

Figure captions

Fig. 1. Bean samples and their cultivation areas.

Fig. 2. Representative mature dry seeds, plants after one month, flowers, entire pods, sectioned pods, entire basal leaf and trifoliate leaf from each accession of Phaseolus spp. studied. The identification codes of bean samples (capital letter) are referred to in Figure 1.

Fig. 3. Molecular analysis. (A) PCoA (Eigenvalue scaling) and (B) UPGMA clustering using 15 SSR markers across 18 different varieties. P. coccineus is shown in red, P. vulgaris commercial varieties in light blue, and P. vulgaris landraces in black. The identification codes of bean samples (capital letter) are referred to in Figure 1.

Fig. 4. Mean shapes of the eighteen bean samples (a), LDA analysis results (b) and the three main morphologies of bean seeds (c). The identification codes of bean samples (capital letter) are referred to in Figure 1.

Fig. 5. MDS (Multidimensional Scaling) biplot of beans samples associated with nutritional and phytochemical parameters. The identification codes of bean samples (capital letter) are referred to in Figure 1. Key: TIA (Trypsin inhibitor activity); FRAP (Ferric Reducing Antioxidant Power); RSO (Raffinose Series Oligosaccharides)

Tables

Table 1. 20 SSRs identified in bean and used for the molecular analysis (modified from Grisi et al., 2007)

Table 2. Results of Grime's CSR functional strategy. Key: C, competitor; S, stress-tolerator; R, ruderal. The identification codes of bean samples (capital letter) are referred to in Figure 1.

Table 3. Nutritional and phytochemical composition of samples beans. The identification codes of bean samples (capital letter) are referred to in Figure 1. Key:

TIA (Trypsin inhibitor activity); FRAP (Ferric Reducing Antioxidant Power); RSO (Raffinose Series Oligosaccharides)

Funding

No funding

Acknowledgments

This research was supported by the “Fagio.Lo” (“Research, Characterization, and Valorization of Traditional Lombard Bean Cultivars”; Operation 10.2.01 “Conservation of Animal and Plant Biodiversity” – PSR 2014–2020) project, and by the Agritech National Research Centre Spoke 7 that received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) - MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4—D.D. 1032 17/06/2022, CN00000022).

Conflict of interest

The authors declare that they have no conflict of interest.

Article 2:

sustainability

MDPI

Article

Nutritional Characterization and Novel Use of "Copafam" Bean (*Phaseolus coccineus* L.) for the Sustainable Development of Mountains Areas

Davide Pedrali [1], Cristina Proserpio [2], Sara Margherita Borgonovi [2], Marco Zuccolo [1,*], Valeria Leoni [1], Gigliola Borgonovo [2], Alessia Maria Bernardi [1], Alessio Scarafoni [2], Ella Pagliarini [2], Annamaria Giorgi [1,3] and Luca Giupponi [1,3]

[1] Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., Università degli Studi di Milano, 25048 Edolo, Italy
[2] Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, 20133 Milan, Italy
[3] Department of Agricultural and Environmental Sciences-Production, Landscape and Agroenergy-DiSAA, Università degli Studi di Milano, 20133 Milan, Italy
* Correspondence: marco.zuccolo@unimi.it

Abstract: Agrobiodiversity conservation includes strategies and actions to be taken to prevent landrace loss, a worldwide problem. Landraces are local varieties that have agricultural, cultural, and historical value but most of these are not studied yet. This research aimed to study the nutritional and phytochemical characteristics of the "Copafam" bean. In addition, the sensory properties and consumers' hedonic ratings in a model food formulation (biscuits) made by this landrace have been examined. The results show that "Copafam" had a high dietary fiber content (34.83 ± 2.48 g/100 g dw) and it resulted in a great source of secondary metabolites as polyphenols (121.36 ± 5.31 mg GAE/g dw), flavonoids (6.51 ± 0.17 mg/kg dw), and anthocyanins (28.11 ± 0.16 mg Cy3 G/kg dw), having remarkable antioxidant activity too. Biscuits made from "Copafam" bean flour were characterized by a darker color and crunchy texture, and it was considered acceptable by consumers. All these characteristics make it a resource of great interest for innovative forms of consumption like fortified foods. This research showed that landraces can represent a great resource for an innovative food industry aiming to preserve agrobiodiversity and promote the sustainable development of mountain areas.

Keywords: agrobiodiversity; landraces; bean; phytochemical; sustainable development; mountain resources; consumer acceptance; sustainability; novel foods; sensory descriptive analysis

Citation: Pedrali, D.; Proserpio, C.; Borgonovi, S.M.; Zuccolo, M.; Leoni, V.; Borgonovo, G.; Bernardi, A.M.; Scarafoni, A.; Pagliarini, E.; Giorgi, A.; et al. Nutritional Characterization and Novel Use of "Copafam" Bean (*Phaseolus coccineus* L.) for the Sustainable Development of Mountains Areas. *Sustainability* **2022**, *14*, 13409. https://doi.org/10.3390/su142013409

Academic Editor: Svein Øivind Solberg

Received: 21 September 2022
Accepted: 14 October 2022
Published: 18 October 2022

1. Introduction

Today there are important processes underway in European mountains: from one side, the process of abandonment by population, from the other side, the newcomers' arrival. These sociocultural processes are concurrent and often in correlation with the loss of biodiversity and agrobiodiversity [1], determined also by environmental issues such as climate change [2]. Agriculture is often seen as a fundamental business for the recovery of abandoned land and the start of a recovery process of marginal areas and the connected biodiversity and agrobiodiversity [3,4].

Plant agrobiodiversity includes wild relatives, landraces, and modern cultivars [5]. Landraces are dynamic populations of cultivated plants that have a historical origin and distinct identity and lack formal crop improvement, as well as often being genetically diverse, locally adapted, and associated with traditional farming systems [6]. Landraces are genetic resources for crop improvement programs [7,8] and a source of food diversity [9].

They are a priceless heritage, however, are undergoing losses worldwide. Landraces adapted to the environment and climate changes can represent a valuable resource for innovative low-input agricultural systems [2,10].

Considering the increasingly important impacts of climate change and the fast changes in the socio-economic contexts on food production and demand, genetic resources are becoming even more important: United Nations (UN) Organization estimates that the growth of food demand, linked to the increase in population (a world population of 10 billion people expected for 2050), will result in the doubling of food production globally [11,12].

In this context, the last century saw the loss of 75% of global agrobiodiversity and today most of the food worldwide is produced by about 10 plant species, as estimated by the Food and Agriculture Organization (FAO) of the United Nations [13,14]. To overcome this issue, European Union (EU) started to act to preserve agrobiodiversity. This resulted in the drawing up of international strategies such as the EU Biodiversity Strategy 2020 [15] and the 2030 Agenda for Sustainable Development [12].

Sustainable policies on food production must be adopted, including improving yields of agricultural land, encouraging forms of circular economy, and adopting more sustainable food models [16].

The integration of biodiversity conservation into key policies for agriculture and forestry plays a major role in Europe's biodiversity, in fact, the EU adopted the European Register of Conservation Varieties, which represents one of the most modern instruments for in situ conservation of landraces [17]. The decline of genetic diversity, as well as the need of promoting and facilitating the use of traditional crop varieties, have been confirmed also in the 2030 EU agenda. The UE states that by 2030 it is necessary to invert the trend of genetic erosion in agriculture by, for example, the conservation and use of traditional breeds and cultivars [18].

The European Union countries are acting to tackle this problem by following European guidelines. In this context, Italy is rich in agrobiodiversity though most of the landraces are not studied yet, leading to the necessity to make efforts in acquiring knowledge [19–22].

Landraces are mainly found in hilly and sub-mountain areas (150–800 m a.s.l.) and among the most numerous families there are *Fabaceae*, together with *Poaceae* and *Solanaceae*, able to grow in a wide altitudinal range (from sea level to over 1000 m a.s.l.) [23].

The species/cultivars of *Fabaceae* family (legumes) are one of the most important crops in the world since they represent one of the main food and income sources in developing countries as well as in marginal areas. Among them, the genus *Phaseolus* includes more than one hundred species cultivated worldwide [24–26]. To promote sustainable vegetable protein production from higher plants (for example, legumes) the application of innovative technologies could be strategic to ensure sufficient, safe, and healthy food for a growing population [27]. Dry beans are an important source of protein, vitamins, minerals, and carbohydrates with a low-fat content; they contain also prebiotics such as fructooligosacchartides (FOS) and are an important source of dietary fiber [25,26,28]. In addition, beans contribute to preventing and controlling some chronic and degenerative diseases like obesity, diabetes, and numerous types of cancer. They contain a wide range of polyphenols compounds, including flavonoids, tannins, anthocyanins, and phenolic acids such as p-cumaric, ferulic, and cinnamic acid [26,29,30]. Beans are primarily consumed as a dry seed but also as green pods or ground; in fact, bean flour is an option for improving the nutritional quality of food (enriching them with protein and fiber) [30,31].

Among the Italian beans, "Copafam" is a cultivar of runner bean (*P. coccineus* L.) that can be grown only in mountain areas [32]. their edible pods and seeds are larger and more colored than those of common beans (*P. vulgaris* L.) [32]. "Copafam" is a traditional cultivar of historical importance and it is now at risk of extinction due to the depopulation and abandonment of mountain areas, and it is today cultivated by just a few farmers [22].

Giupponi and co-workers [32] studied this landrace from the agronomic point of view, however, important nutritional and phytochemical features have not been explored. Moreover, the possibility of developing innovative foods with its flour and the subsequent consumer responses to these new products were not evaluated. The addition of such ingredients in a food matrix leads to several changes in sensory properties [33–35] potentially influencing consumer hedonic responses, which need to be deeply investigated to ensure future success in the market.

The aim of this study was to investigate if this landrace has distinctive nutritional and phytochemical characteristics from other more common and commercial bean varieties as: Borlotto and Cannellino (*P. vulgaris*) and Bianco di Spagna (*P. coccineus*) (Figure 1). Additionally, to explore if "Copafam" could be used to produce innovative and unique goods, the impact of using bean flour on the sensory properties and consumer hedonic ratings in a model food formulation (biscuits) was investigated. The working hypothesis was to evaluate if "Copafam" could be interesting for starting up unique high-quality agri-food chains of low environmental impact that could be strategic for smart, sustainable, and inclusive growth of mountain territories.

Figure 1. Plant material. "Copafam" sample (beans and flour) is reported in the green box, while commercial bean samples are in the grey box.

2. Materials and Methods

2.1. Plant Materials

"Copafam" beans were cultivated and collected in licensed agricultural fields directly from farmers of Camonica Valley (Brescia Province, Northern Italy) in 2021. Three commercial beans (Bianco di Spagna, Cannellino, and Borlotto) were included as a comparison; these beans were bought at a local market. Bean whole flours were obtained by finely grinding the seeds with a commercial blender (IKA A10 basic, Werke GmbH & Co. KG, Staufen, Germany). All plant material was collected complying with national and international mandatory regulations.

2.2. Phytochemical Composition

2.2.1. Extraction

The sample extraction was performed according to the procedure reported by Alcázar-Valle [25]. Briefly, 10 g bean flour (dry weight) + 100 mL of acetone solvent mixture (acetone, water and acetic acid, 70:29:0.5 $v/v/v$) overnight. Then the extracts were centrifuged a 4000 rpm for 15 min (Hermle z300, HERMLE Labortechnik GmbH, Wehingen, Germany), washed with the solvent mixture once and the supernatant was retained. Finally, the extract was concentrated using a rotary evaporator at 45 °C (LABOROTA 4000eco, Heidolph Instruments GmbH & Co., Schwabach, Germany).

The moisture content of the bean flour was determined by oven-drying method at 105 °C according to the AOAC method n° 945.40 [36]. Total nitrogen content was analyzed by the Kjeldahl procedure [36]; the conversion factor used to transform nitrogen into protein was 6.25. Ash content was determined by incineration at 550 °C in a microwave muffle AACC Method [37] (ZE muffle furnace, Ettore Pasquali s.rl., Milano, Italy). Total lipid content was determined by the AOAC [36] procedure. Five grams of flour were extracted in 30 mL chloroform/methanol (2:1, *v*/*v*) with agitation at 0 °C three times. The extract was vacuum filtered through filter paper on a Büchner funnel. This filtrate was dried in a rotary evaporator at 35 °C. The residue was dissolved in 10 mL of chloroform/methanol (2:1, *v*/*v*) after the addition of 2 mL of KCL 0.75%, mixed vigorously, and put in a separatory funnel attending the phase separation. The chloroform layer was removed, dried over anhydrous Na_2SO_4, filtered, and concentrated by a rotary vacuum evaporator at 35 °C. The extracted lipids were weighed to determine the lipid content.

The determination of total starch content, dietary fiber, and RSO (Raffinose-Series Oligosaccharides) was performed using the Megazyme assay kit (K-TSTA-100A, K-RAFGL, respectively) following the manufacturer's instructions.

2.2.2. Extraction for HPLC

The sample extraction was performed according to Rodríguez Madrera [26]. Briefly, 1.5 g of bean flour was extracted with 30 mL ethanol 46% (0.1 formic acid) overnight. After extraction, the solids were separated by centrifugation and the supernatant was dried in a rotary vacuum evaporator at 50 °C and reconstituted with 2 mL of methanol 20% (0.1 formic acid).

2.2.3. Basic Hydrolysis

Basic hydrolysis was performed following the procedure reported by Lin [30]. Briefly, 1 mL of extract was dried and dissolved in 0.3 mL of NaOH 4 N and magnetically stirred for 18 h at room temperature. 0.15 HCL 12 N and 0.55 mL of methanol were added. The solution was filtered through a 0.2 µm pore size membrane filter prior to chromatographic analysis.

The High-Performance Liquid Chromatography (HPLC) system used was an LC Agilent series 1200 (Waldbronn, Germany) consisting of a degasser, a quaternary gradient pump, an auto-sampler, and a MWD detector (Waldbronn, Germany). A Luna® C18 (150 × 4.6 mm) column (Phenomenex, Santa Clara, CA, USA) at 25 °C was used for the chromatographic separation and 10 µL of samples were used for injections. The run time was 50 min, with no post-run time. Solvent (A) was HCOOH 0.1% while solvent (B) was acetonitrile; a constant flow rate of 0.8 mL/min was used. The gradient used was: 0 min 95% A, 5 min 95% A, 10 min 85% A, 40 min 60% A, 42 min 5% A, 45 min 5% A, 50 min 95% A, and the absorbance wavelength was set at 310 nm.

Individual stock solutions of each standard were prepared using absolute ethanol and stored at −20 °C. The working standard mixture solutions were made by diluting the appropriate amount of each stock standard solution to obtain 5 calibration levels (the range concentration of p-cumaric and sinap acid was 2.5–100 mg/mL while the range concentration of ferulic acid was 5–500 mg/mL). The retention times of all the standards were confirmed by individual standard injections. A fortification of random samples was used to check further the retention factors. A standard mixture to check the retention times was injected each working day. LOD (0.5 µg/mL) and LOQ (1 µg/mL) was calculated according S/N ratio 3 and 10, respectively.

2.2.4. Antioxidant Activity

The antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method reported by Brand-Williams [38]. Here, 0.3 mL of bean extracts were mixed with 2.7 mL of a DPPH solution 6×10^{-5} M. This mixture and a DPPH blank were incubated for 30 min in the dark at room temperature. After this time, the absorbance was measured with a UV/Vis spectrophotometer at 517 nm (Varian Cary 50 scan, Agilent, 5301 Stevens Creek Blvd, Santa Clara, CA, USA). The antioxidant activity was calculated as the percentage of RSA (radical scavenging activity) using the formula RSA = $[(AB - AA)/AB] \times 100$, where AB is the absorbance of the DPPH solution and AA is the absorbance of the sample solution.

2.2.5. Polyphenols Content

Folin-Ciocalteu spectrophotometric method was used to analyze total polyphenols [39]. Quantification was performed with gallic acid (mg GAE/g dw) as follows: 0.2 mL of extract was mixed with 0.2 mL of folin reagent and 3 mL of distilled water. Then 0.75 mL of sodium carbonate was added. The mixture was incubated for 8 min at room temperature. Finally, 0.85 mL of distilled water was added, followed a homogenization and incubation in the dark for 2 h at room temperature. The absorbance was measured at 765 nm.

2.2.6. Flavonoids

The determination of total flavonoids was performed by an aluminum chloride test [25]. Quantification was expressed as quercetin equivalents per kg of bean flour (mg QE/kg dw) by mixing 0.05 mL of extract, 0.7 mL of distilled water and 0.25 mL of AlCl3 (133 mg of AlCl3 and 400 mg of sodium acetate in 100 mL of methanol/water/acid acetic (140:50:10, *v*/*v*/*v*)). The absorbance was measured at 415 nm.

2.2.7. Anthocyanins

The anthocyanins analysis was performed following the procedure reported by Abdel-Aal [40]: 3 g of bean flour was mixed in 24 mL ethanol (0.15% HCL 0.1 N). The mixture was shaken for 30 min and centrifuged for 15 min. The supernatant was poured into a 50 mL volumetric flask and made up to volume. The solution was filtered and absorbance was measured at 535 nm against a reagent (solvent) blank. Quantification was expressed as cyanidin 3-glucoside equivalents per kg of bean flour (mg Cy3 G/kg dw).

2.2.8. TIA

Trypsin inhibitor activity (TIA) was performed following the ISO 14902:2001 [41] assay. Briefly, 62.5 mg of bean flour was dissolved in 1.25 mL of extraction buffer (500 mM NaCl + NaOH 10 mM; ratio 1:20) and subsequently centrifuged for 10 min at 13,000 rpm; the supernatant was recovered.

In a water bath at 37 °C, 20 µL of extract, 100 µL of L-BAPA solution (Benzoyl-L-arginine-p-nitroanilide), and 40 µL of distilled water were mixed in a test tube. This was followed by adding 20 µL of trypsin working solution to start the colorimetric reaction. After 10 min, 20 µL of 5.6 M acetic acid was added to stop the reaction. The L-BAPA solution was prepared by diluting 3.2 mg of L-BAPA into 50 µL of DMSO 1%; the volume was adjusted to 5 mL with the working buffer (Tris 50 mM + CaCl2 5 mM pH 8.2). The reaction mixture (total 200 µL) was measured for absorbance at 415 nm by spectrophotometer; the result was expressed in mg/g dw of the sample. For a reference reading, water was used instead of the extract. A sample blank and a reagent blank were also prepared and measured. The inhibition percentage of the sample extract solution was calculated as followed:

$$I = [(At - Abt) - (As - Abs)/(At - Abt)] \times 100 \quad (1)$$

where I is the inhibition percentage, At is the absorbance of the solution with trypsin only, Abt is the absorbance of the blank trypsin only, As is the absorbance of the solution with the sample, and Abs is the absorbance of the blank sample.

The trypsin inhibitor activity is calculated as followed:

$$TIA = (I \times mt \times ds)/(100 \times ms) \quad (2)$$

where mt is the mass of trypsin used in the assay, in milligrams, ds is the dilution factors of the sample, and ms is the mass of the test sample used for the assay.

2.2.9. Phytic Acid

The content of phytic acid was determined using the Megazyme assay kit (K-PHYT, Megazyme International Ireland Ltd., Wicklow, Ireland). The results were expressed as mg of phytic acid per g of bean flour.

2.2.10. Statistical Analysis

Data were analyzed using one-way ANOVA with the Tukey post-hoc test. The assumptions of homogeneity of variances and normality of group data were verified using the Levene and Shapiro–Wilk's tests, respectively. The data were expressed as mean ± standard deviation (SD) and differences were considered statistically significant when $p < 0.05$. The samples were ordered using principal component analysis (PCA) performed by Statgraphics 5.1 (STCC Inc.; Rockville, MD, USA). Hierarchical clustering heatmap and dendrogram were performed on nutritional and phytochemical trades.

2.3. Consumer Study

2.3.1. Participants

A total of 80 subjects (40% men; mean age: 39 ± 15 years) were recruited among the students and employees of the Centre of Applied Studies in the Sustainable Management of the Mountain Environment (Ge.S.Di.Mont.) of the University of Milan and among the population of the neighboring municipalities. Only subjects who like legumes and biscuits, not suffering from food intolerances and allergies were involved. This study was approved by the Ethics Committee of the University of Milan ad was conducted in compliance with the principles laid down in the Declaration of Helsinki. All participants signed a written informed consent to be involved in the study.

2.3.2. Samples

A biscuit control sample (ST) was developed with the following ingredients: 250 g type 1 wheat flour, 85 g brown sugar, 85 g butter, 20 g of eggs, 4 g baking powder, 50 g water, 2 g of baking soda, and 1 g common salt. In addition to the control, other six experimental samples were prepared by replacing wheat flour with 25% and 50% of borlotto flour (Bor25 and Bor50), cannellino flour (Can25 and Can50), and copafam flour (Cop25 and Cop50), respectively. The biscuit samples were baked at 180 °C for 15 min. All samples were prepared by a local bakery "Forneria Pasticceria Salvetti" (Malonno, BS, Italy).

2.3.3. Liking Assessment and Sensory Descriptive Evaluation

Subjects were asked to taste the biscuits and to express their liking using a 10 cm visual analog scale (VAS) anchored by the extremes "extremely disliked" (rated 0) and "extremely liked" (rated 10). Prior to tasting, instructions about the use of the scale were provided to the participants.

In order to obtain a sensory description of the samples, a focus group was preliminarily performed involving 20 untrained subjects to define the appropriate attributes in terms of appearance, odor, taste, flavor, and texture [42]. Secondly, an open discussion was conducted, and the experimenters selected only the most mentioned sensory attributes (frequency of selection at least 40%) to avoid synonyms [43]. Finally, the check-all-that-apply (CATA) questionnaire consisted of a list of 26 sensory attributes: 5 for the appearance (light color, dark color, patchy, uniform, and speckled), 5 for the odor (strong, mild, toasted, legume, and butter), 4 for the taste (sweet, bitter, sour and salty), 6 for the flavor (strong, mild, toasted, legume and butter), and 7 for the texture (sticky, moist, dry, crunchy, crumbly, floury and grainy). The "to assessor" list order allocation scheme was applied to randomize attributes' order [44]. The 80 subjects involved in the experimental session were then asked to select from the list of 26 terms the best ones describing each sample.

2.3.4. Food Neophobia Evaluation

The validated Italian version of the food neophobia questionnaire [45] was applied to investigate the level of reluctance to try and eat unfamiliar foods. The questionnaire consists of 10 statements evaluated using a seven-point Likert scale ranging from "I strongly disagree" (score 1) to "I strongly agree" (score 7). The food neophobia level was calculated, after reversing the negatively worded statements, as a sum of the responses yielding a range of 10–70. Higher scores reflected higher food neophobia levels.

2.3.5. Experimental Procedure

Subjects attended one session and were asked to refrain from consuming anything but water for 2 h before the test. Samples were provided to the participants in plastic plates labeled with three-digit codes in a serving portion of approximately 30 g. Water was available for rinsing the palate between the samples. For each sample, subjects had to evaluate their overall liking and perform a sensory descriptive analysis by means of the check-all-that-apply (CATA) methodology. After testing the first four samples, subjects had to complete the questionnaires. The entire session took approximately 30 min.

2.3.6. Data Analysis

ANOVA model was performed on overall liking scores considering samples (ST, Bor25, Bor50, Can25, Can50, Cop25, and Cop 50) as factor. When a significant difference ($p < 0.05$) was found, Tukey's HSD test was performed as a multiple comparison test.

Cochran's Q test was applied to identify which sensory attributes were discriminating among samples comparing the frequency of mention for each term of the CATA questionnaire. The relationship between samples and sensory attributes was evaluated by means of correspondence analysis (CA).

The internal consistency reliability of the food neophobia scale was evaluated by means of Cronbach's alpha. To investigate the relationship between food neophobic traits and biscuit liking, subjects were categorized (based on the 25th and 75th percentiles) according to their neophobia scores into the following three groups: Neophilic (score < 19); Neutral ($20 \leq$ score ≤ 33); Neophobic (scores > 33). ANOVA model was performed on liking data considering food neophobia traits as a factor.

All analyses were performed using IBMSPSS Statistics for Windows, Version 24 (IBM-Corp., Armonk, NY, USA) and XLSTAT (Version 2019.2.2, Addinsoft., Boston, MA, USA).

3. Results

3.1. Nutritional and Phytochemicals Composition

The nutritional composition (moisture, lipids, ash, protein, starch, RSO, d-glucose, and saccharose) of the different beans cultivars is presented in Figure 2 and the phytochemical characteristics (anthocyanins, dietary fiber, phenolic acids, DPPH, total flavonoid content, (TFC), total phenol content (TPC), Trypsin inhibitor activity (TIA), and phytic acid) are presented in Figure 3. The moisture level was quite homogeneous among all samples (Figure 2a). The protein content (Figure 2c) was the lowest in "Copafam" (21.93 ± 0.41 g/100 g dw; $p < 0.05$; Appendix A) and, in general, samples of *P. vulgaris* (Borlotto, cannellino) showed a high content of this macronutrient compared to those of *P. coccineus* (Bianco di Spagna, "Copafam"). "Copafam", Bianco di Spagna and Cannellino had similar ash contents (over 4%) while Borlotto bean ($3.84 \pm 0.1\%$) had the lowest (Figure 2g). "Copafam" and Borlotto had a lower lipid content compared with the two other varieties ($3.23 \pm 0.15\%$ and $2.83 \pm 0.04\%$, respectively. Figure 2b) and showed on the contrary the highest content in dietary fiber (Figure 3h, 34.83 ± 2.48 and

33.94 ± 0.45 g/100 g dw, respectively). "Copafam" and Cannellino had similar saccharose content (4.42 ± 0.01 and 4.46 ± 0.03 g/100 g dw, respectively. Figure 2e), the lowest among the four samples. The starch level was lower in samples belonging to the *P. coccineus* genus (Figure 2d), while glucose and RSO (Raffinose-Series Oligosaccharides) content was similar among all samples (Figure 2f).

"Copafam" resulted as the best source of polyphenols (121.36 ± 5.31 mg GAE/g dw, Figure 3f) as well as of anthocyanins (28.11 ± 0.16 mg Cy3 G/kg dw, Figure 3b); this landrace and Borlotto bean showed a high level of flavonoids (6.51 ± 0.17 and 7.67 ± 0.5 mg /kg dw, respectively. Figure 3e) and great antioxidant activity too (76.42 ± 1.27 and 77.4 5 ± 0.48%, respectively. Figure 3c). Phytic acid and TIA (antinutrients) were similar for all samples but "Copafam" had the lowest level of tiamin inhibition activity (Figure 3a) and of phytic acid (2.34 ± 0.7 mg/g dw; 1.02 ± 0.01 g /100 g dw, respectively. Figure 3d).

High-Performance Liquid Chromatography (HPLC) analysis of hydrolyzed bean extracts showed similar chromatogram profiles, and the hydroxycinnamic acid constituted the main phenolic compound of all the beans considered (Figure 3g). The samples of P. coccineus revealed a high sinapic acid content and a lower content of ferulic and p-cumaric acid; "Copafam" sample showed the lowest level (16.01 ± 0.45 mg/100 dw) of ferulic acid.

The results of the nutritional and phytochemical analysis were confirmed by heat map (Figure 4a) and Principal Components Analysis (PCA) biplot (Figure 4b). The heat-map (Figure 4a) reflected the differences between the "Copafam" sample and the other ones in terms of a high total phenol content, anthocyanins, fiber, and antioxidant activity and low content of protein, sugar, and antinutrient levels. All samples were well separated and "Copafam" samples were located in the bottom left part of the PCA biplot (Figure 4b), where antioxidant activity, polyphenols, anthocyanin content, and dietary fiber were positioned.

3.2. Consumer Study

The frequency table of terms checked by consumers to describe biscuit samples is reported in Table 1.

Cochran's Q test revealed significant differences in 23 out of 26 terms. The sensory attributes that were not useful in order to discriminate samples were: sour, salty, and moist. Biscuit samples were then generally characterized by low sour and salty tastes as well as low moist texture. The samples with "Copafam" were characterized by a darker color and an uneven appearance compared to the control, probably due to the punctuation detected on the surface of the samples. From an olfactory point of view, consumers identified a mild odor only for the sample with 25% "Copafam", while the 50% sample was found to be more intense compared to biscuit samples without legume flour; Cop25 was mainly described by a butter odor, whereas the samples at 50% were characterized by legume and toasted odors. Both samples were found to be crunchy, crumbly, grainy, and dry.

A significant sample effect ($F = 5.39$; $p < 0.001$) was found on liking scores (Figure 5). All samples were considered acceptable by the consumers except for the biscuit with 50% of Borlotto flour which obtained the lowest score (5.0 ± 0.2). The control sample without legume flour obtained the highest liking score (6.8 ± 0.2) and obtained a comparable score to Cop25 (6.5 ± 0.1) and Bor25 (6.3 ± 0.2). These three formulations were in turn comparable with Can25 (6.2 ± 0.2), Can50 (5.9 ± 0.2), and Cop50 (6.1 ± 0.2).

A biplot of the samples based on sensory descriptive analysis was obtained by means of correspondence analysis (CA) (Figure 6). The CA performed on the total frequency of participants' counts for each attribute resulted in two dimensions accounting for 91.65% of the variance of data. As shown in Figure 6, samples appear to be separated in the plan according to the type of legume.

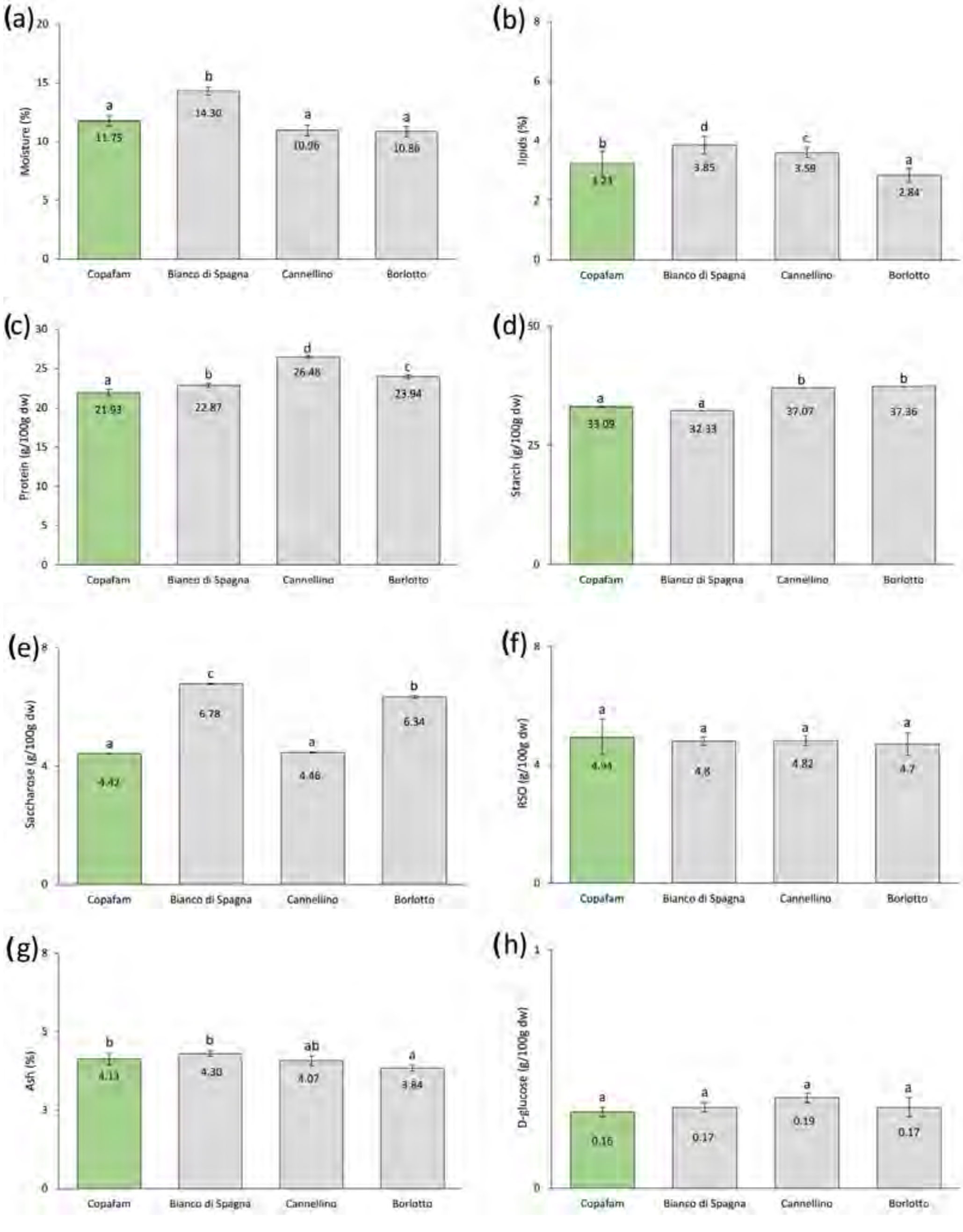


Figure 2. Nutritional features of bean flour. Data with different superscript letters are significantly different, $p < 0.05$. Key: RSO (Raffinose-Series Oligosaccharides). Green column represents "Copafam" bean and the grey columns denote the commercial samples.

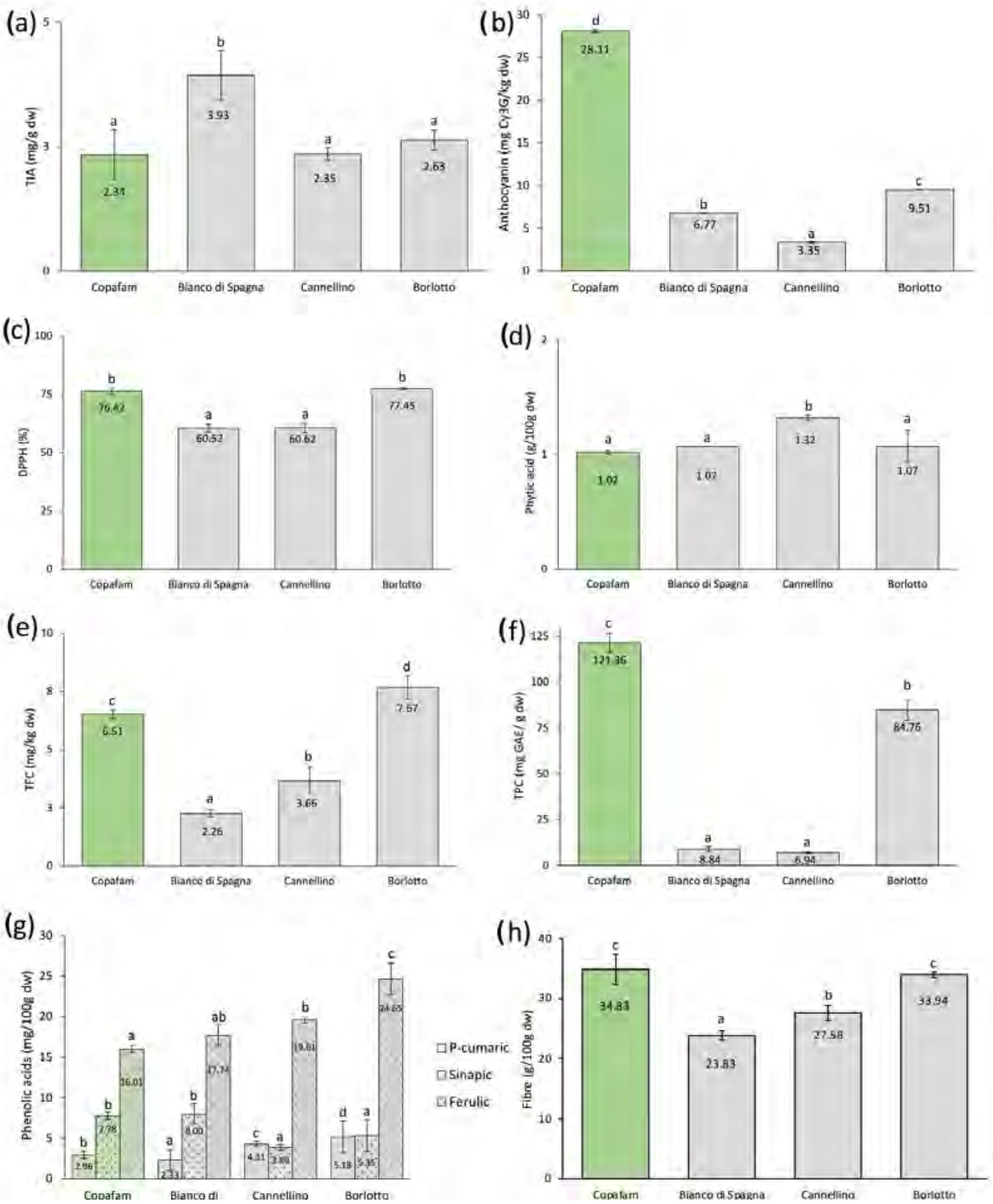


Figure 3. Phytochemical features of bean flour data with different superscript letters are significantly different, $p < 0.05$. Key: TIA (Trypsin inhibitor activity); TPC (total polyphenol component); TFC (total flavonoid content). Green column represents "Copafam" bean and the grey columns denote the commercial samples.

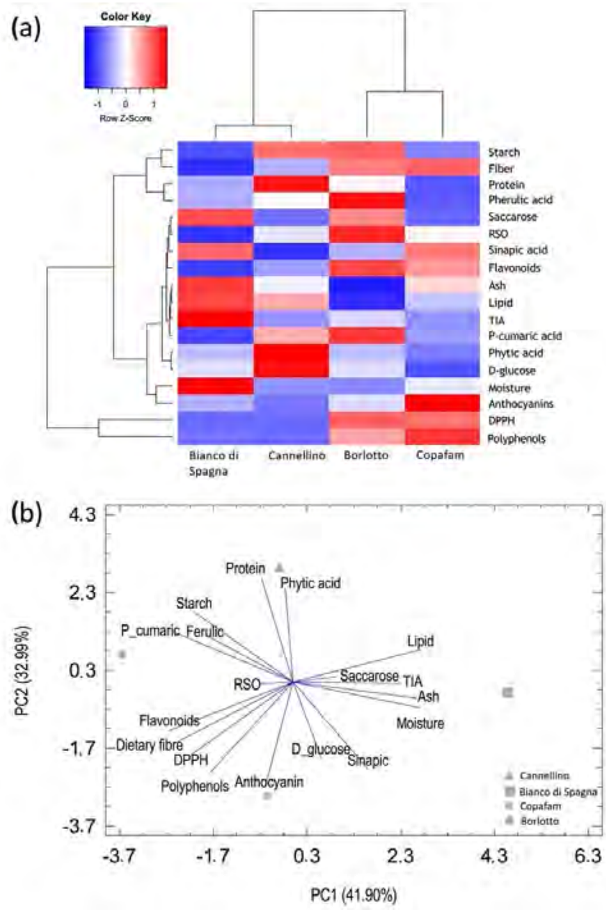


Figure 4. Hierarchical cluster analysis heatmap (**a**) and principal component analysis biplot (**b**) of beans samples associated with nutritional and phytochemical variables. The first two principal components (PCs) explain 74.89% of total variance (PC1 = 41.90%; PC2 = 32.99%). Key: TIA (Trypsin inhibitor activity); RSO (Raffinose-Series Oligosaccharides).

The liking is positioned close to the control sample (ST) and on the upper side of the map where samples with increasing amounts of "Copafam" flour, as well as the biscuit with 25% of Borlotto flour, are positioned. The sensory attributes that were mainly related to the positive hedonic responses were: sweet, mild odor/flavor, butter odor/flavor, and crumbly texture. The sample Bor50 obtained the lowest liking score and was characterized by the terms bitter, legume odor/flavor, and toasted odor/flavor. Cronbach's alpha test revealed satisfactory internal consistency on food neophobia scale (alpha = 0.78). The mean food neophobia value for the population involved was 26.46 $\pm$ 9.7. ANOVA results revealed no significant effect of food neophobia level on hedonic ratings.

Key: ST, biscuit with 100% type 1 wheat flour; Bor25, biscuit with 25% of borlotto flour; Bor50, biscuit with 50% of borlotto flour; Can25, biscuit with 25% of cannellino flour; Can50, biscuit with 50% of cannellino flour; Cop25, biscuit with 25% of "Copafam" flour; Cop50, biscuit with 50% of "Copafam" flour.

Table 1. Frequency counts of check-all-that-apply terms used to describe biscuit samples and results of Cochran's Q test for comparison among the samples.

Sensory Attributes	Samples						
	ST	**Bor25**	**Bor50**	**Can25**	**Can50**	**Cop25**	**Cop50**
Appearance							
Light color ***	73^{d}	26^{b}	3^{a}	73^{d}	49^{c}	19^{ab}	1^{a}
Dark color ***	0^{a}	17^{ab}	59^{c}	0^{a}	5^{ab}	20^{b}	54^{c}
Patchy ***	5^{ab}	18^{bc}	14^{ab}	5^{ab}	3^{0}	31^{cd}	37^{d}
Uniform ***	55^{c}	25^{b}	24^{b}	56^{c}	50^{c}	16^{ab}	5^{a}
Speckled ***	3^{a}	51^{b}	59^{b}	2^{a}	3^{a}	56^{b}	64^{b}
Odor							
Mild ***	40^{c}	31^{abc}	23^{ab}	39^{bc}	34^{abc}	32^{abc}	19^{a}
Strong **	5^{a}	10^{ab}	12^{ab}	6^{ab}	17^{ab}	8^{ab}	18^{b}
Butter ***	41^{c}	32^{bc}	8^{a}	35^{c}	10^{a}	34^{c}	17^{ab}
Legumes ***	3^{a}	7^{ab}	20^{c}	8^{ab}	12^{abc}	7^{ab}	17^{bc}
Toasted ***	2^{ab}	12^{abcd}	21^{d}	1^{a}	14^{bcd}	7^{abc}	19^{cd}
Taste							
Sour n.s.	1^{a}	2^{a}	1^{a}	0^{a}	3^{a}	1^{a}	3^{a}
Bitter ***	0^{a}	2^{a}	20^{c}	1^{a}	5^{ab}	4^{ab}	14^{bc}
Sweet ***	39^{d}	21^{abc}	7^{a}	29^{cd}	23^{bc}	24^{bcd}	13^{ab}
Salty n.s.	6^{a}	11^{a}	6^{a}	6^{a}	6^{a}	4^{a}	6^{a}
Flavor							
Mild ***	47^{b}	30^{ab}	13^{a}	45^{b}	31^{ab}	38^{b}	15^{a}
Strong ***	10^{a}	18^{ab}	27^{b}	9^{a}	18^{ab}	9^{a}	23^{ab}
Butter ***	42^{d}	16^{abc}	2^{a}	28^{cd}	11^{ab}	21^{bc}	4^{a}
Legumes ***	4^{a}	24^{bc}	35^{c}	8^{a}	25^{bc}	15^{ab}	30^{bc}
Toasted ***	2^{a}	9^{ab}	32^{c}	6^{ab}	18^{bc}	9^{ab}	24^{c}
Texture							
Sticky **	2^{a}	2^{a}	1^{a}	10^{b}	3^{ab}	4^{ab}	1^{a}
Crunchy ***	19^{a}	34^{abc}	47^{c}	21^{a}	39^{bc}	30^{ab}	33^{abc}
Floury **	19^{a}	13^{a}	12^{a}	20^{a}	18^{a}	8^{a}	10^{a}
Crumbly ***	52^{b}	48^{b}	25^{a}	48^{b}	41^{ab}	44^{b}	45^{b}
Grainy ***	11^{ab}	11^{ab}	15^{abc}	6^{a}	10^{a}	27^{c}	25^{bc}
Dry **	22^{a}	29^{b}	39^{b}	17^{a}	32^{ab}	30^{ab}	28^{ab}
Moist n.s.	3^{a}	1^{a}	1^{a}	5^{a}	2^{a}	2^{a}	1^{a}

Key: ST, biscuit with 100% type 1 wheat flour; Bor25, biscuit with 25% of borlotto flour; Bor50, biscuit with 50% of borlotto flour; Can25, biscuit with 25% of cannellino flour; Can50, biscuit with 50% of cannellino flour; Cop25, biscuit with 25% of "copafam" flour; Cop50, biscuit with 50% of "Copafam" flour. Different letters show significant differences ($p < 0.05$) according to post hoc test. n.s, not significant; ** $p < 0.01$; *** $p < 0.001$.

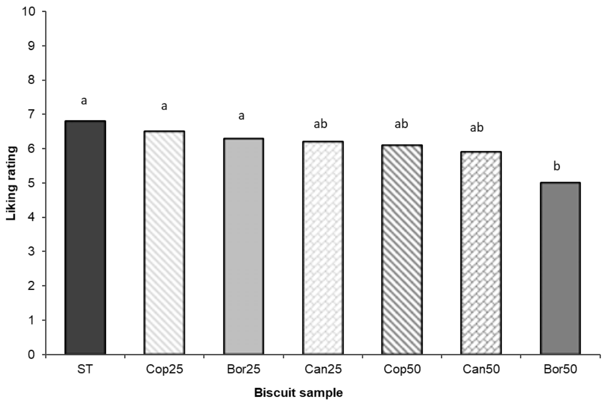


Figure 5. Mean hedonic ratings by samples. Different letters show significant differences ($p < 0.05$) according to post hoc test. Key: ST, biscuit with 100% type 1 wheat flour; Bor25, biscuit with 25% of borlotto flour; Bor50, biscuit with 50% of borlotto flour; Can25, biscuit with 25% of cannellino flour; Can50, biscuit with 50% of cannellino flour; Cop25, biscuit with 25% of "copafam" flour; Cop50, biscuit with 50% of "Copafam" flour. Different letters show significant differences ($p < 0.05$) according to post hoc test.

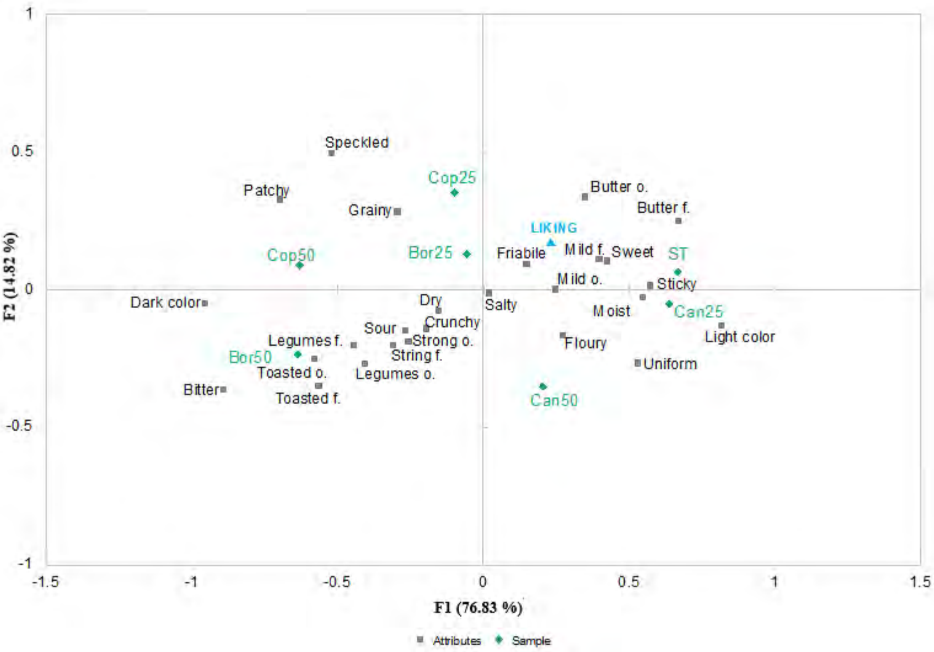


Figure 6. Correspondence analysis from check-all-that-apply data (o, odor; f, flavor).

4. Discussion

This research suggested that the variability among species is greater than the variability between varieties of the same species, in accordance with previous findings [46]. The present results revealed that "Copafam" beans differ from commercial beans in the nutritional and phytochemical composition as well as in the consumer study findings. In fact, the amount of protein was higher in beans belonging to *P. vulgaris* (Borlotto and Cannellino) species while "Copafam" (Figure 2c) showed the lowest content. However, all samples had more than 20% protein content leading beans to be considered a good source of this macronutrient and a possible alternative to red meat, the current principal source of proteins in consumers' diets. Globally, red meat consumption is higher than other protein sources like legumes, fish, poultry, and eggs, despite the well-documented health benefits of legume consumption [47].

The content of lipids was similar among all cultivars considered; the level ranged from 3.19% to 4.49% and "Copafam" was the one with low fat content (Figure 2b). According to Yoshida [48], the colored beans showed the lowest mean values of lipid content. Further, in previous research works, it was found that the principal fatty acids in beans are unsaturated and polyunsaturated, which are important compounds to improve human health [48,49]. Modest lipid and sugar levels (Figure 2e,h) represent the right conditions for maintaining a healthy diet [50]. A WHO guideline recommends people reduce their daily intake of free sugars (such as glucose, fructose, and sucrose) to less than 10% of their total energy intake in order to improve health benefits [51,52]. In fact, WHO estimated that by 2025, approximately 167 million people will become overweight or obese, therefore they suggested restricting high-fat food consumption [53].

The "Copafam" bean showed the highest fiber content (Figure 3h). Dietary fiber, besides reducing blood and cholesterol levels, induces the proliferation of beneficial microbes in the gut (prebiotic properties) and short-chain fatty acids (SCFAs) precursors throughout the fermentation process [28,47]. Alcázar-Valle [25] found in P.coccineus a high percentage of fiber and lower protein content compared to *P. vulgaris* which presented a higher percentage of protein levels, coherently with the findings of our research and the previous results [32].

The results about the seeds' antioxidant properties showed that the "Copafam" variety is characterized by a high DPPH scavenging activity (Figure 3c). This antioxidant action could be directly related to the phenolic compounds profile. Indeed, "Copafam" was the richest in anthocyanin and polyphenol content (Figure 3b and Figure 3f, respectively). Specifically, all the beans examined in this study contain similar hydroxycinnamic acid derivatives as their main phenolic component (Figure 3g). The samples of *P. coccineus* contained higher sinapic acid levels while *P.vulgaris* samples were abundantly composed of ferulic and p-cumaric acid. These results are concurrent with previous findings by Lin [30] and Madrera [28], which reported that hydroxycinnamic acid derivatives constituted the main phenolic components of beans upon alkaline treatment. The bioactive compounds (TPC, TFC, anthocyanin, and antioxidant activity) identified in the beans are associated with nutraceutical proprieties and biological activity in reducing the risk of obesity, chronic diseases, diabetes, and regulation of metabolism [25,28,30].

Moreover, "Copafam" showed the lowest content of phytic acid and a minor trypsin inhibitor activity. A high antinutrient level represents an important nutritional issue since the presence of TIA decreases protein digestibility and a high level of phytic acid, for example, reduces the availability of some essential nutrients like minerals, such as K, Fe, and Zn, and amino acids [25–31].

Due to its nutritional and phytochemical composition, "Copafam" could be considered a good source of protein and of bioactive and functional components; it should be argued that consumers are paying more and more attention to nutritional aspects, and in view of this, the agri-food sector could use "Copafam". In this context, the results obtained by sensory evaluation revealed that the experimental biscuit samples with added bean flour were well accepted by the consumers. In particular, biscuits with "Copafam" flour were found to be comparable to the control sample. It should be considered that the positive hedonic responses to these new food formulations are not so obvious; indeed, the addition of legume flour in a food matrix could negatively modify sensory properties [33,34]. Consumers' food choices and habits are then mainly driven by food preferences and, even if the enrichment in functional compounds adds value to the consumer [54], they are not really inclined to compromise on their diet [55]. Indeed, perceptual factors of a food—namely the characteristics perceived by our senses—still remain the more relevant in defining eating behavior [56,57].

The sensory descriptive analyses revealed that the specific attributes mainly responsible for the low acceptance of the sample with half of the wheat flour substituted with borlotto flour were associated with visual flavor aspects. Indeed, when the legume flavor became too intense and the color shifted from light to dark, consumers seemed to be less satisfied. Previous studies have been performed investigating the amount that could possibly be used to substitute flour with different percentages of legume flour. It has been suggested, for example, that formulations with up to 40% of pulse flours can be used to prepare snacks, such as crackers, without negatively affect their sensory quality [58], whereas other authors depicted a decrease in acceptability scores already at lower concentration [59]. Recently, legume flours from chickpea and green pea were successfully used to enrich a new rice-based snack [35]. According to the present results, positive hedonic responses to the addition of bean flour have been highlighted in cookies [60] as well as in other bakery products, such as tortillas [61], suggesting the real possibility of investing resources in promoting the use of these flours in the development of new products.

Unexpectedly, a significant effect of food neophobia level on liking scores was not revealed, whereas previous research works showed different hedonic responses to new food formulations according to this behavioral variable. Indeed, it is well established that food-neophobic subjects are more diffident in trying novel foods compared to neophilic ones, who tend to have a wide and varied diet [35,62]. These contrasting results could be explained by the type of food products use as the model (biscuit) and the ingredient added (legume flour), which could be perceived as familiar by the consumers involved.

Following the definition of Camacho Villa [6], landraces differ from conventional commercial varieties in terms of lacking formal crop improvement, being locally adapted, and being suitable for low-input traditional farming systems. Agriculture is considered one of the most important drivers of climate change, since innovation bought by the Green Revolution has completely modified the sustainability of processes, leading to an irreversible tendency at adopting conventional and intensive practices. This process is even more important in fragile territories of natural importance as mountains, where a high-input agricultural model (with the introduction of a high amount of input in terms of nutrition, health, and management costs) cannot be applied [2,10]. Landraces are less demanding in environmental and nutritional factors and can adapt to withstand the "typical" conditions (cold, heat, drought, soils, and "poor" foods) of the marginal areas and to organic agriculture [63].

Unfortunately, during the last century, the progressive substitution of landraces with modern varieties led to a dramatic reduction of crop diversity all over the world [2]. In addition, most of these traditional varieties are often found in mountain and hilly areas [23,64] and often they do not have clear origins or known morphometric and phytochemical characteristics, making their enhancement and conservation very difficult. This lack of information, in addition to preventing the conservation of agrobiodiversity, does not even allow the study and enhancement of landraces that could be particularly interesting for starting up unique high-quality agri-food chains of low environmental impact that could be strategic for smart, sustainable and inclusive growth of marginal and mountain territories.

In general, the potential for commercializing landraces is substantial, given the poor number of plants used for food production worldwide. Many underutilized species have the potential for the creation of value chains that could increase farmers' income. To achieve many of the UN SDGs [65], agricultural and health and nutrition experts will need to work more closely together toward a food system that better links agriculture, diet, and human health [63].

"Copafam", for its particular agronomic and nutritional features in addition to its link with the history and gastronomy of a territory [32], could become an important resource in this framework. Currently, this bean is not available commercially and it is cultivated and used only by a few farmers in the Brescia pre-Alps (and neighboring territories), mostly hobbyists. They and Indigenous peoples use "Copafam" in the preparation of typical dishes (e.g., Copafam soup or pasta and beans) [32]. In the future, it could improve the incomes of farmers and restauranteurs in the mountain areas of Lombardy, because consumers are increasingly interested in local agricultural products and traditional foods [32].

At present, in order to conserve and enhance agrobiodiversity and re-establish ties with the territory in terms both of territorial tradition and history of agricultural and food practices, some farmers gathered in consortia and associations. Unfortunately, most of the landraces are still cultivated by individual hobby farmers, in the foothill and mountain areas especially [4,23]. The study and characterization of ancient crop varieties is the first step for their recovery and there are many virtue cases around the world, such as what was carried out recently by Fenzi et al. [19] for the reintroduction of a disappeared landrace of Veneto (called Sponcio maize). The reintroduction of this variety was possible thanks to an intense workflow that started from the discovery and study of lost landraces to the development of innovative approaches by farmers.

A similar strategy was used by Cassani et al. [66] for the landraces of beaked corn: "Nero Spinoso di Valcamonica". The starting point was the genetic and phytochemical characterization of the landrace that appeared as a flavonoid-rich food that accumulates phlobaphenes in the pericarp. Genetic investigations demonstrated further that phlobaphene pigmentation is under the control of a monogenic dominant gene, making this variety an extremely interesting resource as a functional food and a useful tool in future breeding programs. After the nutritional and phytochemical characterization of "nero spinoso" maize landrace, a farmers' consortium with the objective to preserve, produce, and transform this cultivar, was settled up. This traditional cultivar was included in the European Register of Conservation Varieties in order to prevent its loss as well as to preserve the genetic variability. Today the "Nero Spinoso" maize is grown in small plots in the Camonica Valley on a total area of about 30,000 m^2. Before the activity of study and enhancement, it was cultivated in a small isolated field of 100 m^2 by just one farmer, so it was at great risk of genetic erosion (oral communication by Nero Spinoso Consortium).

Involving people in modern sustainable agricultural models, which also include the cultivation of landraces, will require time. The multi-actor approach is really important in revitalizing landraces and creating a value chain, meaning the focal role of partnerships of research centers concerned with the conservation of agrobiodiversity and the sustainable development of mountain regions with the private sector, NGOs, commonalities, etc. It is fundamental to look for support from organizations that deal with training, conservation of the territory and biodiversity, and socio-economic development. This aspect is of fundamental importance at a global level to support the local agri-food sector and therefore to favor the conservation of agrobiodiversity and the sustainable development of the local economy, in the foothill and mountain areas especially.

5. Conclusions

This research aimed at a comprehensive characterization of the "Copafam" landrace bean, comparing it with commercial cultivars to investigate its specificities. In front of a lower protein and lipidic content and higher dietary fiber, "Copafam" resulted as the best source of secondary metabolites such as polyphenols and anthocyanins and showed a high level of flavonoids, having, consequently, an interesting antioxidant activity. All these characteristics make it a resource of great interest for an innovative food industry.

Health is deeply tangled with the food production system and food consumer perception. The most promising legumes could be selected to enhance local production and improve food distribution and consumption strategies. The high content of functional molecules present in the "Copafam" bean could represent innovative forms of consumption like fortified foods to valorize agricultural products for human health. Our research was a preliminary trial opening possibilities for further investigations about alternative uses of "Copafam" bean flour. These "unconventional" uses are also ways to preserve, spread, and enhance that agricultural product. Coordinated actions of national institutions, supported by the EU, as well as of stakeholders, can improve knowledge, access, and use of landrace cultivars to transform them into potential resources for the sustainable development of mountains territories.

Author Contributions: Conceptualization, D.P., L.G., A.G., E.P. and A.S.; formal analysis, D.P., C.P., M.Z., G.B., S.M.B. and V.L.; investigation, D.P., V.L., M.Z., L.G. and A.M.B.; cultivars identification, L.G.; methodology and data curation, D.P., C.P., L.G., G.B., M.Z. and S.M.B.; writing—original draft preparation, D.P., C.P., S.M.B. and V.L.; writing—review and editing, D.P., L.G., V.L. and S.M.B.; supervision, L.G., A.G., E.P. and A.S.; project administration, A.G.; funding acquisition, A.G. and L.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by "Montagne: Living Labs di innovazione per la transizione ecologica e digitale", "PSR Fagio.Lo.– Ricerca, caratterizzazione e valorizzazione di cultivar di fagiolo tradizionali lombarde", "MIND FoodS HUB (Milano Innovation District Food System Hub): Innovative concept for the eco-intensification of agricultural production and for the promotion of dietary patterns for human health and longevity through the creation in MIND of a digital Food System Hub" and "National Center for Technology in Agriculture-AGRITECH" projects.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Milan.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgments: We wish to thank the local "Copafam" bean producers' and the "Forneria Pasticceria Salvetti" bakery for their collaboration.

Conflicts of Interest: The authors declare no competing interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Appendix A

Table A1. One-way ANOVA results of the effect of the bean cultivar on phytochemical and nutritional features.

Source of Variance	Df	Sum of Squares	Mean Square	F-Ratio	p-Value
Moisture	3	233,020	77,673.4	47.25	0.0000
Protein	3	347,634	115,878	137.59	0.0000
Lipid	3	17,450.9	5816.97	71.67	0.0000
TPC	3	2.92×10^{13}	9.73×10^{12}	615.76	0.0000
Anthocyanin	3	1.10×10^{12}	3.68×10^{11}	40,725.9	0.0000
DPPH	3	8.06×10^{11}	2.69×10^{11}	462.68	0.0000
Ash	3	3115.58	1038.53	11.09	0.0032
TFC	3	560,994	186,998	1298.6	0.0000
P-cumaric	3	149,656	49,885.4	108.31	0.0000
Sinapic	3	353,152	117,717	76.85	0.0000
Ferulic	3	1.25×10^{11}	417,903	29.48	0.0001
TIA	3	51,709.6	17,236.5	12.09	0.0024
Phytic acid	3	1630.33	543,444	10.62	0.0037
D-glucose	3	14.25	4.75	2.28	0.1563
Saccarose	3	137,592	45,864.1	4138.11	0.0000
RSO	3	872.25	290.75	0.18	0.9083
Starch	3	620,205	206,735	19.71	0.0005
Dietary fibre	3	2.48×10^{11}	828,181	77.61	0.0000

Key: TIA (Trypsin inhibitor activity); TPC (total polyphenol component); TFC (total flavonoid content); RSO (Raffinose-Series Oligosaccharides).

References

1. Giupponi, L.; Leoni, V. VegeT: An Easy Tool to Classify and Facilitate the Management of Seminatural Grasslands and Dynamically Connected Vegetation of the Alps. *Land* **2020**, *9*, 473. [CrossRef]
2. Raggi, L.; Pacicco, L.C.; Caproni, L.; Álvarez-Muñiz, C.; Annamaa, K.; Barata, A.M.; Batir-Rusu, D.; Díez, M.J.; Heinonen, M.; Holubec, V.; et al. Analysis of landrace cultivation in Europe: A means to support in situ conservation of crop diversity. *Biol. Conserv.* **2022**, *267*, 109460. [CrossRef]
3. Singh, V.; Jardhari, V. Landrace renaissance in the mountains: Experiences of the Beej Bachao Andolan in the Garhwal Himalayan region, India. In *An Exchange of Experiences from South and South East Asia, Proceedings of the International Symposium on Participatory Plant Breeding and Participatory Plant Genetic Resources Enhancement, Pokhara, Nepal, 1–5 May 2000*; Consultative Group on International Agricultural Research (CGIAR), Participatory Research and Gender Analysis (PRGA), Program Coordination Office: Cali, Colombia, 2001; pp. 87–96.
4. Giupponi, L.; Pilu, R.; Scarafoni, A.; Giorgi, A. Plant agro-biodiversity needs protection, study and promotion: Results of research conducted in Lombardy region (Northern Italy). *Biodivers. Conserv.* **2020**, *29*, 409–430. [CrossRef]
5. FAO. *Agricultural Biodiversity, Multifunctional Character of Agriculture and Land Conference, Background Paper 1*; FAO: Maastricht, The Netherlands, 1999.
6. Villa, T.; Maxted, N.; Scholten, M.; Ford-Lloyd, B. Defining and identifying crop landraces. *Plant Genet. Res.* **2005**, *3*, 373–384. [CrossRef]
7. Ceccarelli, S. Landraces: Importance and use in breeding and environmentally friendly agronomic systems. In *Agrobiodiversity Conservation: Securing the Diversity of Crop Wild Relatives and Landraces*; Maxted, N., Ehsan Dulloo, M., Ford-Lloyd, B.V., Frese, L., Iriondo, J.M., Pinheiro de Carvalho, M.A.A., Eds.; CAB International: Oxfordshire, UK, 2012; pp. 3–117.
8. Puglisi, D.; Landoni, M.; Cassani, E.; Toschi, I.; Lucchini, G.; Cesari, V.; Borlini, G.; Scapin, A.; Pilu, R. Traditional farmers' varieties: A valuable source of genetic variability for biofortification programs. *Maydica* **2018**, *63*, 10.
9. Frison, E.A.; Cherfas, J.; Hodgkin, T. Agricultural biodiversity is essential for a sustainable improvement in food and nutrition security. *Sustainability* **2011**, *3*, 238–253. [CrossRef]

10. Bertolino, M.A. *Agri-Cultural Resistance and Local Development: The Cases of Lavender and Rye in the Susa Valley In La Nuova Centralità Della Montagna*; Bolognesi, M., Corrado, F., Eds.; Territorio Rivista di Studi Territorialisti Firenze University Press: Firenze, Italy, 2009.
11. FAO. 2021. Available online: https://www.fao.org/director-general/news/news-article/it/c/1390762/ (accessed on 11 April 2021).
12. United Nations. Transforming Our World: The 2030 Agenda for Sustainable Development. 2015. Available online: https://undocs.org/A/RES/70/1 (accessed on 11 September 2021).
13. FAO. *Building on Gender, Agrobiodiversity and Local Knowledge*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2004.
14. FAO. *Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture. Commission on Genetic Resources for Food and Agriculture*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2010.
15. EU Commission. EUROPE 2020 a Strategy for Smart, Sustainable and Inclusive Growth. 2010. Available online: https://eurlex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52010DC2020 (accessed on 11 September 2020).
16. European Commission. A European Green Deal. Available online: https://ec.europa.eu/info/strategy/priorities-2019-2024/european-green-deal (accessed on 19 November 2021).
17. Spataro, G.; Negri, V. The European seed legislation on conservation varieties: Focus, implementation, present and future impact on landrace on farm conservation. *Genet. Resour. Crop Evol.* **2013**, *60*, 2421–2430. [CrossRef]
18. European Commission. EU COM 380, 20.5.2020. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Commettee of the Regions: EU Biodiversity Strategy for 2030. Bringing Nature Back into Our Lives. Available online: https://ec.europa.eu/info/sites/info/files/communication-annex-eubiodiversity-strategy-2030_en.pdf. (accessed on 19 November 2021).
19. Fenzi, M.; Couix, N. Growing maize landraces in industrialized countries: From the search for seeds to emerge of new practices and value. *Int. J. Agric. Sustain.* **2021**, *20*, 327–345. [CrossRef]
20. Giupponi, L.; Borgonovo, G.; Panseri, S.; Giorgi, A. Multidisciplinary study of a little known landrace of Fagopyrum tataricum Gaertn. of Valtellina (Italian Alps). *Genet. Resour. Crop Evol.* **2019**, *66*, 783–796. [CrossRef]
21. Giupponi, L.; Leoni, V.; Pedrali, D.; Ceciliani, G.; Bassoli, A.; Borgonovo, G. Morphometric and phytochemical characterization and elevation effect on yield of three potato landraces of the Ligurian Apennines (Northern Italy). *J. Appl. Bot. Food* **2020**, *93*, 234–243. [CrossRef]
22. Giupponi, L.; Leoni, V.; Colombo, F.; Cassani, E.; Hejna, M.; Rossi, L.; Pilu, R. Characterization of "Mais delle Fiorine" (*Zea mays* L.) and nutritional, morphometric and genetic comparison with other maize landraces of Lombardy region (Northern Italy). *Genet. Resour. Crop Evol.* **2021**, *68*, 2075–2091. [CrossRef]
23. Giupponi, L.; Pedrali, D.; Leoni, V.; Rodari, A.; Giorgi, A. The Analysis of Italian Plant Agrobiodiversity Databases Reveals That Hilly and Sub-Mountain Areas Are Hotspots of Herbaceous Landraces. *Diversity* **2021**, *13*, 70. [CrossRef]
24. Aquino-Bolaños, E.N.; Garzón-García, A.; Alba-Jiménez, J.; Chávez-Servia, J.; Vera-Guzmán, A.; Carrillo-Rodríguez, J.; Santos-Basurto, M. Physicochemical Characterization and Functional Potential of *Phaseolus vulgaris* L. and *Phaseolus coccineus* L. Landrace Green Beans. *Agronomy* **2021**, *11*, 803. [CrossRef]
25. Alcázar-Valle, M.; Lugo-Cervantes, E.; Mojica, L.; Morales-Hernández, N.; Reyes-Ramírez, H.; Enríquez-Vara, J.N.; García-Morales, S. Bioactive Compounds, Antioxidant Activity, and Antinutritional Content of Legumes: A Comparison between Four Phaseolus Species. *Molecules* **2020**, *25*, 3528. [CrossRef]
26. Rodríguez Madrera, R.; Negrillo, A.C.; Valles, B.S.; Ferreira Fernández, J.J. Phenolic Content and Antioxidant Activity in Seeds of Common Bean (*Phaseolus vulgaris* L.). *Foods* **2021**, *10*, 864. [CrossRef]
27. National Recovery and Resilience Plan (NRRP). Available online: https://www.governo.it/sites/governo.it/files/PNRR.pdf (accessed on 19 November 2021).
28. Rodríguez Madrera, R.; Valles, B.S. Development and validation of ultrasound assisted extraction (UAE) and HPLC-DAD method for determination of polyphenols in dry beans (*Phaseolus vulgaris*). *J. Food Compost. Anal.* **2020**, *85*, 10334. [CrossRef]
29. Finetti, F.; Biagi, M.; Ercoli, J.; Macrì, G.; Miraldi, E.; Trabalzini, L. *Phaseolus vulgaris* L. var. Venanzio Grown in Tuscany: Chemical Composition and In Vitro Investigation of Potential Effects on Colorectal Cancer. *Antioxidant* **2020**, *9*, 1181. [CrossRef]
30. Lin, L.Z.; Harnly, M.J.; Pastor-Corrales, M.S.; Luthria, D.L. The polyphenolic profiles of common bean (*Phaseolus vulgaris* L.). *Food Chem.* **2008**, *107*, 399–410. [CrossRef]
31. Correia Bento, J.A.; Ribeiro, P.R.V.; e Silva, L.M.A.; Filho, E.G.A.; Bassinello, P.Z.; de Brito, E.S.; Caliari, M.; Júnior, M.S.S. Chemical profile of colorful bean (*Phaseolus vulgaris* L.) flours: Changes influenced by the cooking method. *Food Chem.* **2021**, *356*, 129718. [CrossRef]
32. Giupponi, L.; Tamburini, A.; Giorgi, A. Prospects for Broader Cultivation and Commercialization of Copafam, a Local Variety of *Phaseolus coccineus* L. in the Brescia Pre-Alps. *Mt. Res. Dev.* **2018**, *38*, 24–34. [CrossRef]
33. Bortnowska, G.; Krudos, A.; Schube, V.; Krawczyńska, W.; Krzemińska, N.; Mojka, K. Effects of waxy rice and tapioca starches on the physicochemical and sensory properties of white sauces enriched with functional fibre. *Food Chem.* **2016**, *202*, 31–39. [CrossRef] [PubMed]
34. Wang, Y.; Trani, A.; Knaapila, A.; Hietala, S.; Coda, R.; Katina, K.; Maina, N.H. The effect of in situ produced dextran on flavour and texture perception of wholegrain sorghum bread. *Food Hydrocoll.* **2020**, *106*, 105913. [CrossRef]
35. Proserpio, C.; Bresciani, A.; Marti, A.; Pagliarini, E. Legume Flour or Bran: Sustainable, Fiber-Rich Ingredients for Extruded Snacks? *Foods* **2020**, *9*, 1680. [CrossRef]

36. AOAC International. AOAC Method n_9991.36; 945.18; 993.40. In *Official Methods of Analysis of AOAC International*, 18th ed.; Horwitz, W., Latimer, G.W., Eds.; AOAC International: Gaithersburg, ML, USA, 2005.
37. American Association of Cereal Chemists (AACC). *Approved Methods of Analysis; Method n_ 08-01.01.* 11th ed.; AACC International: St. Paul, MN, USA, 1999.
38. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol.* **1995**, *28*, 25–30. [CrossRef]
39. Saura-Calixto, L.; Bravo, F. Charactererization of Dietary Fiber and the In Vitro Indigestible Fraction of Grape Pomace. *Am. J. Enol. Vitic.* **1998**, *49*, 135–141.
40. Abdel-Aal, E.-S.M.; Hucl, P. A Rapid Method for Quantifying Total Anthocyanins in Blue Aleurone and Purple Pericarp Wheats. *Cereal Chem.* **1999**, *76*, 350–354. [CrossRef]
41. *ISO 14902:2001*; Animal Feeding Stuffs—Determination of Trypsin Inhibitor Activity of Soya Products. International Standardization Organization: Geneva, Swizzerland, 2010.
42. Ares, G.; Deliza, R.; Barreiro, C.; Giménez, A.; Gámbaro, A. Comparison of two sensory profiling techniques based on consumer perception. *Food Qual. Prefer.* **2010**, *21*, 417–426. [CrossRef]
43. Jaeger, S.R.; Chheang, S.L.; Yin, J.; Bava, C.M.; Gimenez, A.; Vidal, L.; Ares, G. Check-all-that-apply (CATA) questions for sensory product characterization by consumers: Investigations into the number of terms used in CATA questions. *Food Qual. Prefer.* **2015**, *42*, 154–164. [CrossRef]
44. Meyners, M.; Castura, J.C. Randomization of CATA attributes: Should attribute lists be allocated to assessors or to samples? *Food Qual. Prefer.* **2016**, *48*, 210–215. [CrossRef]
45. Laureati, M.; Spinelli, S.; Monteleone, E.; Dinnella, C.; Prescott, J.; Cattaneo, C.; Proserpio, C.; De Toffoli, A.; Gasperi, F.; Endrizzi, I.; et al. Associations between food neophobia and responsiveness to "warning" chemosensory sensations in food products in a large population sample. *Food Qual. Prefer.* **2018**, *68*, 113–124. [CrossRef]
46. Capistrán-Carabarin, A.; Aquino-Bolaños, E.N.; García-Díaz, Y.D.; Chávez-Servia, J.L.; Vera-Guzmán, A.M.; Carrillo-Rodríguez, J.C. Complementarity in Phenolic Compounds and the Antioxidant Activities of *Phaseolus coccineus* L. and *P. vulgaris* L. Landraces. *Foods* **2019**, *8*, 295. [CrossRef] [PubMed]
47. Conti, M.V.; Guzzetti, L.; Panzeri, D.; De Giuseppe, R.; Coccetti, P.; Labra, M.; Cena, H. Bioactive compounds in legumes: Implications for sustainable nutrition and health in the elderly population. *Trends Food Sci. Technol.* **2021**, *117*, 139–147. [CrossRef]
48. Yoshida, H.; Yoshida, N.; Tomiyama-Sakamoto, Y.; Mizushina, Y. Characteristic distributions of fatty acids in different lipids from Jack beans (*Canavalia gladiata* DC.). *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 787–793. [CrossRef]
49. Doan, L.P.; Nguyen, T.T.; Pham, M.Q.; Tran, Q.T.; Pham, Q.L.; Tran, D.Q.; Than, V.T.; Bach, L.G. Extraction processes, identification of fatty acids, tocopherols, sterols and phenolic constituents and antioxidant evaluation of seed oils from five fabaceae species. *Processes* **2019**, *7*, 456. [CrossRef]
50. WHO. 2020. Available online: https://www.who.int/news-room/fact-sheets/detail/healthy-diet (accessed on 20 April 2022).
51. WHO. *Diet, Nutrition and the Prevention of Chronic Diseases: Report of a Joint WHO/FAO Expert Consultation*; WHO Technical Report Series, No. 916; World Health Organization: Geneva, Switzerland, 2003.
52. WHO. *Guideline: Sugars Intake for Adults and Children*; World Health Organization: Geneva, Switzerland, 2015.
53. WHO. 2022. Available online: https://www.who.int/news/item/04-03-2022-world-obesity-day-2022-accelerating-action-to-stop-obesity (accessed on 20 April 2022).
54. Alava, C.; Verdú, S.; Barat, J.M.; Grau, R. Enrichment of chips with fibre from a tiger-nut (*Cyperus esculentus*) milk co-product at 'source of fibre foods' and 'high fibre content foods' levels: Impact on processing, physico-chemical and sensory properties. *Int. J. Food Sci.* **2019**, *54*, 908–915. [CrossRef]
55. Jaeger, S.R.; Axten, L.G.; Wohlers, M.W.; Sun-Waterhouse, D. Polyphenol-rich beverages: Insights from sensory and consumer science. *J. Sci. Food Agric.* **2009**, *89*, 2356–2363. [CrossRef]
56. Kushwah, S.; Dhir, A.; Sagar, M.; Gupta, B. Determinants of organic food consumption. A systematic literature review on motives and barriers. *Appetite* **2019**, *143*, 104402. [CrossRef]
57. Tuorila, H.; Hartmann, C. Consumer responses to novel and unfamiliar foods. *Curr. Opin. Food Sci.* **2020**, *33*, 1–8. [CrossRef]
58. Millar, K.A. Effect of pulse flours on the physiochemical characteristics and sensory acceptance of baked crackers. *Int. J. Food Sci.* **2017**, *52*, 1155–1163. [CrossRef]
59. Venkatachalam, K.; Nagarajan, M. Physicochemical and sensory properties of savory crackers incorporating green gram flour to partially or wholly replace wheat flour. *Ital. J. Food Sci.* **2017**, *29*, 599. [CrossRef]
60. Simons, C.W.; Hall, C., III. Consumer acceptability of gluten-free cookies containing raw cooked and germinated pinto bean flours. *Food Sci. Nutr.* **2018**, *6*, 77–84. [CrossRef] [PubMed]
61. Anton, A.A.; Lukow, O.M.; Fulcher, R.G.; Arntfield, S.D. Shelf stability and sensory properties of flour tortillas fortified with pinto bean (*Phaseolus vulgaris* L.) flour: Effects of hydrocolloid addition. *LWT-Food Sci. Technol.* **2009**, *42*, 23–29. [CrossRef]
62. Siegrist, M.; Hartmann, C.; Keller, C. Antecedents of food neophobia and its association with eating behavior and food choices. *Food Qual. Prefer.* **2013**, *30*, 293–298. [CrossRef]
63. Rudebjer, P.G.; Schagen, B.V.; Chakeredza, S.; Njoroge, K.; Kamau, H.; Baena, M. *Teaching Agrobiodiversity: A Curriculum Guide for Higher Education*; Bioversity International: Rome, Italy, 2011.
64. Veteto, J.R. Seeds of Persistence: Agrobiodiversity in the American Mountain South. *CAFÉ* **2014**, *36*, 17–27. [CrossRef]

65. United Nations, Department of Economic and Social Affairs—Sustainable Development. Make the SDG a Reality, Discover the Goals. Available online: https://sdgs.un.org/#goal_section (accessed on 9 December 2021).
66. Cassani, E.; Puglisi, D.; Cantaluppi, E.; Landoni, M.; Giupponi, L.; Giorgi, A.; Pilu, R. Genetic studies regarding the control of seed pigmentation of an ancient European pointed maize (*Zea mays* L.) rich in phlobaphenes: The "Nero Spinoso" from the Camonica valley. *Genet. Resour. Crop Evol.* **2017**, *64*, 761–773. [CrossRef]

Article 3:

antioxidants

MDPI

Review

Binary Alginate-Whey Protein Hydrogels for Antioxidant Encapsulation

Davide Pedrali [1,2,3], Alessio Scarafoni [1], Anna Giorgi [2,3] and Vera Lavelli [1,*]

[1] Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Via Celoria 2, 20133 Milan, Italy
[2] Department of Agricultural and Environmental Sciences-Production, Landscape and Agroenergy (DiSAA), University of Milan, Via Celoria 2, 20133 Milan, Italy; anna.giorgi@unimi.it
[3] Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas (CRC Ge.S.Di.Mont.), University of Milan, 25048 Edolo, Italy
* Correspondence: vera.lavelli@unimi.it; Tel.: +39-02-50319172

Abstract: Encapsulation of antioxidants in hydrogels, i.e., three-dimensional networks that retain a significant fraction of water, is a strategy to increase their stability and bioaccessibility. In fact, low oxygen diffusivity in the viscous gelled phase decreases the rate of oxidation. Moreover, some hydrocolloids such as alginate and whey proteins provide a pH-dependent dissolution mechanism, allowing the retention of encapsulated compounds in the gastric environment and their release in the intestine, where they can be absorbed. This paper reviews the information on alginate-whey protein interactions and on the strategies to use binary mixtures of these polymers for antioxidant encapsulation. Results showed that alginate and whey proteins strongly interact, forming hydrogels that can be modulated by alginate molecular mass, mannuronic acid: guluronic acid ratio, pH, Ca^{2+} or transglutaminase addition. Hydrogels of alginate and whey proteins, in the forms of beads, microparticles, microcapsules, and nanocapsules, generally provide better encapsulation efficiency and release properties for antioxidants with respect to the hydrogel of alginate alone. The main challenges for future studies are to extend knowledge on the interactions among three components, namely alginate, whey proteins, and the encapsulated bioactive compounds, and to investigate the stability of these structures under food processing conditions. This knowledge will represent the rationale basis for the development of structures that can be tailored to specific food applications.

Keywords: alginate; whey; β-lactoglobulin; hydrogel; encapsulation; antioxidant; in vitro digestion

check for updates

Citation: Pedrali, D.; Scarafoni, A.; Giorgi, A.; Lavelli, V. Binary Alginate-Whey Protein Hydrogels for Antioxidant Encapsulation. *Antioxidants* **2023**, *12*, 1192. https://doi.org/10.3390/antiox12061192

Academic Editor: Stanley Omaye

Received: 31 March 2023
Revised: 15 May 2023
Accepted: 29 May 2023
Published: 31 May 2023

1. Introduction

Micro- and nanostructures such as molecular complexes, oil-in-water (O/W) emulsions, mycelles, and liposomes have been designed for encapsulation of both hydrophilic and lipophilic low molecular mass antioxidants in the liquid phase, in order to improve their solubility as well as stability and bioaccessibility [1–3]. In fact, food processing promotes antioxidant exposure to heat [4], light [5], and moisture [6], which results in oxidative degradation. Moreover, the bioaccessibility of antioxidants studied by in vitro simulation of mouth, stomach, and intestine digestion is generally low [7,8]. Besides encapsulation in liquid systems, research studies are also focusing on encapsulation strategies for antioxidants in hydrogels, which can be defined as three-dimensional networks that retain a significant fraction of water within their structure. In fact, encapsulation of antioxidants in hydrogels is expected to provide higher stability with respect to encapsulation in liquid systems, due to the diminished molecular diffusivity in a viscous gelled phase. For instance, the oxygen diffusion rate is 2.48×10^9 m^2 s^{-1} in water, while it decreases to 1.06×10^9 m^2 s^{-1} in miglyol, a model viscous oil [9], thus decreasing the rate of oxidative reactions. Furthermore, different food applications require a delivery device for antioxidants that prevents their release in the superior digestive tract and permits their release in the intestine at a predetermined rate. To this aim, various hydrogels are promising delivery systems since they provide a pH-dependent release mechanism.

Alginate is one of the most common food hydrocolloids. It is extracted primarily from brown algae such as *Laminaria* sp., *Durvillaea* sp., and *Sargassum* sp. Alginate is a linear anionic polysaccharide chain composed of 1,4-glycosidic bond-linked α-$_{\text{L}}$-guluronic acid (G) residues and β-Dmannuronic acid (M), occurring as MM-, GG-, and MG-GM-block structures [10]. The molecular mass of alginate generally falls in the range of 32–400 kDa depending on the source, as well as on processing. For instance, alginate can be depolymerized effectively using high intensity ultrasounds (200 W, 24 kHz, 30 min at temperatures of 25 or 75 °C) and different amplitudes (50 or 100%) [11]. Alginate can form hydrogels in the presence of cations, which can shield the electrostatic repulsion among carboxyl groups through direct binding by ionic bonds. Alginate gelation processes with H^+ or Ca^{2+} are the most common in food applications. The H^+-type alginate gels can be formed when the pH of alginate solutions is below the pKa of the uronic acid residues, which are 3.65 and 3.38 for G and M, respectively, in 0.1 M NaCl [12]. Conversely, the Ca^{2+} gelation can be performed in a wide pH range, but the resulting gels have different properties. In particular, the Ca^{2+} alginate gel obtained at pH 3.8 (which is above but close to the pKa of alginate) has stronger chain interactions and hence a denser and interconnected microstructure than Ca^{2+} alginate gels obtained at a pH of 5.0 and 6.8 [13].

Due to the pH sensitivity of alginate, this polymer is used for controlled release of entrapped molecules in the intestine. The kinetic of release of encapsulated compounds is studied under in vitro digestion conditions. The mechanical properties of the hydrogels, such as the elastic modulus (Young's modulus), have also been investigated at different stages of in vitro digestion, since they are closely associated to the release properties. For Ca^{2+} alginate gels, in simulated saliva, an increase in Young's modulus is observed due to partial exchange of Ca^{2+} with Na^+. In simulated gastric fluid (SGF), the Young's modulus increases further and alginate gels shrink due to the protonation of free carboxylic groups and decreased repulsive forces between polymer chains. Conversely, in simulated intestinal fluid (SIF), the Young's modulus decreases since alginate gels swell and break, due to the ion exchange between Ca^{2+} in gels and Na^+ in digestive juice and increased repulsive forces between alginate monomers at high pH [14]. Consistent with the swelling behavior of alginate, the release of encapsulated compounds is generally faster in SIF than in SGF.

However, for hydrophilic antioxidants, the encapsulation efficiency in alginate hydrogel is critical. In fact, the alginate gel matrix is porous, which can lead to spontaneous diffusion and loss of hydrophilic antioxidants. Moreover, shrinkage of alginate microcapsules in SGF causes the gel syneresis along with the expulsion of water-soluble materials from the gel networks [15]. In addition, alginate is a hydrophilic polysaccharide and has poor emulsifying capacity because it lacks hydrophobic groups [16]. Hence, for lipophilic antioxidant encapsulation in alginate hydrogel, a lipid environment and a surfactant are necessary. Therefore, combining alginate hydrogels with a complementary polymer is a strategy to improve its encapsulation properties.

To this aim, β-lactoglobulin is a biopolymer that can extend alginate applications. It is the main component of whey, a byproduct of cheese-making, and it is used as purified protein or is present as the main protein in whey protein isolate (WPI, protein content 90%) or whey protein concentrate (WPC, protein content 50–80%) [17]. β-lactoglobulin is a globular protein of 162 amino acid residues, with a monomer molecular mass of 18.3 kDa, two disulfide bridges, and a free thiol group (SH). β-lactoglobulin displays intrinsic encapsulation ability for low molecular mass lipophilic compounds, primarily due to its conical central cavity (the calyx or β-barrel) that provides the main ligand binding site [18]. Moreover, both β-lactoglobulin and WPI can form hydrogels upon heating at 80 °C for approximately 30 min, which represents the ultimate result of a cascade of physicochemical events, including protein unfolding, aggregation, and gelation. Whey protein gel structure is stabilized by both covalent (disulfide) bonds and noncovalent protein–protein interactions, such as hydrophobic and ionic interactions and hydrogen bonding [19,20]. In salt-free solutions, opaque particulate gels composed of random aggregates occur at pH values near the isoelectric point (pI) of β-lactoglobulin (pH 4–6), whereas transparent fine-stranded gels are obtained by shifting the pH far from the pI [21]. Indeed, WPI hydrogels display pH-sensitive swelling behavior with minimum swelling ratio near the isoelectric point (pI) [20]. Ca^{2+}-driven gelation of β-lactoglobulin or WPI can also be achieved through two consecutive steps: the preparation of a heat-denatured protein suspension, and chilling and addition of $CaCl_2$ which enables cross-linking of proteins and thus promotes gelation. This latter process is called cold gelation because protein denaturation occurs before gelation and is preferred for encapsulation of heat-sensitive compounds [22].

Beside intrinsic encapsulation properties and gelling ability, WPI can act as a surfactant, because of its amphiphilic nature. Hence, WPI rapidly adsorbs to the emulsion interface where it self-aggregates and forms continuous and homogeneous membranes around the oil droplets through intermolecular β-sheets interactions. Then, gelation of these structures can be promoted by both Ca^{2+} or heat treatment [23].

To extend the properties that alginate and WPI/β-lactoglobulin display when used as single hydrocolloids, binary combinations of these polymers have been designed. This review analyzes the strategies to combine alginate and WPI/β-lactoglobulin with the aims to summarize the current knowledge regarding the nature of interaction between these polymers and to discuss the advantages of using both polymers for encapsulation of hydrophilic and lipophilic antioxidants. This will lead to identify future research needs to optimize food applications of antioxidants encapsulated in these hydrogels.

2. Materials and Methods

Literature Study

The literature study was performed using two databases (Scopus and Web of Science). The search included the following keywords: "alginate" and "whey". Papers reporting the development of an encapsulation technology for antioxidants were selected, while those related to the encapsulation of microorganisms, protein, and enzymes were not included in the focus of this review.

3. Results and Discussion

3.1. Alginate-Whey Protein/β-Lactoglobulin Interactions

The analysis of the ζ-potential of polymer solutions was applied to predict the interactions between alginate and WPI [24] or alginate and β-lactoglobulin [25] in the pH range 2–7. The ζ-potential of both the WPI solution and β-lactoglobulin solution changed from negative at pH 7.0 to positive at pH 2.0, with a point of zero charge around pH 4.7, which corresponds to protein pI. The ζ-potential of the alginate solution remained negative across the whole pH range studied, changing from strongly negative at pH 7 to slightly negative at pH 2 [24,25]. It would be expected that these biopolymers should be attracted to each other at pH values where they have opposite charges, but repel each other where they have the same charge, i.e., above pH 4.7. However, alginate and WPI/β-lactoglobulin were associated up to pH 5.5, which can be attributed to the binding of anionic groups on the alginate molecules to cationic patches on the surfaces of the protein molecules [24,25]. The interactions between alginate and WPI were then studied after Ca^{2+} addition to promote gelation. As a result, at pH values of 3.0, 5.0 or 7.0, the amount of protein retained in the hydrogel was found to increase from 11.6% at pH 7.0, to 19.1% at pH 5, to 58.6% at pH 3.0. Moreover, the release of protein in phosphate buffer occurred relatively slowly at pH 3.0, while it was rapid at pH 5.0 and 7.0. This trend was related to the strength of the electrostatic interactions between the protein and alginate molecules [24]. The pH-dependent complex formation between alginate and pure β-lactoglobulin was also studied by transmission electron microscopy, and maximum complex formation was found to occur at pH 4.2 [26]. The binding enthalpy at pH 4.2 was found to be −411 kJ/mol while entropy variation was −1.3 kJ/mol [26]. The exothermicity was attributed to electrostatic interactions between β-lactoglobulin and alginate, which surpasses the energy changes associated to the reduction in the number of hydrogen bonds with water, consequent to protein–alginate interaction.

In a further study, using hetero-nuclear single quantum coherence NMR spectroscopy, two different alginate binding sites were identified for monomeric β-lactoglobulin (isoform A) at pH 2.65; in contrast, only one site was observed at pH 4.0, where β-lactoglobulin occurs as a dimer, confirming that the alginate–protein network strongly depends on pH [27]. The stoichiometry of the complex and the apparent enthalpy variation also depend on G to M ratio. In fact, the β-lactoglobulin binding capacity is higher for alginate with higher M content [28]. For instance, for three alginate chains with the same average molecular mass of about 300 kDa and M/G ratios of 1.8, 1.1, and 0.6, the stoichiometry of the complexes at pH 4.0 was calculated by isothermal calorimetry, resulting 47.9, 43.4, and 28.3 mol β-lactoglobulin/mol alginate (6.8, 6.3, and 4.7 g of β-lactoglobulin per g of alginate), with enthalpy values of −83.4, −76.3, and −73.6 kJ/mol, respectively [28]. The effect of the molecular mass of alginate on the thermodynamic parameters of β-lactoglobulin—alginate complex was also studied considering a system made of binary mixtures of β-lactoglobulin and either low molecular mass alginate (40 kDa) or high molecular mass alginate (280 kDa) or an alginate trisaccharide. The dissociation constant Kd was approximately 10-fold higher for low molecular mass alginate-β-lactoglobulin complex than high molecular mass alginate-β-lactoglobulin complex and 3 orders of magnitude higher for an alginate trisaccharide-β-lactoglobulin complex, as determined by isothermal titration calorimetry. From this approach, it was found that the higher the molecular mass of alginate, the higher the enthalpy for binding with β-lactoglobulin [29].

Besides the occurrence of charge interactions between alginate and β-lactoglobulin, hydrophobic interactions can also be established. Raman spectroscopy analysis revealed that the interactions between WPI and alginate cause conformational changes in WPI; in particular, the ordered structures of α-helixes and β-sheets of WPI were strengthened at pH 4.0 and 5.0 [30,31]. In a further study, the effect of β-lactoglobulin cross-linking using transglutaminase on the binding with alginate was investigated. Under the reaction conditions chosen, cross-linked β-lactoglobulin molecular mass spanned 18 to >240 kDa, while alginate used for the study had a molecular mass of 139 kDa. Crosslinking of β-lactoglobulin changed the number of molecules of protein per molecule of alginate in the complex from 35 to 43. Moreover, there was a moderate increase in the apparent free energy from −42 to −34 kJ/mol and a sharp increase in entropy from 72 to 87 J/mol, as measured at pH 3.0, reflecting changes in the nature of interactions with respect to native β-lactoglobulin. This trend was explained with hypothesizing that hydrophobic interactions occurred among cross-linked β-lactoglobulin and alginate. Indeed, in alginate, the uronate ring side opposite of the carboxylic acid is hydrophobic and CD spectroscopy revealed that cross-linked β-lactoglobulin was structurally similar to heat-treated β-lactoglobulin, suggesting that the protein was unfolded. It was concluded that cross-linking of β-lactoglobulin or whey protein mixtures prior to interaction with alginate can expand the applications of the binary mixtures of these polymers [32].

Research so far performed has proven that strong interactions occur between alginate and WPI/β-lactoglobulin, which can be modulated by M:G ratio, alginate molecular mass, pH, Ca^{2+}, and transaminase. This information has stimulated the development of binary matrices made of these polymers. Whey protein/β-lactoglobuin and alginate mixtures were studied for the production of edible coating and film. Interestingly, the β-lactoglobulin binding properties were maintained in the dry film and after film re-dissolution, which would allow the development of new carriers for food bioactive compounds [31]. The presence of β-lactoglobulin improved the oxygen barrier properties of the film [33] but decreased the tensile strength, and hence further studies are required before using these edible films for application in the food industry [31,33]. A variety of gelled structures made of alginate and WPI/β-lactoglobulin can be obtained, which are referred to with different denominations depending on the size and structure, such as beads (or spheres), capsules, microparticles, and nanocomplexes [33].

Beads or spheres are hydrogel matrices containing a dispersed bioactive compound, having a spherical shape and diameters in the range of millimeters. Depending on their polarity, the bioactive compounds dispersed in the hydrogel matrix of the beads can be directly solubilized in water and entrapped in the gel network or dissolved in lipid droplets, which in turns are entrapped in the gel network [34]. For hydrogels that are designed for possible applications into food products, acceptability is largely dependent on their perception within the mouth which, in turn, is affected by their size. Typically, particles larger than 50–100 μm can be detected as individual entities and give a gritty perception [35]. Hence, there is an interest in reducing the size of the hydrogels. Hydrogels with sizes in the range of 50–100 μm are called microparticles or microcapsules, depending on their inner structure. The microparticles generally refer to hydrogels containing a dispersed bioactive compound, which are irregular in shape [34]. The microcapsules are spheres that comprise a gelled membrane surrounding a liquid core, containing the bioactive compounds that can be either hydrophilic or hydrophobic. Nanoencapsulation provides some advantages with respect to microencapsulation due to the high area:volume ratio, resulting in high bioaccessibility of the encapsulated compounds.

In the following paragraphs, the strategies to encapsulate hydrophilic and lipophilic antioxidants in alginate-WPI/β-lactoglobulin hydrogels are described, which can lead to optimized delivery of these compounds in the food matrices.

3.2. Encapsulation of Hydrophilic Antioxidants in Alginate-Whey Protein/β-Lactoglobulin Hydrogels

3.2.1. Encapsulation in Beads

Considering small hydrophilic antioxidant compounds, encapsulation in alginate hydrogel is challenging due to the porous nature of the network and the possible loss of these compounds for spontaneous diffusion and during gel shrinkage. Hence, the design of the network by combining alginate with another hydrocolloid such as WPI or β-lactoglobulin is crucial. In fact, at the low pH of SGF, carboxyl groups of alginate are protonized and hence the electrostatic repulsions among these groups lessened, favoring matrix shrinkage. In contrast, WPI chains (isoelectric point of 5.2) are positively charged, causing repulsive forces among matrix chains. Moreover, alginate slows pepsin attach to WPI. On the contrary, the ionic environment of SIF causes relaxation of the gel, making pancreatin attack easier on WPI and consequently favoring a fast release of encapsulated compounds [36]. As discussed before, alginate–protein hydrogel can be formed when these polymers have opposite charges but also when they have the same charge, in presence of Ca^{2+} [30,37]. The simplest protocol to encapsulate hydrophilic bioactive compounds includes preparation of a mixture of alginate, bioactive compound, and WPI. This solution is then dripped in $CaCl_2$ for external gelation [38]. This protocol was applied to encapsulate the water-soluble extract of dandelion (*Taraxacum officinale*) leaf extract, which is composed of hydroxycinnamic acids, among which chlorogenic, caftaric, chicoric, and caffeic acids were identified [39]. The dandelion extract was added both to the polymer solution before gelation and to the $CaCl_2$ solution (Figure 1, Table 1). The WPI–alginate beads so far obtained were compared with beads of pure alginate in terms of average dimensions, encapsulation efficiency, and in vitro digestion behavior. The average particle size of plain alginate beads was 2 mm, while that of beads with WPI was 1.76 mm [39]. Encapsulation efficiency for total phenolics was reported to be 82% in pure alginate and 93% in alginate added with WPI. On the other hand, the recovery of polyphenols was related to the amount added to the alginate/WPI solution before gelation, while the amount added to the $CaCl_2$ solution was not considered. This is probably the reason for the observed high efficiency of encapsulation. The retained antioxidant capacity of dandelion leaf extract, determined as the ability to scavenge the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical was lower than the recovery of antioxidants, namely 61% in plain alginate and 81% in alginate added with WPI. FT-IR results confirmed the occurrence of interactions between dandelion polyphenols and the employed carriers, which may explain the decrease in antioxidant activity of the encapsulated compounds compared to the free extract, as the encapsulated bioactive compounds are involved in binding with the carriers. A slight decrease in bioactivity of phenolics encapsulated in alginate had already been observed and attributed to their interactions with the carrier [40]. The release profile of polyphenols in the beads formed by WPI and alginate showed a longer delay in SIG in the presence of two polymers than in the presence of alginate alone, but the exact percentages released were not reported. In the same study, carob or cocoa powders were also used as copolymers of alginate and found to provide less efficiency of encapsulation for phenolics but better delayed release in the digestive tract than WPI [39].

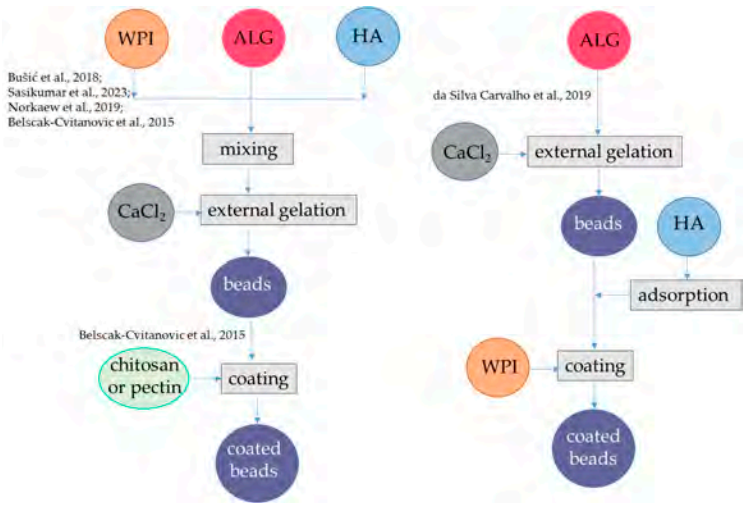


Figure 1. Steps to encapsulate hydrophilic antioxidants into alginate-whey protein beads. The procedures were applied to hydroxycinnamic acids from dandelion [39], flavanols from blood juice [41], anthocyanins from black rice [42], anthocyanins from jussara [43], and flavanols from green tea [44]. ALG, alginate; HA, hydrophilic antioxidants; WPI, whey protein isolate. The concentrations of polymers and antioxidants are indicated in Table 1.

Using a similar approach, polyphenols from blood fruits (*Haematocarpus validus*), which are mainly composed by epigallocatechin, gallocatechin, gallic acid, catechin, 2-coumaric acid, and rutin, were encapsulated in alginate-β-lactoglobulin microbeads at pH 5.0, achieving an encapsulation yield of 84% (Figure 1, Table 1). This system led to a release of 14% of phenolics in SGF and 80% of phenolics in SIF. Moreover, the photo-oxidative stability of encapsulated phenolics was proven upon exposure to ultraviolet light (UV-C) at λ 253.7 for 90 min for sterilization [41]. Conversely, the same approach was not satisfactory to encapsulate anthocyanins. In fact, alginate-WPI beads containing water-soluble black rice (*Oryza sativa*) polyphenols extract as bioactive compounds were produced by external gelation (Figure 1, Table 1). The black rice extract was mainly composed by two anthocyanins, namely, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside. The particle size of the alginate-WPI microbeads was approximately 0.945 mm. The anthocyanin retention was low, i.e., 30%, which was attributed to the loss occurring during the gel formation of the alginate beads in the $CaCl_2$ solution because these bioactive compounds are highly water-soluble. Conversely, in the same study it was found that almost 100% of anthocyanins were retained upon spray-drying with maltodextrin. The antioxidant activity showed the same trend as anthocyanin content, with higher value in the spray-dried maltodextin system than in aginate-WPI beads. The release of anthocyanins from the spray-dried maltodextrin was also better than the release from the alginate-WPI hydrogel. In fact, at the end of the in vitro gastrointestinal digestion, the alginate-WPI microbeads still had a dark purple color, indicating that a high portion of anthocyanins might have strong binding with the beads [42]. An alternative procedure was applied to encapsulate anthocyanins using negatively charged alginate and positively charged WPI (below the pI), which can form a bilayer (Figure 1, Table 1). Plain alginate beads with average diameters of about 1 mm were firstly produced at pH 6.5 and then immersed in a solution containing hydrophilic bioactive compounds, namely anthocyanins from jussara (*Euterpe edulis*) extract, which are mainly represented by cyanidin 3-*O*-rutinoside and cyanidin 3-*O*-glucoside. Due to the porous nature of the alginate hydrogel, the bioactive compounds could be adsorbed to the hydrogel structure. To prevent the diffusion of the bioactive compounds, coating with different polymers such as WPI, chitosan, and gelatin was applied at pH 3.5. Interestingly, the stability of encapsulated anthocyanins during refrigerated storage was investigated and results showed that the coating process using WPI was effective in protecting these compounds from degradation, with about 80% of retention after 1 month compared to 50% retention in the microbeads without WPI. The encapsulation efficiency was not reported, but it was shown that the antioxidant activity as measured with the ORAC (Oxygen Radical Absorbance Capacity) assay was higher for the uncoated alginate beads than for the coated alginate beads. One reason for this behavior could be that the coating polymer could interact with anthocyanin and decrease their antioxidant activity. However, the interaction of anthocyanin with the copolymer was not so strong to markedly delete their release in SGF. Indeed, the alginate beads released 76% anthocyanins, while the beads coated with chitosan, WPI, and gelatin released 73, 71, and 70%, of anthocyanins, respectively. In the SIF, the integrity of the beads was lost upon 20 min and the remaining anthocyanins were released [43].

The basic external gelation procedure can also be modified by introducing a coating step of alginate-WPI beads with other polymers [44]. Initially, a solution of alginate (molecular mass: 80–120 kDa) and WPI (soy protein, casein, bovine serum albumin, or hemp proteins were also used as an alternative to WPI) was blended in a green tea (*Camellia sinensis*) water extract and then the mixture was dripped into a $CaCl_2$ solution containing the same green tea extract (Figure 1, Table 1). The composition of the green tea extract was not specified, while the ratio between flavan-3-ol monomers and polymers is relevant for the encapsulation efficiency in hydrogel, since the monomers are less retained in the matrix [45]. The hydrogel beads obtained were studied as such or coated with either a chitosan or a pectin solution prepared in $CaCl_2$ and green tea extract at pH 2.65. The median diameter d(0.5) was in the range 0.573–1.124 mm, with the highest sizes for the pectin- and chitosan-coated beads. WPI addition to alginate increased the encapsulation efficiency for flavanols from 48 to 60%, and coating with pectin (but not with chitosan) further increased the encapsulation efficiency to 83%. As expected, the changes in the retained antioxidant activity followed the same pattern as that of flavan-3-ols. For the plain alginate-protein beads, the release of flavan 3-ols during in vitro digestion was complete and very rapid (10 min) in SGF, regardless of the type of proteins. However, coating wet beads pectin (but not with chitosan) enabled to prolong the release of flavan-3-ols in SGF, even if after 2 h of incubation in the SGF 50% of flavan-3-ols were released. A complete release of these compounds occurred in the SIF [44].

3.2.2. Encapsulation in Microparticles

The internal gelation technique was used to produce microparticles of alginate and WPI [46]. In contrast to particles formed via external gelation, the gel structure produced by internal gelation is more homogenous [47]. Internal gelation was applied to encapsulate the polyphenol extract of dandelion in alginate-WPI (Figure 2, Table 1), in comparison with the external gelation procedure applied using only alginate [48]. A water phase was prepared using alginate, WPI, and dandelion extract, mainly composed of hydroxycinnamic acids as indicated above. Then, $CaCO_3$ was dispersed in the water phase. Sunflower oil containing Tween-80 as a surfactant and β-carotene was emulsified with the water phase. To promote internal gelation, sunflower oil containing glacial acetic acid was then added. Subsequently, the oil was removed by centrifugation and the microparticles were washed with ethanol. The microparticles obtained had average particle size of 0.3 mm, which was lower than that observed with the external gelation procedure (i.e., 2 mm).

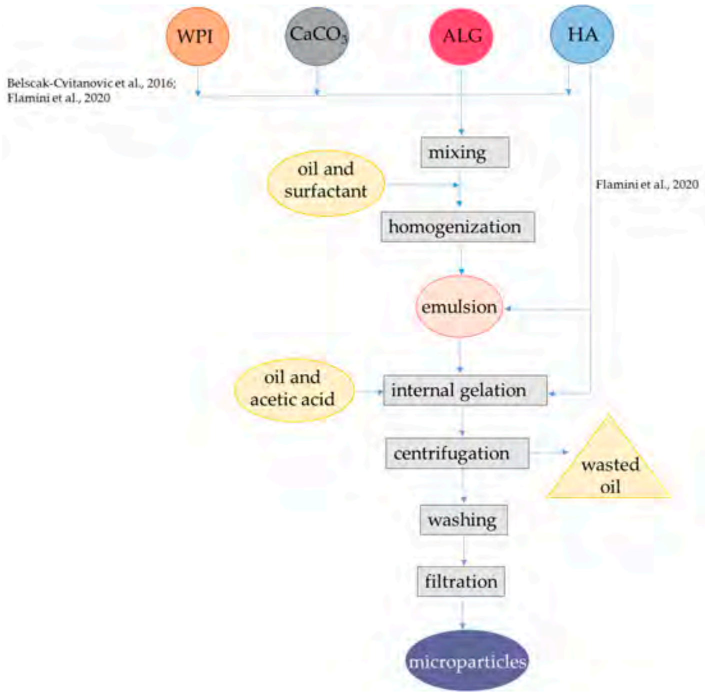


Figure 2. Steps to encapsulate hydrophilic antioxidants into alginate-whey protein microparticles. The procedures were applied to hydroxycinnamic acids from dandelion [48], oleuropein, hydroxytyrosol, and other phenolic compounds from olive oil leaf [49]. ALG, alginate; HA, hydrophilic antioxidants; WPI, whey protein isolate. The concentrations of polymers and antioxidants are indicated in Table 1.

Encapsulation efficiency for total hydroxycinnamic acids was about 80%, which was higher than that obtained by the external gelation procedure with alginate alone (i.e., 60%). Lower radical scavenging capacity as measured with the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was observed for internal/emulsion produced microparticles in comparison to plain alginate obtained by external/hydrophilic encapsulation, which might be due to the interaction between hydroxycinnamic acids and WPI. Indeed, hydrolysis of the microparticles and subsequent HPLC analysis coupled with FT-IR monitoring revealed that specific interactions occurred between hydroxycinnamic acids and the carrier polymers, especially in presence of WPI. Interestingly, WPI enabled β-carotene retention in the system, but the retention percentage was not reported. The high affinity of β-lactoglobulin for carotenoids, especially β-carotene had already been confirmed [50,51]. Results from the in vitro digestion showed that the release of hydroxycinnamic acids in SGF was lower than the release observed in SIF, while in the absence of WPI, the release of these compounds was not delayed [48]. The interactions with the carrier polymers can explain the slow release in SGF of encapsulated hydroxycinnamic acids. However, no information was provided on β-carotene release. The same procedure was also applied using different binary mixtures of polymers as the carrier materials for encapsulation, namely, hydroxypropyl methylcellulose in combination with alginate or pectin with either WPI or hydroxypropyl methylcellulose. Interestingly, the use of WPI in combination with alginate was the optimal carrier system for maximizing the encapsulation efficiencies of total polyphenols and hydroxycinnamic acids [48]. In one similar encapsulation procedure WPI, casein and pectin were compared as co-polymers of alginate to be used to encapsulate via internal gelation the phenolic-rich olive (*Olea europaea sativa*) leaf extract, which mainly consists of oleuropein and hydroxytyrosol [49]. In this latter study, Span 80 was used instead of Tween-80 and β-carotene was not added to the oil. In order to enhance the encapsulation efficiency, the same amount of extract used in the encapsulant solution was added in both the inversion and recovery media solutions. The mean particle size (D4,3) of the microparticles was in the range 48.2–65.1 µm. The low dimension of the microparticles could be related to the presence of the phenolic-rich olive leaves extract that is known to be surface active compounds [52–54]. The use of alginate in combination with WPI and casein improved the encapsulation efficiency from 20% up to 60%, while pectin increased encapsulation efficiency to 80%. This improvement was attributed to the development of interactions between alginate and WPI as well as between WPI and olive leaf phenolics. Interestingly, the alginate-WPI microparticles showed an enhanced antioxidant activity by the ABTS radical cation with respect to that expected based on their phenolic content, despite the occurrence of protein-polyphenols interactions, as confirmed by FT-IR spectra. Regarding the release of bioactive compounds, the microcapsules with WPI and casein showed higher swelling and release rates at pH 6.0 compared to pH 4.5, while those with pectin showed a fast release both at pH 4.5 and at pH 6.0 [49].

3.3. Encapsulation of Lipophilic Antioxidants in Alginate-Whey Protein/β-Lactoglobulin Hydrogels

3.3.1. Encapsulation in Beads

The encapsulation of lipophilic antioxidants in alginate-protein beads can be achieved using a carrier oil phase, which along with proteins acting as emulsifiers—forms small oil droplets that can be dispersed in the alginate hydrogel. When β-lactoglobulin is used as an emulsifier to stabilize oil droplets, coating of the droplets with alginate can be obtained from pH 3.0 to pH 6.0 at low ionic strengths, i.e., 100 mM NaCl. The alginate-coated WPI-oil droplets have better stability to flocculation. At pH 6 and 7, coating does not occur because of the strong electrostatic repulsion between the anionic alginate and anionic protein on the droplet surface [55,56]. However, in the presence of Ca^{2+}, coating of β-lactoglobulin-stabilized emulsion can occur in a wide pH range, also above pH 6.0 where both polymers are negatively charged [57,58]. Following this latter approach, in one study, a primary emulsion was formed with sunflower oil containing α-tocopherol, resveratrol dissolved in ethanol, and denatured WPI at pH 7.0. Then, the emulsion was mixed with alginate at pH 7.0 and dripped into $CaCl_2$ for gelation (Figure 3, Table 1). The oil droplet size ranged from 80 to 360 nm depending on the oil content (varying from 0.1% to 2%) and the WPI content (from 0.4 to 2%), while the gel beads carrying the oil droplets had diameters between 1.977 and 2.152 mm. The bioactive compounds showed different locations in the beads. In fact, α-tocopherol was dissolved in the oil phase while amphiphilic resveratrol was bound to WPI at the oil-water interface. Indeed, the interaction between resveratrol and β-lactoglobulin had already been proven [59,60]. The recovery of resveratrol in all WPI emulsions was >96% while recovery of α-tocopherol was about 85% when the contents of sunflower oil were 0.5% and 1% and >90% at higher oil contents. WPI content at 1% was effective in the protection of resveratrol during storage, with 60% remaining after 60 d at 25 °C. The stability of α-tocopherol was also improved, and its content was about 25% after 60 d [58]. The content of released resveratrol was about 91% and 97% after gastric digestion for 0.5 and 2 h, respectively. The release of α-tocopherol from all emulsion beads was basically close to or <10% after gastric digestion for 2 h and about 20% or less after gastrointestinal digestion for 6 h [58]. To encapsulate an oil phase containing lycopene extracted from tomato (*Lycopersicum eculentum*), alginate and WPI were used as carriers and gelation was performed at pH 7.0, in presence of Ca^{2+} as described above [14]. On the other hand, the amount of oil used to encapsulate lycopene emulsion was 10-fold higher than that used to encapsulate α-tocopherol emulsion (Figure 2, Table 1), which could be the reason for the higher bioaccessibility found for lycopene than for α-tocopherol, while the encapsulation efficiency for lycopene was not reported. The Authors found that the sizes of oil droplets delivering lycopene in gel beads (average size 3 mm) were significantly larger than the pores of hydrogel matrix, which are between 5 and 200 nm [61]. Therefore, lycopene and oil droplets were released from the gel beads due to structural degradation after swelling during the intestinal phase. Interestingly, the presence of WPI delayed the release of lycopene in the intestinal phase of digestion, probably due to a slower swelling rate of the beads. Lycopene bioaccessibility was found to be nearly 80% [14].

3.3.2. Encapsulation in Microcapsules

Alginate-β-lactoglobulin microcapsules were obtained by using transglutaminase (Figure 3, Table 1). According to this procedure, β-lactoglobulin-alginate hydrogel with particle size in the range of 5 μm were produced at pH 4.5 using diluted polymer solution and transglutaminase as a crosslinking agent in place of Ca^{2+} to encapsulate black pepper (*Piper nigrum*) essential oil [62]. The black pepper essential oil contains different terpenes, among which the prevalent are β-caryophyllene, limonene, sabinene, β-pinene, and α-pinene. For encapsulation, a primary emulsion was formed with black pepper essential oil and β-lactoglobulin, then alginate was added, and the pH was adjusted to 4.5. Transglutaminase solution was added to induce crosslinking. Encapsulation efficiency of 80% was observed when β-lactoglobulin:alginate ratio was 17:1 and core:wall ratio was 2:1. FT-IR analysis revealed that the black pepper essential oil modified the tertiary structure of β-lactoglobulin, resulting from rearrangement of hydrophobic interactions, hydrogen, and ionic bonds. The microcapsule released about 30% of the encapsulated compounds under oral conditions while no release was observed under gastric conditions and up to 100% of the encapsulated compounds were released in the intestine, contributing 31% bioaccessibility for the essential oil [62]. Similar results were observed using lactoferrin in place of β-lactoglobulin [63].

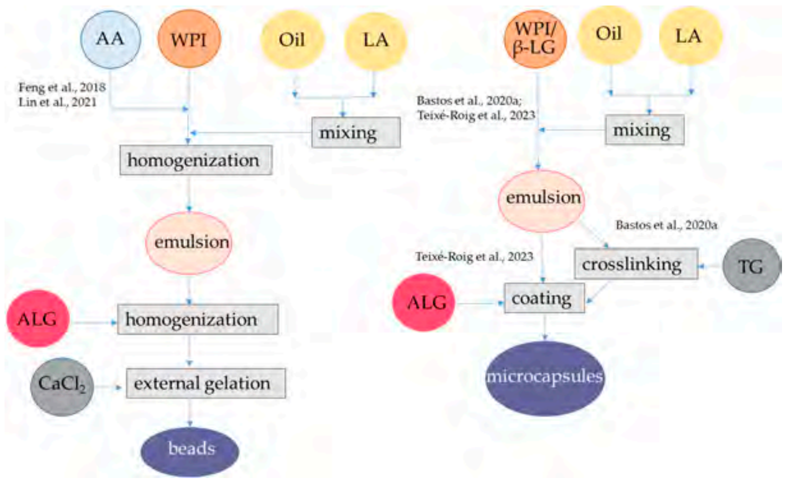


Figure 3. Steps to encapsulate lipophilic antioxidants into alginate-whey protein beads and microcapsules. The procedures were applied to α-tocopherol and resveratrol [58], lycopene [14], black pepper essential oil [62], and curcumin [64]. AA, amphiphilic antioxidants; ALG, alginate; β-LG, β-lactoglobulin; LA, lipophilic antioxidants; TG, transglutaminase; WPI, whey protein isolate. The concentrations of polymers and antioxidants are indicated in Table 1.

Further studies evidenced that in the presence of 0.5% alginate, the gastric digestion of crosslinked WPI simulated in vitro is effectively reduced from 29% to 8%. The protection by alginate towards protein digestion was attributed to the charge interactions between polymers. Notably, digestion was also slowed in the intestinal phase even though the alginate protein particles were dissociating due to the pH of 7.0 of the SIF [65]. In another approach aimed at obtaining microcapsules to encapsulate curcumin, a carrier oil was used to dissolve curcumin and then added with WPI and alginate [64]. The particle size ranged between 0.487 and 2.251 μm, depending on alginate concentration. The addition of alginate did not affect the encapsulation efficiency, which was about 95%. When adding alginate, ζ-potential values of the microcapsules became more negative, and this is probably the reason for higher physical stability. On the other hand, with increasing alginate concentration from 0 to 1.5%, the bioaccessibility of curcumin decreased from 70% to 30% [64].

3.3.3. Encapsulation in Nanoparticles

A nanoencapsulation strategy based on binary all material combination WPI-alginate was proposed using a carrier oil for lipophilic antioxidants. A primary emulsion was obtained with olive oil containing curcumin and WPI, at pH 7.0 by ultrasonication. Then, the pH of the primary emulsion was adjusted to 5, added with alginate, and sonicated further. Hence, the alginate layer was formed by electrostatic interaction with WPI, without Ca^{2+} addition. The average size of the primary emulsion droplets containing curcumin was 359 nm, whereas in the case of the secondary emulsion, it was increased to 841 nm. The encapsulation efficiency was 100%. In vitro digestion showed that in the gastric conditions, the emulsions remained stable, thereby not allowing the curcumin to be released from the emulsified droplets. Maximum releases of curcumin of almost 63 and 71% were attained after 2 h for the primary and secondary emulsion, respectively, and thereafter, it remained constant [66].

Alternatively, nanocapsules of β-lactoglobulin/WPI and alginate were also designed to encapsulate lipophilic antioxidants without the need of an oil phase, based on the affinity of β-lactoglobulin for small hydrophobic compounds. In one approach, β-lactoglobulin was added with caffeine and heated at 80 °C for 30 min, in order to allow heat-induced gelation. Then, the nanocomplexes obtained were coated with alginate in the presence of $CaCl_2$. Additional layers of alginate coating were applied by repeating the procedure of alginate and $CaCl_2$ addition up to four times. Encapsulation efficiency was not reported. This study demonstrated that the swelling and release behavior of the whey protein hydrogels can be changed easily with different layers of alginate coating. However, the release behavior under simulated digestion conditions was not investigated [20]. One point to notice is that bioactive compounds entrapped in integer nanoparticles may have decreased bioactivity. In fact, nanocomplexes of alginate, WPI, and thyme (*Thymus vulgaris*) oil with particle size of approximately 200 nm had decreased antimicrobial capacity, then free thyme oil [67,68]. Moreover, the heat-induced gelation procedure for encapsulation in WPI and alginate is only suitable for heat-stable bioactive compounds. In a further approach, these limitations were solved since β-lactoglobulin was first preheated at 80 °C for 30 min, cooled, and then was mixed with α-tocopherol as bioactive compound at 2:1 ratio. Then, the nanocomplexes formed were coated with alginate and $CaCl_2$ (Figure 4, Table 1). Encapsulation efficiency was approximatively 20%. The positive effects of β-lactoglobulin and alginate interplay was observed in the release behavior under simulated digestion conditions, since evidence was provided that alginate-coated protein particles prolong the release of α-tocopherol till SIF conditions. In fact, the α-tocopherol retained in SGF was 55% and complete release occurred in SIF [69].

Self-assembly between alginate and whey protein (with no Ca^{2+} addition) can also be applied to create carrier nanostructures. A spontaneous association of oppositely charged alginate and β-lactoglobulin can occur at low concentrations (<4.5%); this phenomenon is also called complex coacervation [70]. This process was applied to encapsulate either quercetin or curcumin. These bioactive compounds were mixed with β-lactoglobulin at 1:1 ratio, which resulted in the formation of a nanocomplex. Then, alginate (molecular mass 200 kDa with M:G ratio of 0.6) was added at pH 4.0 in order to coat the nanocomplex

(Figure 4, Table 1), obtaining an average particle size between 143 and 167 nm, depending on alginate concentration. The efficiency of encapsulation was above 90%. As observed previously [69], the release profile of the bioactive compounds from the nanoparticles under simulated digestion was improved due to the presence of alginate, since <3.5% release occurred during 6 h in SIF, while 77% release was observed during 12 h in SIF [71,72]. The physical stability of nanoparticles was investigated upon storage at pH 4.0 for 30 d at 25 °C, and upon HTST treatment at 75 °C for 30 s. Interesting, the β-lactoglobulin complexes were poorly stable upon both storage at pH 4.0 and heating, due to protein aggregation. Conversely, the physical stability of the nanoparticles made with both β-lactoglobulin and alginate was high, which was attributed to the anionic alginate shell that inhibited aggregation. Similarly, alginate shell provided a better chemical stability of quercetin and curcumin during storage at 45 °C for 18 d with respect to β-lactoglobulin alone, with about 50% of retention of both quercetin and curcumin [71,72]. In a further study, self-assembly of alginate and WPI was found to occur also at pH 5.0. In fact, the isoelectric point of WPI is 5.0 and hence the net charge is zero; there can still be some functional groups, including lysine residues, that are positively charged and can electrostatically interact with alginate. When pH was decreased to 4.5 or lower, strong electrostatic interactions occurred between WPI and alginate, leading to the formation of large insoluble aggregates. Conversely, above pH 5.5, both WI and alginate were negatively charged, and no electrostatic absorption occurred. Hence, at pH 5.0, alginate–WPI nanoparticles had an average size of 268 nm and were able to absorb curcumin added in ethanol. It was supposed that hydrophobic interactions were the main driving forces to promote curcumin binding with WPI, with encapsulation efficiency up to 85%. The WPI–alginate nanocomplex proved to be physically stable in high sucrose and NaCl concentration, and also at 90 °C up to 120 min [73].

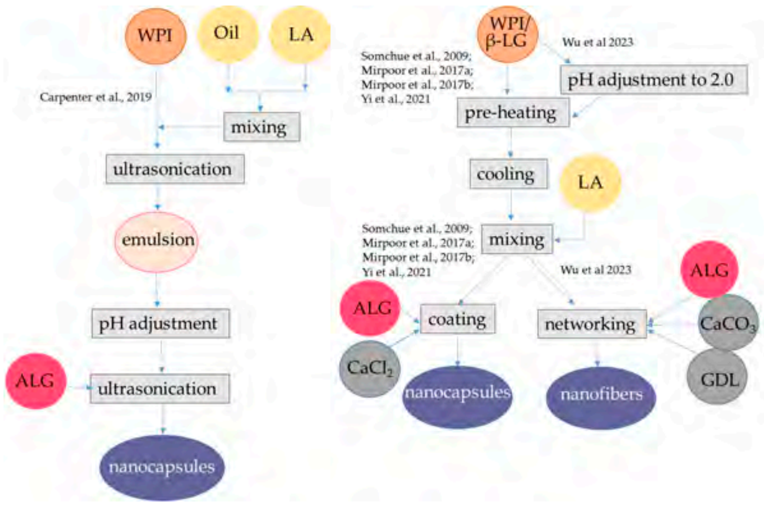


Figure 4. Steps to encapsulate lipophilic antioxidants into alginate-whey protein nanocapsules and nanofibers. The procedures were applied to α-tocopherol and resveratrol [69], quercetin [71], and curcumin [14,66,72,73]. ALG, alginate; β-LG, β-lactoglobulin; GDL, glucono δ-lactone; WPI, whey protein isolate. The concentrations of polymers and antioxidants are indicated in Table 1.

In a different approach, WPI-alginate nanofibers were produced to encapsulate curcumin [74]. To obtain nanofibers, WPI was denatured by heating to 80 °C, at pH 2.0. Under these conditions, the native spherical protein was converted into a partially folded intermediate form containing β-sheets. Then, oligomers formed along the direction perpendicular to the β-sheets, forming protein nanofibers [75,76]. After formation of WPI nanofibers, the pH was adjusted to 6.5 and curcumin dissolved in ethanol was added. To form the binary microfibers, alginate, gluconate δ-lactone, and $CaCO_3$ were finally added.

Table 1. Formulation of alginate-WPI/β-lactoglobulin hydrogels to encapsulate hydrophilic and lipophilic antioxidants *.

Structure-Wall Materials	Crosslinker	Core	Reference
Beads ALG 2.4%; WPI 4%	$CaCl_2$ 2 or 3%	hydroxycinnamic acids extract from dandelion in water 1.2%	[39]
Beads: ALG 1.5%; β-LG 1%	$CaCl_2$ 4%	phenolics from blood fruit extract in 80% aqueous ethanol 10%	[41]
Beads: ALG 0.5%; WPI 0.5%	$CaCl_2$ 22%	anthocyanin extract from black rice in ethanol 0.25%	[42]
Beads: ALG 2%; WPC 1%	$CaCl_2$ 2%	anthocyanin extract from jussara in water (n.p.)	[43]
Beads: ALG 1.28–1.6%; WPI 2%	$CaCl_2$ 2%	flavanol extract from green tea in water (n.p.)	[44]
Microparticles: ALG 1.6%; WPI 2%	$CaCO_3$ 0.5% acetic acid 5% in SO	hydroxycinnamic acids extract from dandelion in water (n.p.)	[48]
Microparticles: ALG 2%; WPI 2%	Ca-citrate 2% acetic acid 1.25% in SO	phenolics from olive oil leaf extracted in water 0.5%	[49]
Beads: ALG 1.4%; WPI 0.1–1%	$CaCl_2$ 22%	SO 0.1–2%, α-tocopherol 1% in SO resveratrol in ethanol (n.p)	[58]
Beads: ALG 0.4%; WPI 2%	$CaCl_2$ 2%	SO% 10% lycopene from tomato 0.015% in SO	[14]
Microcapsules: ALG + β-LG 0.45–1.8%	TG 0.25%	terpens of black pepper essential oil 0.45–1.8%	[62]
Microcapsules: ALG 0–1.5%; WPI 0–1.5%	-	CO 5% curcumin 0.1% in CO	[64]
Nanocapsules: ALG 0.2%; WPI 0.44%	-	OO 4.9% curcumin 0.022% in OO	[66]
Nanocapsules: ALG n.p; β-LG 0.5–2%	$CaCl_2$ 1.1–11%	α-tocopherol 0.4–7%	[69]
Nanocapsules: ALG 0.05 or 0.019%; β-LG 0.025%	-	curcumin 0.0005%	[71]
Nanocapsules: ALG 0.05 or 0.019%; β-LG 0.025%	-	quercetin 0.0004%	[72]
Nanocapsules: ALG 0.16–0.05%; WPI 0.83–0.5%	-	curcumin 0.01%	[73]
Nanofibers ALG 1%; WPI 1–6%	$CaCO_3$ 0.1% GDL 28.14 mM	curcumin 0.125%	[74]

* ALG, alginate; β-LG, β-lactoglobulin; CO, corn oil; n.p., concentration not provided; GDL, glucono δ-lactone; OO, olive oil; SO, sunflower oil; TG, transglutaminase; WPI, whey protein isolate; WPC, whey protein concentrate.

At this step, the hydrolysis of gluconate δ-lactone causes a slow release of Ca^{2+} from $CaCO_3$, which then promotes the formation of a double network hydrogel between alginate and WPI [74]. The encapsulation efficiency was 91.6%. However, the release during in vitro digestion was not studied.

4. Conclusions

Interactions between alginate and WPI/β-lactoglobulin have been proven, which can be modulated by M:G ratio, protein denaturation, pH, and crosslinking with Ca^{2+} or transglutaminase. Interestingly, binary hydrogels can be formed both in a pH range where electrostatic forces occur due to opposite charge of these polymers and in a pH range where the two polymers are negatively charged, favored by the presence of Ca^{2+} ions.

Hydrophilic antioxidants can be encapsulated in alginate and WPI/β-lactoglobulin beads or microcapsules. In general, for hydrophilic antioxidants, the binary hydrogels improve the encapsulation efficiency with respect to the alginate hydrogel alone, probably due to the interactions between WPI/β-lactoglobulin and alginate, which allow the formation of a compact network. A delayed release in SIF from the beads and microparticles was observed for some hydrophilic antioxidant compounds.

Lipophilic antioxidants can be dissolved into an oil phase, emulsified by WPI/β-lactoglobulin and then coated with alginate in the presence of Ca^{2+} or transglutaminase in the form of beads or microcapsules. This approach leads to high encapsulation efficiency, but the release in SGF and SIF is low unless a high amount of oil (10%) is present. Alternatively, lipophilic compounds can be directly loaded into β-lactoglobulin followed by coating with alginate, without an oily phase, which leads to nanocapsules with high encapsulation efficiency and high release in SIF.

One point to notice is that in most of the studies so far performed, no information is reported regarding relevant factors such as alginate molecular mass, M:G ratio, and pH of gelation, which would be useful to correlate with the observed encapsulation efficiency and release properties. Additionally, for both hydrophilic and lipophilic bioactive compounds, interactions with the carrier polymers can cause a decreased antioxidant activity.

In this context, the main challenge for the future studies is to extend knowledge on the interactions among three components, namely alginate, WPI, and the encapsulated bioactive compound. This will represent the rationale basis for the development of structures that can be tailored to specific food applications. Moreover, knowledge on the physical and chemical stability of the structures under different conditions relevant to food processing and storage would help a better design of applications.

Author Contributions: Conceptualization, V.L. and D.P.; methodology, V.L. and D.P.; investigation, data curation, writing, review and editing, D.P., A.S., A.G. and V.L.; supervision, A.G. and V.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Aguiar, J.; Estevinho, B.N.; Santos, L. Microencapsulation of natural antioxidants for food application—The specific case of coffee antioxidants—A review. *Trends Food Sci. Technol.* **2016**, *58*, 21–39. [CrossRef]
2. Bennacef, C.; Desobry-Banon, S.; Probst, L.; Desobry, S. Advances on alginate use for spherification to encapsulate biomolecules. *Food Hydrocoll.* **2021**, *118*, 106782. [CrossRef]
3. Lavelli, V.; Sereikaite, J. Kinetic study of encapsulated β-carotene degradation in aqueous environments: A review. *Foods* **2022**, *11*, 317. [CrossRef] [PubMed]
4. Ling, J.K.U.; Jeevanandam, J.H.S.; Chan, J.Y.S.; Nandong, J. Thermal degradation of antioxidant compounds: Effects of parameters, thermal degradation kinetics, and formulation strategies. *Food Bioproc. Tech.* **2022**, *15*, 1919–1935. [CrossRef]
5. Vargas-Ramella, M.; Pateiro, M.; Gavahian, M.; Franco, D.; Zhang, W.; Khaneghah, A.M.; Guerrero-Sanchez, Y.; Lorenzo, J.M. Impact of pulsed light processing technology on phenolic compounds of fruits and vegetables. *Trends Food Sci. Technol.* **2021**, *115*, 1–11. [CrossRef]
6. Corey, M.E.; Kerr, W.L.; Mulligan, J.H.; Lavelli, V. Phytochemical stability in dried apple and green tea functional products as related to moisture properties. *LWT Food Sci. Technol.* **2011**, *44*, 67–74. [CrossRef]

7. Mota, J.C.; Almeida, P.P.; Freitas, M.Q.; Stockler-Pinto, M.B.; Guimarães, J.T. Far from being a simple question: The complexity between in vitro and in vivo responses from nutrients and bioactive compounds with antioxidant potential. *Food Chem.* **2023**, *402*, 134351. [CrossRef]
8. McClements, D.J.; Peng, S.-F. Current status in our understanding of physicochemical basis of bioaccessibility. *Curr. Opin. Food Sci.* **2020**, *31*, 57–62. [CrossRef]
9. Pénicaud, C.; Guilbert, S.; Peyron, S.; Gontard, N.; Guillard, V. Oxygen transfer in foods using oxygen luminescence sensors: Influence of oxygen partial pressure and food nature and composition. *Food Chem.* **2010**, *123*, 1275–1281. [CrossRef]
10. Ramdhan, T.; Ching, S.H.; Prakash, S.; Bhandari, B. Physical and mechanical properties of alginate based composite gels. *Trends Food Sci. Technol.* **2020**, *106*, 150–159. [CrossRef]
11. Hosseini, S.M.H.; Emam-Djomeh, Z.; Razavi, S.H.; Moosavi-Movahedi, A.A.; Saboury, A.A.; Atri, M.S.; Van der Meeren, P. β-Lactoglobulin sodium alginate interaction as affected by polysaccharide depolymerization using high intensity ultrasound. *Food Hydrocoll.* **2013**, *32*, 235–244. [CrossRef]
12. Hu, C.; Lu, W.; Mata, A.; Nishinari, K.; Fang, Y. Ions-induced gelation of alginate: Mechanisms and applications. *Int. J. Biol. Macromol.* **2021**, *177*, 578–588. [CrossRef]
13. Zazzalia, I.; Calvo, T.R.A.; Ruíz-Henestros, V.M.P.; Santagapita, P.R.; Perullini, M. Effects of pH, extrusion tip size and storage protocol on the structural properties of Ca(II)-alginate beads. *Carbohydr. Polym.* **2019**, *206*, 749–756. [CrossRef] [PubMed]
14. Lin, D.; Kelly, A.L.; Maidannyk, V.; Miao, S. Effect of structuring emulsion gels by whey or soy protein isolate on the structure, mechanical properties, and in vitro digestion of alginate-based emulsion gel beads. *Food Hydrocoll.* **2021**, *110*, 106165. [CrossRef]
15. Zheng, H.; Zhou, Z.; Chen, Y.; Huang, J.; Xiong, F. pH-Sensitive alginate/soy protein microspheres as drug transporter. *J. Appl. Polym. Sci.* **2007**, *106*, 1034–1041. [CrossRef]
16. Lee, K.Y.; Mooney, D.J. Alginate: Properties and biomedical applications. *Prog. Polym. Sci.* **2012**, *37*, 106–126. [CrossRef] [PubMed]
17. Lavelli, V.; Beccalli, M.P. Cheese whey recycling in the perspective of the circular economy: Modeling processes and the supply chain to design the involvement of the small and medium enterprises. *Trends Food Sci. Technol.* **2022**, *126*, 86–98. [CrossRef]
18. Hosseini, S.M.H.; Emam-Djomeh, Z.; Sabatino, P.; Van der Meeren, P. Nanocomplexes arising from protein-polysaccharide electrostatic interaction as a promising carrier for nutraceutical compound. *Food Hydrocoll.* **2015**, *50*, 16–26. [CrossRef]
19. Morr, C.V.; Ha, E.W.Y. Whey protein concentrates and isolates: Processing and functional properties. *Crit. Rev. Food Sci. Nutr.* **1993**, *33*, 431–476. [CrossRef]
20. Gunasekaran, S.; Ko, S.; Xiao, L. Use of whey proteins for encapsulation and controlled delivery applications. *J. Food Eng.* **2007**, *83*, 31–40. [CrossRef]
21. Lefevre, T.; Subirade, M. Molecular differences in the formation and structure of fine-stranded and particulate β-lactoglobulin gels. *Biopolymers* **2000**, *54*, 578–586. [CrossRef] [PubMed]
22. Barbut, S.; Foegeding, E.A. Ca^{2+}-induced gelation of pre-heated whey protein isolate. *J. Food Sci.* **1993**, *58*, 867–871. [CrossRef]
23. Sok Line, V.L.; Remondetto, G.E.; Subirade, M. Cold gelation of β-lactoglobulin oil-in-water emulsions. *Food Hydrocoll.* **2005**, *19*, 269–278. [CrossRef]
24. Zhang, Z.; Zhang, R.; Zou, L.; McClements, D.J. Protein encapsulation in alginate hydrogel beads: Effect of pH on microgel stability, protein retention and protein release. *Food Hydrocoll.* **2016**, *58*, 308–315. [CrossRef]
25. Harnsilawata, T.; Pongsawatmanita, R.; McClements, D.J. Characterization of β-lactoglobulin–sodium alginate interactions in aqueous solutions: A calorimetry, light scattering, electrophoretic mobility and solubility study. *Food Hydrocoll.* **2006**, *20*, 577–585. [CrossRef]
26. Gorji, E.G.; Waheed, A.; Ludwig, R.; Toca-Herrera, J.L.; Schleining, G.; Gorji, S.G. Complex coacervation of milk proteins with sodium alginate. *J. Agric. Food Chem.* **2018**, *66*, 3210–3220. [CrossRef]
27. Stender, E.G.P.; Birch, J.; Kjeldsen, C.; Nielsen, L.D.; Duus, J.O.; Kragelund, B.B.; Svensson, B. Alginate trisaccharide binding sites on the surface of β-lactoglobulin identified by NMR spectroscopy: Implications for molecular network formation. *ACS Omega* **2019**, *4*, 6165–6174. [CrossRef]
28. Madsen, M.; Westh, P.; Khan, S.; Ipsen, R.; Almdal, K.; Aachmann, F.L.; Svensson, B. Impact of alginate mannuronic-guluronic acid contents and pH on protein binding capacity and complex size. *Biomacromolecules* **2021**, *22*, 649–660. [CrossRef]
29. Stender, E.G.P.; Khan, S.; Ipsen, R.; Madsen, F.; Hagglund, P.; Hachem, M.A.; Almdal, K.; Westh, P.; Svensson, B. Effect of alginate size, mannuronic/guluronic acid content and pH on particle size, thermodynamics and composition of complexes with β-lactoglobulin. *Food Hydrocoll.* **2018**, *75*, 157–163. [CrossRef]
30. Liu, X.; Qin, X.; Wang, Y.; Zhong, J. Physicochemical properties and formation mechanism of whey protein isolate-sodium alginate complexes: Experimental and computational study. *Food Hydrocoll.* **2022**, *131*, 107786. [CrossRef]
31. Baez, G.D.; Llopart, E.E.; Berino, R.P.; Moro, A.; Roxana, A.; Verdini, P.A.; Busti, N.; Delorenzi, J. Characterisation of β-lactoglobulin/sodium alginate dry films. *J. Food Sci. Technol.* **2022**, *57*, 242–248. [CrossRef]
32. Madsen, M.; Khan, S.; Kunstmann, S.; Aachmann, F.L.; Ipsen, R.; Westh, P.; Emanuelsson, C.; Svensson, B. Unaided efficient transglutaminase cross-linking of whey proteins strongly impacts the formation and structure of protein alginate particles. *Food Chem.* **2022**, *5*, 100137. [CrossRef] [PubMed]
33. Chakravartula, S.A.N.; Soccio, M.; Lotti, N.; Balestra, F.; Dalla Rosa, M.; Siracusa, V. Characterization of composite edible films based on pectin/alginate/whey protein concentrate. *Materials* **2019**, *12*, 2454. [CrossRef]

34. Leong, J.-Y.; Lam, W.-H.; Ho, K.-W.; Voo, W.-P.; Lee, M.F.-X.; Lim, H.-P.; Lim, S.-L.; Tey, B.-T.; Poncelet, D.; Chan, E.-S. Advances in fabricating spherical alginate hydrogels with controlled particle designs by ionotropic gelation as encapsulation systems. *Particuology* **2016**, *24*, 44–60. [CrossRef]
35. Joye, I.J.; McClements, D.J. Biopolymer-based nanoparticles and microparticles: Fabrication, characterization, and application. *Curr. Opin. Colloid Interface Sci.* **2014**, *19*, 417–427. [CrossRef]
36. Hébrard, G.; Hoffart, V.; Cardot, J.-M.; Subirade, M.; Alric, M.; Beyssac, E. Investigation of coated whey protein/alginate beads as sustained release dosage form in simulated gastrointestinal environment. *Drug Dev. Ind. Pharm.* **2009**, *35*, 1103–1112. [CrossRef]
37. Ching, S.H.; Bhandari, B.; Webb, R.; Bansal, N. Visualizing the interaction between sodium caseinate and calcium alginate microgel particles. *Food Hydrocoll.* **2015**, *43*, 165–171. [CrossRef]
38. Wichchukit, S.; Oztop, M.H.; McCarthy, M.J.; McCarthy, K.L. Whey protein/alginate beads as carriers of a bioactive component. *Food Hydrocoll.* **2013**, *33*, 66–73. [CrossRef]
39. Bušić, A.; Belščak-Cvitanović, A.; Vojvodić Cebin, A.; Karlović, S.; Kovač, V.; Špoljarić, I.; Mršić, G.; Komes, D. Structuring new alginate network aimed for delivery of dandelion (*Taraxacum officinale* L.) polyphenols using ionic gelation and new filler materials. *Food Res. Int.* **2018**, *111*, 244–255. [CrossRef]
40. Lavelli, V.; Sri Harsha, P.S.C. Microencapsulation of grape skin phenolics for pH controlled release of antiglycation agents. *Food Res. Int.* **2019**, *119*, 822–828. [CrossRef]
41. Sasikumar, R.; Sharma, P.; Jaiswal, A.K. Alginate and β-lactoglobulin matrix as wall materials for encapsulation of polyphenols to improve efficiency and stability. *Int. J. Food Eng.* **2023**, *19*, 1–13. [CrossRef]
42. Norkaew, O.; Thitisut, P.; Mahatheeranont, S.; Pawin, B.; Sookwong, P.; Yodpitak, S.; Lungkaphind, A. Effect of wall materials on some physicochemical properties and release characteristics of encapsulated black rice anthocyanin microcapsules. *Food Chem.* **2019**, *294*, 493–502. [CrossRef]
43. da Silva Carvalho, A.G.; Teixeira da CostaMachado, M.; de Freitas, H.D.; Barros, Q.; Cazarin, C.B.B.; Maróstica, M.R.J.; Dupas Hubinger, M. Anthocyanins from jussara (*Euterpe edulis* Martius) extract carried by calcium alginate beads pre-prepared using ionic gelation. *Powder Technol.* **2019**, *345*, 283–291. [CrossRef]
44. Belscak-Cvitanovic, A.; Dordevic, V.; Karlovic, S.; Pavlovic, V.; Komes, D.; Jezek, D.; Bugarski, B.; Nedovic, V. Protein-reinforced and chitosan-pectin coated alginate microparticles for delivery of flavan-3-ol antioxidants and caffeine from green tea extract. *Food Hydrocoll.* **2015**, *51*, 361–374. [CrossRef]
45. Pedrali, D.; Barbarito, S.; Lavelli, V. Encapsulation of grape seed phenolics from winemaking byproducts in hydrogel microbeads—Impact of food matrix and processing on the inhibitory activity towards α-glucosidase. *LWT Food Sci. Technol.* **2020**, *133*, 109952. [CrossRef]
46. Chen, L.; Subirade, M. Effect of preparation conditions on the nutrient release properties of alginate-whey protein granular microspheres. *Eur. J. Pharm. Biopharm.* **2007**, *65*, 354–362. [CrossRef] [PubMed]
47. Poncelet, D.; De Smet, B.P.; Beaulieu, C.; Huguet, M.L.; Fournier, A.; Neufeld, R.J. Production of alginate beads by emulsification/internal gelation. II. Physicochemistry. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 644–650. [CrossRef]
48. Belscak-Cvitanovic, A.; Bušić, A.; Barisic, L.; Vrsaljko, D.; Karlovic, S.; Spoljari, I.; Vojvodi, A.; Mrsic, G.; Komes, D. Emulsion templated microencapsulation of dandelion (*Taraxacum officinale* L.) polyphenols and β-carotene by ionotropic gelation of alginate and pectin. *Food Hydrocoll.* **2016**, *57*, 139–152. [CrossRef]
49. Flammini, F.; Di Mattia, C.; Nardella, M.; Chiarini, M.; Valbonetti, L.; Neri, L.; Difonzo, G.; Pittia, P. Structuring alginate beads with different biopolymers for the development of functional ingredients loaded with olive leaves phenolic extract. *Food Hydrocoll.* **2020**, *108*, 105849. [CrossRef]
50. Cornacchia, L.; Roos, Y.H. Stability of β-Carotene in protein-stabilized oil-in-water delivery systems. *J. Agric. Food Chem.* **2011**, *59*, 7013–7020. [CrossRef]
51. Mensi, A.; Choiset, Y.; Rabesona, H.; Haertle, T.; Borel, P.; Chobert, J.M. Interactions of betalactoglobulin variants A and B with vitamin A. Competitive binding of retinoids and carotenoids. *J. Agric. Food Chem.* **2013**, *61*, 4114–4119. [CrossRef] [PubMed]
52. Di Mattia, C.; Balestra, F.; Sacchetti, G.; Neri, L.; Mastrocola, D.; Pittia, P. Physical and structural properties of extra-virgin olive oil based mayonnaise. *LWT Food Sci. Technol.* **2015**, *62*, 764–770. [CrossRef]
53. Flammini, F.; Di Mattia, C.; Difonzo, G.; Neri, L.; Faieta, M.; Caponio, F.; Pittia, P. From by-product to food ingredient: Evaluation of compositional and technological properties of olive-leaf phenolic extracts. *J. Sci. Food Agric.* **2019**, *99*, 6620–6627. [CrossRef] [PubMed]
54. Giacintucci, V.; Di Mattia, C.; Sacchetti, G.; Neri, L.; Pittia, P. Role of olive oil phenolics in physical properties and stability of mayonnaise-like emulsions. *Food Chem.* **2016**, *213*, 369–377. [CrossRef]
55. Pongsawatmanit, R.; Harnsilawat, T.; McClements, D.J. Influence of alginate, pH and ultrasound treatment on palm oil-in-water emulsions stabilized by β-lactoglobulin. *Colloids Surf.* **2006**, *287*, 59–67. [CrossRef]
56. Harnsilawat, T.; Pongsawatmanit, R.; McClements, D.J. Influence of pH and ionic strength on formation and stability of emulsions containing oil droplets coated by β-lactoglobulin-alginate interfaces. *Biomacromolecules* **2006**, *7*, 2052–2058. [CrossRef]
57. Fioramonti, S.A.; Martinez, M.J.; Pilosof, A.M.R.; Rubiolo, A.C.; Santiago, L.G. Multilayer emulsions as a strategy for linseed oil microencapsulation: Effect of pH and alginate concentration. *Food Hydrocoll.* **2015**, *43*, 8–17. [CrossRef]
58. Feng, W.; Yue, C.; Wusigale; Ni, Y.; Liang, L. Preparation and characterization of emulsion-filled gel beads for the encapsulation and protection of resveratrol and α-tocopherol. *Food Res. Int.* **2018**, *108*, 161–171. [CrossRef]

59. Liang, L.; Tajmirriahi, H.A.; Subirade, M. Interaction of β-lactoglobulin with resveratrol and its biological implications. *Biomacromolecules* **2008**, *9*, 50–56. [CrossRef]
60. Hemar, Y.; Gerbeaud, M.; Oliver, C.M.; Augustin, M.A. Investigation into the interaction between resveratrol and whey proteins using fluorescence spectroscopy. *Int. J. Food Sci.* **2011**, *46*, 2137–2144. [CrossRef]
61. George, M.; Abrham, T.E. Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan—A review. *J. Control. Release* **2006**, *114*, 1–14. [CrossRef]
62. Bastos, L.P.H.; de Sá Costa, B.; Siqueira, R.P.; Garcia-Rojas, E.E. Complex coacervates of β-lactoglobulin/sodium alginate for the microencapsulation of black pepper (*Piper nigrum* L.) essential oil: Simulated gastrointestinal conditions and modeling release kinetics. *Int. J. Biol. Macromol.* **2020**, *160*, 861–870. [CrossRef]
63. Bastos, L.P.H.; dos Santosb, C.H.C.; de Carvalho, M.G.; Garcia-Rojasa, E.E. Encapsulation of the black pepper (*Piper nigrum* L.) essential oil by lactoferrin-sodium alginate complex coacervates: Structural characterization and simulated gastrointestinal conditions. *Food Chem.* **2020**, *316*, 126345. [CrossRef]
64. Teixé-Roig, J.; Oms-Oliu, G.; Odriozola-Serrano, I.; Martín-Belloso, O. Enhancing the gastrointesinal stability of curcumin by using sodium alginate-based nanoemulsions containing natural emulsifiers. *Int. J. Mol. Sci.* **2023**, *24*, 498. [CrossRef] [PubMed]
65. Madsen, M.; Rønne, E.M.; Li, R.; Greco, I.; Ipsen, R.; Svensson, B. Simulated gastrointestinal digestion of protein alginate complexes: Effects of whey protein cross-linking and the composition and degradation of alginate. *Food Funct.* **2022**, *13*, 8375. [CrossRef] [PubMed]
66. Carpenter, J.; George, S.; Saharan, V.K. Curcumin encapsulation in multilayer oil-in-water emulsion: Synthesis using ultrasonication and studies on stability and antioxidant and release activities. *Langmuir* **2019**, *35*, 10866–10876. [CrossRef]
67. Volic, M.; Pecinar, I.; Micic, D.; Đorđevic, V.; Pesic, R.; Nedovic, V.; Obradovic, N. Design and characterization of whey protein nanocarriers for thyme essential oil encapsulation obtained by freeze-drying. *Food Chem.* **2022**, *386*, 132749. [CrossRef] [PubMed]
68. Volić, M.; Pajić-Lijaković, I.; Djordjević, V.; Knežević-Jugović, Z.; Pećinar, I.; Stevanović-Dajić, Z.; Veljović, D.; Hadnadjev, M.; Bugarskia, B. Alginate/soy protein system for essential oil encapsulation with intestinal delivery. *Carbohydr. Polym.* **2018**, *200*, 15–24. [CrossRef] [PubMed]
69. Somchue, W.; Sermsri, W.; Shiowatana, J.; Siripinyanond, A. Encapsulation of α-tocopherol in protein-based delivery particles. *Food Res. Int.* **2009**, *42*, 909–914. [CrossRef]
70. Ganzevles, R.A.; Stuart, M.A.C.; van Vliet, T.; de Jongh, H.H. Use of polysaccharides to control protein adsorption to the air-water interface. *Food Hydrocoll.* **2006**, *20*, 872–878. [CrossRef]
71. Mirpoor, S.F.; Hashem Hosseini, S.H.; Nekoei, A.R. Efficient delivery of quercetin after binding to beta-lactoglobulin followed by formation soft-condensed core-shell nanostructures. *Food Chem.* **2017**, *233*, 282–289. [CrossRef] [PubMed]
72. Mirpoor, S.F.; Hashem Hosseini, S.H.; Yousefi, G.H. Mixed biopolymer nanocomplexes conferred physicochemical stability and sustained release behavior to introduced curcumin. *Food Hydrocoll.* **2017**, *71*, 216–224. [CrossRef]
73. Yi, J.; Peng, G.; Zheng, S.; Wen, Z.; Gan, C.; Fan, Y. Fabrication of whey protein isolate-sodium alginate nanocomplex for curcumin solubilization and stabilization in a model fat-free beverage. *Food Chem.* **2021**, *348*, 129102. [CrossRef] [PubMed]
74. Wu, S.; Wang, L.; Zhao, Y.; Chen, B.; Qiu, D.; Sun, P.; Shao, P.; Feng, S. Fabrication of high strength cold-set sodium alginate/whey protein nanofiber double network hydrogels and their interaction with curcumin. *Food Res. Int.* **2023**, *165*, 112490. [CrossRef]
75. Mankar, S.; Anoop, A.; Sen, S.; Maji, S.K. Nanomaterials: Amyloids reflect their brighter side. *Nano Rev.* **2011**, *2*, 6032. [CrossRef]
76. Williams, A.H.; Roh, S.; Jacob, A.R.; Stoyanov, S.D.; Hsiao, L.; Velev, O.D. Printable homocomposite hydrogels with synergistically reinforced molecular-colloidal networks. *Nat. Commun.* **2021**, *12*, 2834. [CrossRef]

6. Case study II: “Carciofo di Malegno”

6.1 *Introduction*

Italy boasts a rich agrobiodiversity, particularly in its mountainous and hilly regions. Recent data report 40 artichoke (*Cynara cardunculus* L.; family: Asteraceae; chorotype: steno-Mediterranean (Pignatti 2017) landraces cultivated on farms, but only one, “Carciofo di Malegno”, is traditionally grown in the Southern Alps, outside the Mediterranean basin (Figure 4). This landrace is cultivated in the municipality of Malegno (Camonica Valley, Brescia Province, Lombardy; latitude 45°57′06″N; longitude 10°16′30″E), where its flower heads were already appreciated in the early 20th century (Fappani 2023). Local farmers describe “Carciofo di Malegno” as smaller, more bitter, and tastier than commercial varieties (personal communication), and propagation is traditionally done by planting suckers in autumn or spring.

Figure 4. “Carciofo di Malegno” landrace

The plant exhibits the morphological features of globe artichoke (*Cynara cardunculus* L. subsp. *scolymus* (L.) Hayek), as identified using “Flora d’Italia” dichotomous keys (Pignatti 2017). Globe artichoke is valued not only for its high fiber and mineral content, but also for its traditional use in folk medicine for hepatoprotective, choleretic, diuretic, and lipid-lowering activities (Lattanzio et al., 2009; Pérez-Esteve et al., 2018). Its health-promoting properties are linked to high polyphenol content (Fratianni et al., 2014; Lattanzio et al., 2009), particularly caffeoylquinic acids (CQAs), and O-glycosylated flavonoids such as apigenin and luteolin derivatives (Gouveia & Castilho, 2012). Among CQAs, chlorogenic acid (5-O-caffeoylquinic acid) is the most abundant, while cynarin (1,3-O-dicaffeoylquinic acid) is better known for its bioactivity and occurs in both leaves and flower heads.

Despite its uniqueness, “Carciofo di Malegno” has never been the subject of valorization or protection programs, and no scientific studies have characterized it to date.

This research aims to provide a comprehensive characterization of the “Carciofo di Malegno” landrace, focusing on its morphological traits, an essential requirement for its registration in the National Register of Agrobiodiversity, and its phytochemical profile, which holds relevance for both producers and consumers. This landrace was compared with other four commercial artichokes.

In addition, the study investigates the current and future geographical areas suitable for the cultivation of this landrace within the southern Alpine region. Identifying the ecological niche of traditional varieties, particularly those propagated vegetatively such as artichoke, is crucial for anticipating suitable cultivation zones under changing climatic conditions. Such efforts could provide essential tools (e.g., ecological niche modeling maps) to guide in situ conservation and support sustainable development and agrobiodiversity preservation in marginal areas.

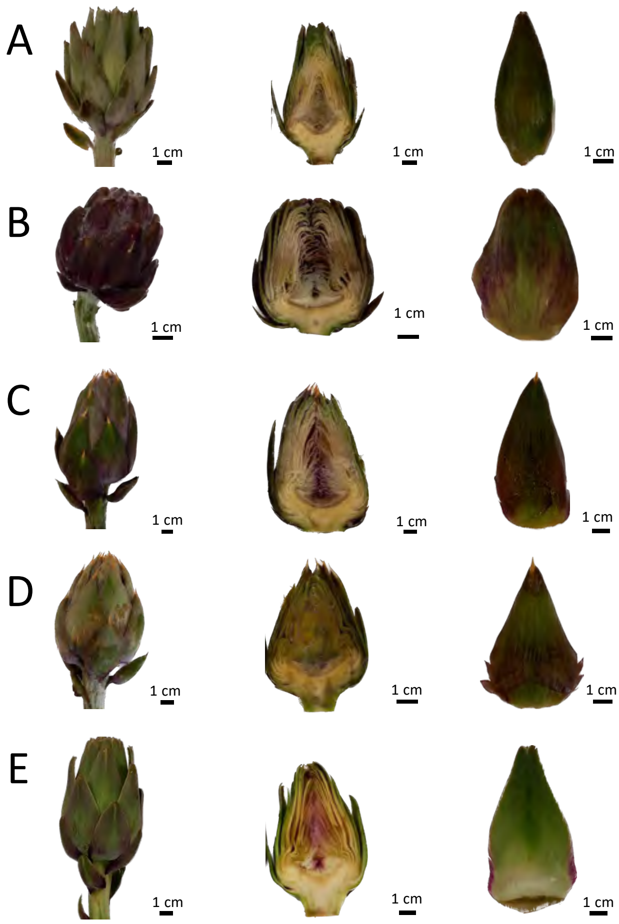


Figure 5. The “Carciofo di Malegno” (A) and four commercial globe artichoke (B–E).

6.2 *Characterization*

Morphometric analysis revealed that the landrace was distinct from all other artichoke samples analyzed (Figure 5). Due to its spiny shape, this landrace appeared to be morphologically closer to the samples classified within the “Spinosi” group while it differed markedly the rounder profile typical of the “Romaneschi” group.

Modern geometric morphometric (GMM) techniques, such as outline analysis, represent an innovative and effective approach for describing and comparing biological shapes and forms.

These methods are increasingly applied across diverse disciplines, including plant biology, as they enable the quantification of size and shape by analyzing the relative positions of landmarks and sets of points approximating curves (outlines) and surfaces. identifying both interspecific differences and intraspecific variation useful for the definition of morphotypes and ecotypes. As highlighted in previous studies, geometric morphometrics are particularly useful for the characterization and identification of landraces, and they have the potential to supplement, or even partially replace, the conventional morphological descriptors used in Distinctness, Uniformity, and Stability (DUS) testing.

GMM techniques are not only effective and objective but also low-cost, minimizing operator bias. They require only basic equipment, such as a digital scanner for image acquisition, freely available software for shape analysis, and standard statistical tools.

Currently, DUS testing, regulated by the International Union for the Protection of New Varieties of Plants (UPOV) and the Community Plant Variety Office, is mandatory for the registration of cultivars, including both conservation varieties and those listed in national agrobiodiversity registers. The possibility of creating open-access libraries or datasets of cultivar shapes for direct import into GMM software would greatly facilitate landrace comparison and improve the precision of new variety characterization. At present, the standards used in DUS forms are still based on hand-drawn illustrations, and many researchers or breeders lack access to reference varieties for direct morphological comparison.

The globe artichoke is valued not only as a culinary vegetable but also as a medicinal plant and a source of secondary metabolites with beneficial health properties. It is particularly rich in phenolic compounds, with caffeoylquinic acids being the most prominent. Among them, chlorogenic acid is the most abundant, while cynarine has attracted considerable attention for its health-promoting effects (Lattanzio et al., 2009).

In this study, the capitula of the "Carciofo di Malegno" exhibited chlorogenic acid and cynarine contents comparable to those found in commercial cultivars. In all analyzed samples, the edible parts of the capitula (i.e., the receptacle and inner fleshy bracts) contained higher levels of these two caffeoylquinic derivatives than the non-edible parts, in agreement with previous reports (Lombardo et al., 2010; Pérez-Esteve et al., 2018). The stems of artichokes A (1032.9 ± 294.9 mg × 100 g^{-1} DW), B (1414.0 ± 325.2 mg × 100 g^{-1} DW), and C (1222.2 ± 196.2 mg × 100 g^{-1} DW) showed higher chlorogenic acid levels than the heads, whereas stems of cultivars D and E had significantly lower levels (76.0 ± 3.0 and 445.9 ± 22.6 mg × 100 g^{-1} DW, respectively). This discrepancy may be related to tissue aging, as the stems of D and E displayed a higher degree of lignification. It is well known that the accumulation of caffeoylquinic acids is closely linked to tissue physiology, with a decline associated with increased lignification (Lattanzio et al., 2009; Lombardo et al., 2010).

Generally, the stems of all analyzed cultivars had lower cynarine content than the edible parts. However, “Carciofo di Malegno” (7.4 ± 1.2 mg × 100 g−1 DW), and sample C showed similar concentrations while cultivar B had even higher values (12.5 ± 0.7 mg × 100 g−1 DW). These findings indicate the potential of these cultivars for recovering valuable caffeoylquinic acids from artichoke stems, which are typically discarded as waste.

Beyond chlorogenic acid and cynarine, artichokes also contain various other caffeoylquinic derivatives (Lattanzio et al., 2009). To estimate the total content, caffeic acid was quantified following basic hydrolysis, serving as an indirect measure of total caffeoylquinic acids. The distribution pattern of caffeic acid across samples mirrored that of chlorogenic acid (an expected result), given that chlorogenic acid is the major precursor of caffeic acid upon hydrolysis (De Falco et al., 2015).

Luteolin and apigenin are the principal flavonoids in globe artichoke, commonly occurring in glycosylated forms (De Falco et al., 2015). To quantify these compounds, extracts from each sample were subjected to acid hydrolysis to release the aglycones. In “Carciofo di Malegno”, luteolin was present in significant amounts in both the stems and the edible parts of the capitula (9.4 ± 1.5 mg × 100 g−1 DW, respectively). Conversely, apigenin was predominantly found in both the edible and non-edible parts of the capitula, but not in the stems. The levels of both flavonoids in “Carciofo di Malegno” were comparable to those observed in the other cultivars. These results suggest that the receptacle and inner bracts of “Carciofo di Malegno” may serve as an excellent dietary source of these flavonoids. Moreover, the stems and outer (non-edible) bracts, typically considered as agricultural waste, may be repurposed in the herbal sector to produce flavonoid-rich extracts, supporting a circular economy approach.

From a phytochemical standpoint, “Carciofo di Malegno” demonstrates comparable properties to those of commercial cultivars. Its bioactive profile offers potential for the development of innovative functional products with health benefits.

These findings support the promotion of “Carciofo di Malegno” as part of new local supply chains aimed at the sustainable development of mountain regions. At the same time, such initiatives can contribute to the *in situ* conservation of this landrace. Indeed, much of today’s crop diversity is maintained ex situ in gene banks or breeders' collections, rather than preserved on-farm (*in situ*) (FAO 2005).

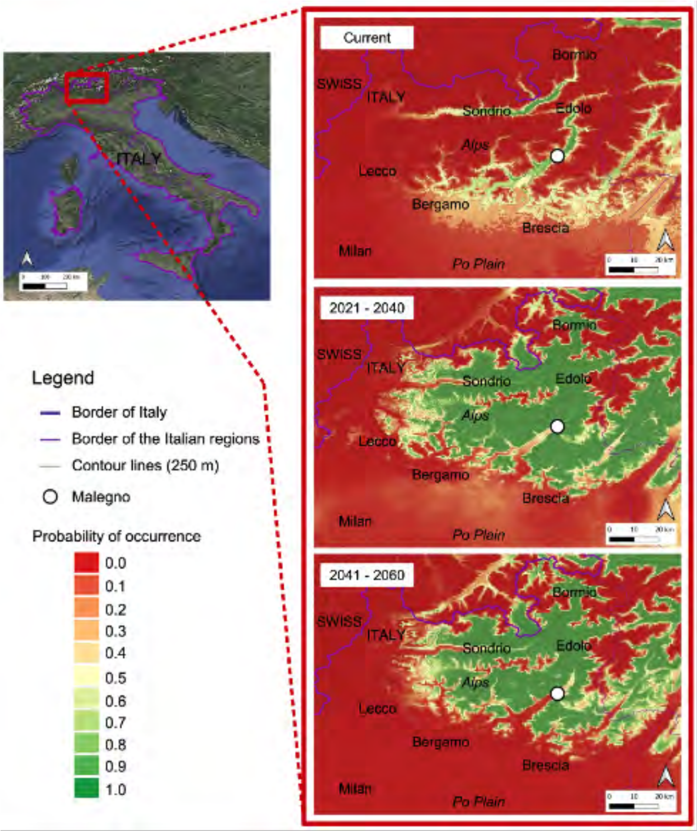


Figure 6. Projections of the spatial distribution of "Carciofo di Malegno" according to MaxEnt across the north of Italy at present with occurrence points and at horizon 2021–2040 and 2041–2060.

To contrast the general trend of declining cultivation of traditional varieties, the potential distribution of the "Carciofo di Malegno" was modelled using the MaxEnt algorithm combined with R software. This ecological niche modelling approach was applied to estimate the current and future suitability of northern Italy for the cultivation of this landrace under various climate change scenarios (Figure 6).

Based on the current climatic conditions, results indicated that "Carciofo di Malegno" is adapted to a relatively limited area of Lombardy, particularly the valley floors of Camonica Valley and Valtellina. However, projections under the SSP1-4.5 climate scenario (2021–2040) suggest that the suitable cultivation area may expand significantly, encompassing large parts of the pre-Alpine and Alpine zones of Lombardy. This predicted upward shift in altitudinal range is consistent with the expected increase in average temperatures due to global warming. Consequently, while higher elevations may become increasingly suitable for cultivation, the traditional area of origin (Malegno and the lower valley floors) may become climatically unsuitable in the coming decades. This raises concerns about the *in situ* conservation of the landrace and about the legal and administrative frameworks for its protection. Indeed, current instruments such as the European Register of Conservation Varieties (Spataro & Negri, 2013) and the Italian Register of Agrobiodiversity protect not only the landrace itself but also its traditional cultivation area. Thus, any potential translocation of "Carciofo di Malegno" outside

its historical territory might conflict with the existing definitions of landrace protection and valorization.
To address this issue, it would be advisable to consider periodic updates to conservation registers, potentially introducing a distinction between the historical cultivation area and newly suitable areas identified under shifting environmental conditions. A flexible, adaptive approach would ensure the long-term sustainability of conservation strategies while enabling continued cultivation of traditional varieties in climatically favourable zones.
Projections for the 2041–2060 period under the same SSP1-4.5 scenario indicate a reduction in the potential cultivation area compared to the previous horizon, suggesting that rising temperatures may surpass the thermal optimum for the species in some zones. As a result, understanding the phenotypic plasticity of "Carciofo di Malegno" (its ability to adjust to changing environmental conditions) becomes a key priority (Sultan 1995). Currently, little is known about the adaptive capacity of this landrace, as is the case for many traditional varieties. In this context, "Carciofo di Malegno" could also serve as a valuable genetic resource for breeding programs, particularly aimed at developing cold-tolerant cultivars. Its proven capacity to grow and complete its life cycle in a mountain valley with significantly lower temperatures than Mediterranean regions may support the development of new commercial hybrids suitable for non-traditional environments (Azeez et al., 2018; Bonasia et al., 2023; Spataro & Negri, 2013). This would not only enhance the resilience of artichoke cultivation in Italy but could also extend production to mountainous regions such as the Apennines and Alps.
Overall, the integration of ecological modelling, phytochemical analysis, and genetic characterization underscores the multifaceted value of the "Carciofo di Malegno" as a landrace worthy of conservation and valorization. Promoting its cultivation, both within and beyond its traditional territory, could enhance agrobiodiversity conservation, support climate change adaptation, and foster the sustainable development of mountain regions through the establishment of localized value chains.

6.3 *Valorization*

Based on information and interviews conducted with local growers, primarily hobbyists, currently cultivating the "Carciofo di Malegno", it is estimated that only a few hundred plants remain, distributed across small plots (such as terraces, home gardens, and vegetable patches) located within the municipal territory of Malegno. The total cultivated surface area is estimated to be less than one hectare.
Italy has taken significant steps to protect its agrobiodiversity, both *in situ* and *ex situ*. To enroll a landrace in National Register of Agrobiodiversity, several documents are required such as a morphological description of the plant genetic resource, historical documentation

proving its traditional cultivation, a reference germplasm bank conserving its propagation material, and a list of “custodian farmers” committed to its on-farm conservation producing in-purity seeds.

The scientific research conducted by the UNIMONT research team has contributed significantly to this process. Phytochemical analyses revealed the valuable nutritional and functional potential of “Carciofo di Malegno”, suggesting promising avenues for its economic valorization. Furthermore, the UPOV characterization forms were completed, and a formal application was submitted to include “Carciofo di Malegno” in the National Register. This would formally recognize and protect the landrace, with potential positive effects on its cultivated area, thereby enhancing in situ conservation efforts and promoting its valorization through the development of novel agri-food products (Alicandri et al., 2024).

This landrace was also included as one of the case studies within the national AGRITECH research project – Spoke 7, titled “Integrated models for the development of marginal areas to promote multifunctional production systems enhancing agroecological and socio-economic sustainability”, which contributed to further exploring its potential for innovation and sustainability in mountain agri-food systems. As part of the dissemination activities, a public seminar was held to share the research outcomes with the local community and farmers. During this event, a sensory evaluation was conducted on prototypes developed using the leaves of “Carciofo di Malegno”, further emphasizing its potential for diversified use and market development.

In particular, a collaboration was established between UNIMONT (the alpine hub of the University of Milan), the Tevini Distillery (Edolo), and the Municipality of Malegno with the aim of enhancing the value of “Carciofo di Malegno” leaves, traditionally regarded as agricultural waste, by transforming them into a functional ingredient for alcoholic beverages (Figure 7). The Tevini Distillery developed a hydroalcoholic extract from the artichoke leaves, which served as the base for three spirit prototypes. These prototypes were subjected to public sensory evaluation at the UNIMONT research centre. One prototype was really appreciated by citizen and farmers, and it was further developed for commercialization generating added economic value and contributing to the sustainable development of the Camonica Valley. This initiative not only exemplifies a circular economy model by valorizing by-products but also illustrates how local landraces such as “Carciofo di Malegno” can be leveraged for innovative agri-food and herbal applications. Demonstrating the economic potential of cultivating and commercializing such landraces is essential to encouraging their on-farm conservation by local farmers, promoting both biodiversity conservation and rural development through the creation of high-value, niche agri-food products.

Figure 7. Network for the valorization of the "Carciofo di Malegno" byproduct

6.4 *Conclusion and future activities*

Although the "Carciofo di Malegno" is smaller in size compared to commercial cultivars and less competitive in industrial markets, its phytochemical richness, morphological distinctiveness, and traditional origin make it highly suitable for niche food production and herbal applications. The valorization of this landrace through both agronomic and phytochemical pathways supports the development of innovative functional products, aligning with growing consumer demand for health-promoting and locally sourced foods. Moreover, its promotion could foster the creation of short, local supply chains, thereby enhancing the sustainable development of mountain areas. Encouraging on-farm (*in situ*) conservation of this variety would also counterbalance the prevalent trend of maintaining crop diversity primarily *ex situ* in gene banks.

In this context, the "Carciofo di Malegno" emerges not only as a genetic and cultural resource worth preserving but also as a case study demonstrating how traditional landraces can be re-integrated into contemporary agriculture through scientific characterization and strategic valorization using its byproduct.

However, the lack of in-depth scientific data, particularly on its genetics but also on other aspects of its chemical composition and bioactivity, represents a gap that hinders the promotion of its conservation and the valorization of both the landrace and the territory where it is traditionally cultivated, and could therefore represent potential directions for future research activities.

6.5 *Bibliography*

Alicandri, E., Paolacci, A. R., Coluccia, L., Marcomeni, M., Taviani, P., & Ciaffi, M. (2024). Exploring molecular, morphological, and biochemical diversity of Phaeolus vulgaris landraces cultivated in the Aniene Valley (Lazio region, Italy). Current Plant Biology, 39, 100374. https://doi.org/10.1016/j.cpb.2024.100374

Azeez, M.A.; Adubi, A.O.; Durodola, F.A. Landraces and Crop Genetic Improvement. In *Rediscovery of Landraces as a Resource for the Future*; InTechOpen: London, UK, 2018.

Bonasia, A., Conversa, G., Lazzizera, C., & Elia, A. (2023). Characterization of Targeted Phenolic Compounds in Globe Artichoke Heads and Waste from Vegetatively and "Seed"-Propagated Genotypes. Plants, 12(13), 2579. https://doi.org/10.3390/plants12132579

De Falco, B., Incerti, G., Amato, M., & Lanzotti, V. (2015). Artichoke: Botanical, agronomical, phytochemical, and pharmacological overview. Phytochemistry Reviews, 14(6), 993–1018. https://doi.org/10.1007/s11101-015-9428-y

Enciclopedia Bresciana di Antonio Fappani. Available online: http://www.enciclopediabresciana.it/enciclopedia/index.php? title=MALEGNO_(2) (accessed on 12 June 2023).

FAO. *Building on Gender, Agrobiodiversity an Local Knowledge—A Training Manual*; FAO: Rome, Italy, 2005.

Fratianni, F., Pepe, R., & Nazzaro, F. (2014). Polyphenol Composition, Antioxidant, Antimicrobial and Quorum Quenching Activity of the "Carciofo di Montoro" (<i>Cynara cardunculus</i> var. <i>scolymus</i>) Global Artichoke of the Campania Region, Southern Italy. Food and Nutrition Sciences, 05(21), 2053–2062. https://doi.org/10.4236/fns.2014.521217

Gouveia, S. C., & Castilho, P. C. (2012). Phenolic composition and antioxidant capacity of cultivated artichoke, Madeira cardoon and artichoke-based dietary supplements. Food Research International, 48(2), 712–724. https://doi.org/10.1016/j.foodres.2012.05.029

Lattanzio, V., Kroon, P. A., Linsalata, V., & Cardinali, A. (2009). Globe artichoke: A functional food and source of nutraceutical ingredients. Journal of Functional Foods, 1(2), 131–144. https://doi.org/10.1016/j.jff.2009.01.002

Lombardo, S., Pandino, G., Mauromicale, G., Knödler, M., Carle, R., & Schieber, A. (2010). Influence of genotype, harvest time and plant part on polyphenolic composition of globe artichoke [Cynara cardunculus L. var. Scolymus (L.) Fiori]. Food Chemistry, 119(3), 1175–1181. https://doi.org/10.1016/j.foodchem.2009.08.033

Pérez-Esteve, É., Sałata, A., Barat, J. M., Stępniowska, A., López-Galarza, S., & Nurzyńska-Wierdak, R. (2018). POLYPHENOLIC COMPOSITION OF SPANISH CULTIVARS OF GLOBE ARTICHOKE (Cynara cardunculus L. var. Scolymus (L.) Fiori). Acta Scientiarum Polonorum Hortorum Cultus, 17(2), 177–184. https://doi.org/10.24326/asphc.2018.2.15

Pignatti, S.; Guarino, R.; La Rosa, M. Flora d'Italia, 2nd ed.; Edagricole: Bologna, Italy, 2017.

Spataro, G., & Negri, V. (2013). The European seed legislation on conservation varieties: Focus, implementation, present and future impact on landrace on farm conservation. Genetic Resources and Crop Evolution, 60(8), 2421–2430. https://doi.org/10.1007/s10722-013-0009-x

Sultan, S.E. (1995) Phenotypic plasticity and plant adaptation. Acta Bot. Neerl., 44, 363–383.

Article 4:

plants

MDPI

Article

Characterization and Future Distribution Prospects of "*Carciofo di Malegno*" Landrace for Its In Situ Conservation

Davide Pedrali , Marco Zuccolo , Luca Giupponi *, Stefano Sala and Annamaria Giorgi

Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy; davide.pedrali@unimi.it (D.P.); marco.zuccolo@unimi.it (M.Z.); stefano.sala1@unimi.it (S.S.); anna.giorgi@unimi.it (A.G.)

* Correspondence: luca.giupponi@unimi.it

Abstract: "Carciofo di Malegno" is a little-known landrace of *Cynara cardunculus* subsp. *scolymus* cultivated in Camonica Valley (northern Italy). The morphological and phytochemical characteristics of this landrace were investigated; furthermore, a species distribution model (MaxEnt algorithm) was used to explore its ecological niche and the geographical area where it could be grown in the future. Due to its spiky shape, "Carciofo di Malegno" was distinct from any other artichoke sample considered, and it appears to be similar to those belonging to the "Spinosi" group. The concentration of chlorogenic acid (497.2 ± 116.0 mg/100 g DW) and cynarine (7.4 ± 1.2 mg/100 g DW) in "Carciofo di Malegno" was comparable to that of the commercial cultivars. In "Carciofo di Malegno," luteolin was detected in a significant amount (9.4 ± 1.5 mg/100 g DW) only in the stems and in the edible parts of the capitula. A MaxEnt distribution model showed that in the coming decades (2040–2060s), the cultivation of this landrace could expand to the pre-Alps and Alps of Lombardy. Climate change may promote the diffusion of "Carciofo di Malegno", contributing to preservation and the enhancement of this landrace and generating sustainable income opportunities in mountain areas through exploring new food or medicinal applications.

Keywords: agro-biodiversity; *Cynara cardunculus* subsp. *scolymus*; outline analysis; Camonica Valley; chlorogenic acid; cynarine; species distribution models; MaxEnt; Southern Alps

check for updates

Citation: Pedrali, D.; Zuccolo, M.; Giupponi, L.; Sala, S.; Giorgi, A. Characterization and Future Distribution Prospects of "*Carciofo di Malegno*" Landrace for Its In Situ Conservation. *Plants* **2024**, *13*, 680. https://doi.org/10.3390/plants13050680

Academic Editor: Panayiotis Dimitrakopoulos

Received: 19 January 2024
Revised: 21 February 2024
Accepted: 27 February 2024
Published: 28 February 2024

1. Introduction

Nowadays there has been a significant loss of global agrobiodiversity with an estimated 75% decline [1,2]. Plant agrobiodiversity includes wild relatives, landraces, and modern cultivars of agricultural and food interest. Landraces are traditional crop varieties, locally adapted, that can hold "immense" value in terms of their agronomic and phytochemical-nutritional characteristics, as well as their ability to adapt to climatic change [3,4]. Many landraces exhibit unique profiles of secondary metabolites, antioxidants, and micronutrients, making them valuable resources for crop improvement and human health, but only a few of them have been conserved in situ (on-farm) and/or ex situ (in germplasm banks) [5–9].

The European Union (EU) has taken action to preserve agrobiodiversity with international strategies such as the EU Biodiversity Strategy 2020 [10], the National Recovery and Resilience Plan (PNRR) [11], and the 2030 Agenda for Sustainable Development [12]. These policies place the emphasis on the need to adopt innovative and sustainable solutions including the conservation and use of traditional cultivars and encouraging circular economy forms to invert the trend of genetic erosion in agriculture.

Italy has recognized the importance of preserving its agrobiodiversity and has taken various actions to promote the conservation of landraces both in situ (on-farm) and ex situ. In particular, Italy has established the National Register of Agrobiodiversity (Ministerial Decree 2019/39407; https://rica.crea.gov.it/APP/anb/ accessed on 1 September 2023) with the objective of protecting and promoting agricultural and food resources from the risk of extinction and genetic erosion. This register was established in December 2019 in accordance with Law No. 194/2015 ("Provisions for the conservation and enhancement of biodiversity of agricultural and food interest"). It operates under the supervision of the Ministry of Agriculture and Forestry Policies and is currently being set up and implemented. To register a landrace in the National Register of Agrobiodiversity, it is necessary to submit a series of documents to the relevant regional offices. These documents include the morphological description of the plant genetic resource, historical documentation proving its connection to the traditional cultivation area, a germplasm bank that conserves the seeds (or other propagation material) ex situ, and a list of farmers to preserve/cultivate it in its agroecosystem (on-farm conservation).

To implement conservation strategies on farms, it is essential to involve and incentivize "custodian farmers" who are responsible for in-purity seed production in the geographical area where landraces are traditionally cultivated. One strategy to engage custodian farmers, without relying on state economic subsidies, is to help them understand that cultivating/conserving and commercializing landraces can generate income [10]. In fact, some landraces possess phytochemical and nutritional characteristics of high value as well as exclusive sensorial aspects [5,13], making them highly sought after by consumers; this makes them valuable raw materials for the food and herbal industries. Unfortunately, only a few landraces have been thoroughly characterized [7,14]. This task is crucial for increasing knowledge about plant agrobiodiversity, promoting in situ conservation of landraces, and creating agri-food supply chains that foster sustainable development of territories [4].

For the same purpose, it would also be necessary to analyze the ecological niches of landraces, especially those that are propagated vegetatively, in order to understand where they could be cultivated/preserved in the near future based on environmental modifications due to climate change. Indeed, many cultivated plants are propagated vegetatively by humans (such as potatoes, garlic, asparagus, and artichoke), without producing and using seeds [15]. This prevents the evolution of populations and consequently hinders adaptation to the rapidly changing climate [13,16].

Italy is rich in agrobiodiversity and has a significant number of landraces, especially in mountainous and hilly areas [7,14]. According to recent census data [7], Italy has 40 landraces of artichoke (*Cynara cardunculus* L.; family: Asteraceae; chorotype: steno-mediterranean, [17]) cultivated on farms, of which only one ("*Carciofo di Malegno*") is traditionally cultivated in the Southern Alps outside the Mediterranean basin (Figure 1). In the territory of the municipality of Malegno (Camonica Valley, Brescia Province, Lombardy region; latitude 45°57′06″N; longitude 10°16′30″E), a traditional cultivar of artichoke called "*Carciofo di Malegno*" is grown, and its flower heads were already appreciated in the early 20th century [18]. The inhabitants of Malegno have cultivated this landrace for decades, passing down their knowledge and expertise from one generation to the next. The local farmers declare that the "*Carciofo di Malegno*" is tastier, more bitter, and smaller than the artichoke commercial varieties [personal communication]. This landrace is traditionally propagated vegetatively by collecting and planting (in autumn or spring) the suckers produced by the plants [personal communication]. The "*Carciofo di Malegno*" exhibits the morphological characteristics of a globe artichoke of *Cynara cardunculus* L. subsp. *scolymus* (L.) Hayek (identified by the authors using "Flora d'Italia" dichotomous keys) [17].

The globe artichoke is considered an excellent source of fiber and minerals but also receives a place in folk medicine as a traditional herbal remedy for its beneficial properties, including hepatoprotective, choleretic, diuretic, and lipid-lowering activities [16,19]. Indeed, apart from its nutritional values, globe artichoke is characterized by a high content of polyphenols that are considered strictly related to its recognized healthy properties [19,20].

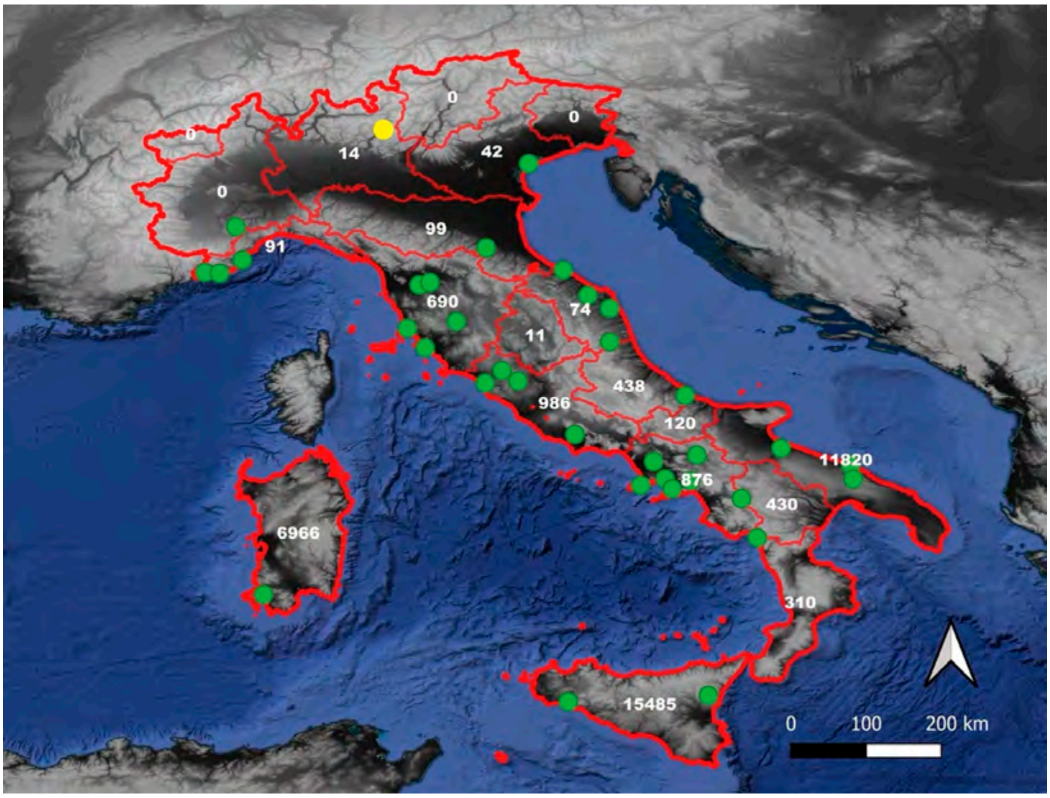


Figure 1. Distribution maps of artichoke landraces in Italy: "*Carciofo di Malegno*" (yellow point) and other artichoke landraces (green points). Cultivated hectares of artichoke in each region (white numbers).

These compounds provide many health benefits in human life, including antioxidant, anti-inflammatory, cardio-protective, anticancer, anti-aging, and antimicrobial activities [21,22], and it is recognized that a diet rich in polyphenols may be associated with a reduced risk of chronic diseases as diabetes and cardiovascular, cerebrovascular, and neurodegenerative diseases [22,23]. In the globe artichoke, the main represented polyphenols are the compounds belonging to the family of caffeoylquinic acids (CQAs) together with *O*-glycosylated flavonoids like apigenin and luteolin derivatives [24] Several mono- and di-caffeoylquinic acids derivatives have been identified in globe artichoke extracts, and, among them, chlorogenic acid (5-*O*-caffeoylquinic acid) is the major caffeoylquinic acid derivative. Conversely, cynarin (1,3-*O*-dicaffeoylquinic acid), despite not being the most abundant, is the better-known derivative of this class of globe artichoke [19]. Cynarin has been identified both in capitula and leaves extracts and has been recognized as the compound responsible for the choleretic and cholesterol-lowering activities of the globe artichoke [19,25]. Regarding the "*Carciofo di Malegno*", no studies have been conducted to characterize it (as it has never been involved in protection and valorization programs). It would be interesting to describe this landrace morphologically, ethnobotanically, ecologically, and phytochemically as well as discover its ecological niche to comprehend its potential present and future cultivation/conservation sites.

This research aims to characterize "*Carciofo di Malegno*" from a morphological perspective (essential for its registration in the National Register of Agrobiodiversity) and its phytochemical properties (essential for farmers and consumers). In particular, the morphological aspect was investigated by morphometric analysis while the phytochemical composition was analyzed using HPLC equipment. Furthermore, the study aims to determine the current and future geographical areas where the "*Carciofo di Malegno*" can be grown in the Southern Alps, providing tools (maps of the ecological niche modeling) that can be useful for its in situ conservation for those involved in actions for agrobiodiversity protection and sustainable development of the territory.

2. Materials and Methods

2.1. Plant Material

Figure 2 shows the five different globe artichoke genotypes included in this study: the "*Carciofo di Malegno*" (A) and four commercial globe artichokes (B, C, D, and E).

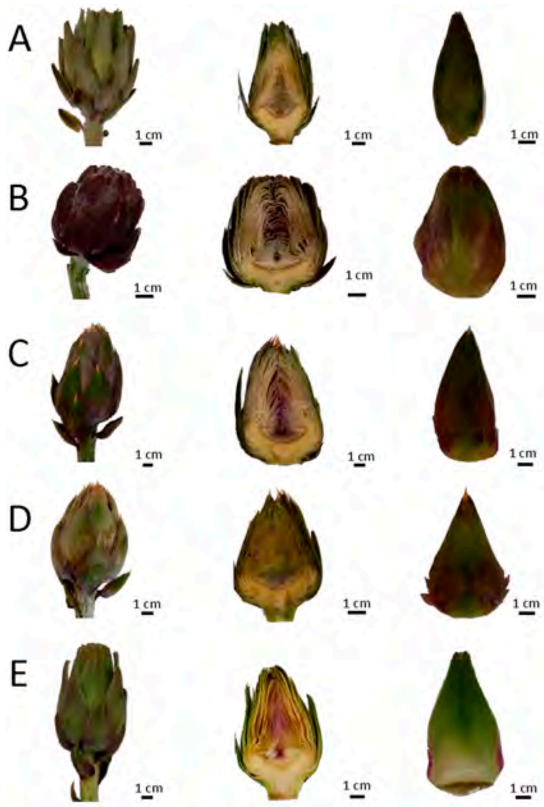


Figure 2. The "*Carciofo di Malegno*" (**A**) and four commercial globe artichoke (**B**–**E**).

The "*Carciofo di Malegno*" (A) was collected from a local farmer, Felice Pezzoni, growing it in the Commonality of Malegno (Camonica Valley; latitude 45°57′06″ N; longitude 10°16′30″ E), who reported cultivating it from the 40s. The capitula for the analysis were collected from the plants in the field. Twenty capitula were collected manually with about 10 cm of stem. The four commercial globe artichokes (B–E) were collected from an Ortofrutta La Sicilia spadafora local shop that sells a different variety of Italian artichoke; this shop receives fresh artichokes every day. Genotype B was a "Romaneschi" type artichoke [26] and was originally grown in Sicily while both artichokes C and D belonged to the "Spinosi" group [26] and came, respectively, from Sardinia and Sicily. Artichoke E belonged to the

"Catanesi" group [26] and came from Apulia. For each artichoke, 20 capitula were collected, and the stems were cut at about 10 cm of length.

In the laboratory, the capitula of all the artichokes were washed, examined to eliminate the damaged samples, and weighted (XB A220, Precisa Gravimetrics AG, Dietikon, Switzerland). Twenty capitula for each genotype were randomly selected for the morphometric analysis. The remaining were divided into three parts: non-edible flower head parts, stem, and receptacle with the inner bracts. The stems were cut at about 0.5–1 cm below the receptacle, weighted (Table 1, Figure 2), and subsequently cut in 5–6 parts. The capitula were turned removing the outer bract together with the upper spiny part of the flower head. The outer bracts were removed up to the fifth turn for the four commercial globe artichokes (B–E) and up to the third turn for the "*Carciofo di Malegno*" (A). The outer bracts and the upper spiny parts were weighted together as non-edible flower head parts.

Table 1. Origin, shape, color, and consumable part of artichoke sample. Stem, non-edible, and edible parameters are indicated with mean ± standard deviation. The identification codes of artichoke samples (capital letter) are referred to in Figure 2.

Code	Origin	Thorn/Mucro	Head Shape	External Color	Stem (g)	Non-Edible (g)	Edible (g)
A	Malegno (BS)	Mucro	Conical	Green	8.73 ± 0.63	22.13 ± 4.39	35.12 ± 3.94
B	Sicily	Short thorn/absent	Spherical	Violet with green tones	35.01 ± 7.33	106.19 ± 10.13	109.29 ± 15.98
C	Sicily	Thorn	Conical	Green with violet tones	36.04 ± 10.23	59. 67 ± 8.52	8.51 ± 10.28
D	Sardinia	Thorn	Conical	Green with violet tones	26.18 ± 1.05	44.65 ± 7.02	87.99 ± 10.44
E	Apulia	Short thorn/absent	Ovoidal	Green with violet tones	30.12 ± 2.49	47.07 ± 7.96	85.67 ± 5.84

Each part was frozen at −80 °C using a freezer (Haier Biomedical, Qingdao, P.R. China) and then milled with a kitchen mixer (Moulinex) to obtain a coarse powder. The powder was stored in a sealed container (50 mL, centrifuge tube) at −80 °C before the extraction.

2.2. Morphometric Analysis

Thirty samples for "*Carciofo di Malegno*" (A) and 30 artichokes for the commercial variety (B–E) randomly collected from local markets were used for the outline analysis (elliptical Fourier descriptors analysis) [27]. Each sample was placed on a white support table and photographed using a Canon EOS 2000D digital camera positioned perpendicular to the support surface. The images of the artichokes were processed using Adobe Photoshop CC2017 software: the shadows were removed, and the images were transformed to black and white. The outline coordinates were extracted with Momocs 1.4.0 [28,29] in an R environment [30] and converted into Fourier coefficients considering 9 and 15 harmonics (bracts and capitula, respectively) that gathered at least 99% of the total harmonic power [28]. In order to control left/right asymmetry, the artichokes were flipped in the same direction (considering as apex the most acute part of the artichoke), and then a landmark was defined at their base as a starting point for importing outline coordinates. The contours were centered, and the outline analysis was performed without numerical normalization.

Principal component analysis (PCA) was conducted on the matrix of coefficients to reduce dimensionality, and the samples were plotted on the first two axes (principal components). Linear discriminant analysis of principal components (DAPC) [31] was performed. The mean shape of the artichokes of each genotype was reconstructed using the MSHAPES function of Momocs, and multivariate analysis of variance (MANOVA) was performed to evaluate the differences in artichoke shape (bracts and capitula) among the five cultivars. Finally, pairwise MANOVA was used to highlight the differences between artichokes.

2.3. Phytochemical Analysis

All the solvents, reagents, and analytical standards were purchased from Merck (Milan, Italy).

The extracts for the HPLC assays were obtained by a two-step solvent extraction procedure adapting literature procedure [16,20]. An exactly weighed sample (1.5 g) of frozen artichoke powder was transferred in a screw-top cap conical-bottom centrifuge tube and extracted twice with methanol (1:10 g/mL *w*/*v* ratio) using an ultrasonic bath Digital Ultrasonic Cleaner MH020S (Vevor, USA) for 30 min at room temperature [20,24]. The supernatants were collected by centrifugation (Hermle z300, HERMLE Labortechnik GmbH, Wehingen, Germany) at 4000 rpm for 10 min. The residue was then extracted twice with 70% ethanol (1:10 g/mL *w*/*v* ratio) and sonicated for 30 min at room temperature. The collected supernatants were pooled together and evaporated to dryness under reduced pressure (rotary evaporator, LABOROTA 4000eco, Heidolph Instruments GmbH & Co., Schwabach, Germany) at 45 °C. The residue was re-dissolved in 60% methanol to a final volume of 5 mL. The resulting phenolic extract was stored at −20 °C overnight and then centrifuged at 4000 rpm for 10 min. A 1 mL aliquot of the extract was filtered through a nylon 0.2 µm Millex®-GN syringe filter prior to the chromatographic analysis.

An aliquot of the phenolic extract was subjected to acidic hydrolysis following the procedure developed by Nuutila et al. [32], with minor modifications. Briefly, 480 µL aliquot of the phenolic extract was mixed with 120 µL of 6 N hydrochloric acid and heated in a screw-top capped tube at 80 °C for 2 h. After cooling to room temperature, the mixture was diluted with 400 µL of methanol, sonicated, and filtered through a nylon 0.2 µm Millex®-GN syringe filter prior to the chromatographic analysis.

An aliquot of the phenolic extract was subjected to alkaline hydrolysis following the procedure developed by Lin et al. [33], with minor modifications. Briefly, 1 mL aliquot of the phenolic extract was taken to dryness under air flow. After that, the residue was dissolved with 300 µL of 4 N aqueous sodium hydroxide and incubated at room temperature for 18 h. After that, the mixture was acidified to pH by the addition of 150 µL of 12 N hydrochloric acid, diluted with 550 µL of methanol, and filtered through a nylon 0.2 µm Millex®-GN syringe filter prior to the chromatographic analysis.

The High-Performance Liquid Chromatography (HPLC) analysis was performed using a LC Agilent series 1200 apparatus (Waldbronn, Germany) consisting of a degasser, a quaternary gradient pump, an auto-sampler, and an MWD detector (Waldbronn, Germany). A Luna® 5 µm C18 (150 × 4.6 mm) column (Phenomenex, Santa Clara, CA, USA) at 40 °C was used for this analysis. Sample injections were made at 10 µL for all samples and standards; the run time was 40 min. A binary gradient comprising 0.1% aqueous formic acid (*v*/*v*) (A) and acetonitrile (B) at a flow rate of 0.8 mL min^{-1} was used as the mobile phase. The gradient profile was as follows: 0 min, 5% B; 20 min, 25% B; and 30 min, 95% B; 40 min, 5%B. Absorbance wavelength was 330 ± 10 and 370 ± 10 nm, for the caffeoylquinic acids and for flavonoids, respectively.

The standards used were chlorogenic acid, cynarine, apigenin, and luteolin. Individual stock solutions of each standard were prepared using methanol at 1 mg/mL and stored at −20 °C. The working standard solutions were made by diluting the appropriate amount of each stock standard solution (1000 µg/mL) to obtain 5 calibration levels: chlorogenic acid, 25–800 µg/mL; cynarine, 0.5–6.25 µg/mL; caffeic acid, 12.4–400 µg/mL; apigenin, 1.042–50 µg/mL; and luteolin, 4.17–100 µg/mL.

Phytochemical data was analyzed using a one-way ANOVA test using R 3.2.1 software [30] to highlight the significant differences ($p < 0.05$) attributable to each genotype. All the results were expressed to dried weight of plant material.

2.4. Prediction of the Potential Distribution of "*Carciofo di Malegno*"

The fields where the "*Carciofo di Malegno*" is cultivated (and where it was cultivated in the latest decades) were identified by consulting the local farmers and were georeferenced using a GPS device (Garmin Etrex 32×). Twenty-five georeferenced points (occurrence points) were collected to assess the spatial distribution of "*Carciofo di Malegno*" in Malegno whose coordinates were imported into R in CSV format.

Nineteen bioclimatic variables (Table 2) were retrieved as predictors to model the potential environmental niche of "*Carciofo di Malegno*" based on its occurrence dataset. In particular, the bioclimatic layers were obtained from the World Climate Database (WorldClim 2.1, http://worldclim.org, accessed on 25 September 2023.) at a spatial resolution of 0.5 arc-s. All the bioclimatic variables were used to establish the distribution model of "*Carciofo di Malegno*" under the current conditions (2016–2020) and future global warming scenarios (2021–2040 and 2041–2060).

Table 2. Environmental variables used for modeling the potential distribution of "*Carciofo di Malegno*".

Code/Unit	Bioclimatic Variable
BIO1 (°C)	Annual Mean Temperature
BIO2 (°C)	Mean Diurnal Range (Mean of monthly (max temp–min temp)
BIO3 (-)	Isothermality (BIO2/BIO7 × 100)
BIO4 (°C)	Temperature Seasonality (standard deviation × 100)
BIO5 (°C)	Max Temperature of Warmest Month
BIO6 (°C)	Min Temperature of Coldest Month
BIO7 (°C)	Temperature Annual Range (BIO5-BIO6)
BIO8 (°C)	Mean Temperature of Wettest Quarter
BIO9 (°C)	Mean Temperature of Driest Quarter
BIO10 (°C)	Mean Temperature of Warmest Quarter
BIO11 (°C)	Mean Temperature of Coldest Quarter
BIO12 (mm)	Annual Precipitation
BIO13 (mm)	Precipitation of Wettest Month
BIO14 (mm)	Precipitation of Driest Month
BIO15 (-)	Precipitation Seasonality (Coefficient of Variation)
BIO16 (mm)	Precipitation of Wettest Quarter
BIO17 (mm)	Precipitation of Driest Quarter
BIO18 (mm)	Precipitation of Warmest Quarter
BIO19 (mm)	Precipitation of Coldest Quarter

The latest iteration of climatic scenarios, used for the Sixth Phase of the Coupled Model Intercomparison Project (CMIP6, 2016–2021) and featured in the IPCC Sixth Assessment Report (AR6) [34], is based on a set of Shared Socio-economic Pathways (SSPs). The SSP-based scenarios are the most complex created to date and span a range from very ambitious mitigation (SSP1—sustainable development) to ongoing growth in emissions (SSP5—fossil-fueled development). The SSP2-4.5 scenario ("Middle of the road scenario") was used in this research as it is an intermediate scenario compared to the two mentioned above. According to this scenario, the CO_2 emissions will start to fall mid-century (without reaching net-zero by 2100), and the temperatures will rise 2.7 °C by the end of the century.

One global climate model (CNRM-CM6-1) was obtained from the WorldClim database for the future scenarios of the periods 2021–2040 and 2041–2060. The CNRM-CM6-1 is the recent fully coupled atmosphere-ocean general circulation model of the sixth generation jointly (developed by Centre National de Recherches Météorologiques and CMIP6) that replaced and improved the CNRM-CM5.1 model [33].

In this research, all models were run using the MaxEnt algorithm in R environment. The relative importance/weight of each bioclimatic preditor for the distribution model was assessed using the Jackknife test [35], and the response curves for each of the environmental variables were generated.

The accuracy of the resulting model was evaluated by computing the Area under the Curve (AUC) of the Receiver Operating characteristic Curve (ROC). AUC values range from 0 to 1, and the higher the value of AUC, the better the performance of the model.

The output of the MaxEnt application is a georeferenced raster file, indexing the environmental suitability of "*Carciofo di Malegno*" with values ranging from 0 (unsuitable) to 1 (optimal). All the raster files generated in this study were imported into QGIS 3.28 (http://qgis.osgeo.org) to produce the distribution maps of the "*Carciofo di Malegno*" for the current and future scenarios.

3. Results

3.1. Shape of the Flower Capitulum and Bracts

Figure 3a,b shows the PCA biplot of the Fourier coefficients calculated for the five artichokes' capitula and the results of the Linear Discriminant Analysis of principal components (LDA), respectively. Along the first principal component (PC1 = 35.2%), the reconstructions of the capitula's shape become rounder, while along the PC2 (22.6%), the apexes become more acute.

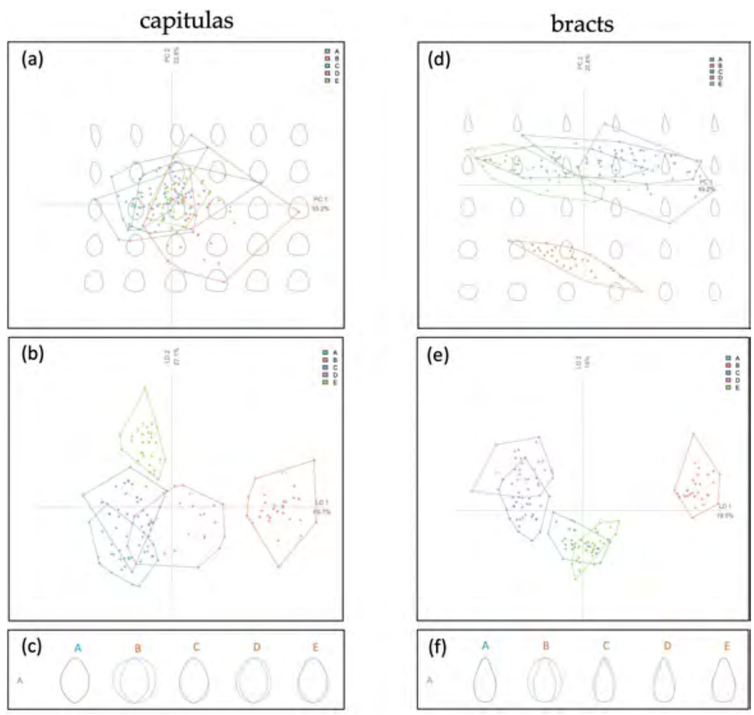


Figure 3. PCA biplot of artichokes' capitula of the five genotypes (**a**); linear discriminant analysis of principal components (LDA) biplot of the artichokes' capitula (**b**). (**c**) Mean shape of artichokes' capitula of the five genotypes. (**d**) PCA biplot of artichokes' bracts of the five genotypes; (**e**) linear discriminant analysis of principal components (LDA) biplot of the artichokes' bracts. (**f**) Mean shape of artichokes' capitula of the five genotypes. Grey figures in the background (**a**,**d**) show reconstructions of artichoke shape (capitula and bracts, respectively) according to each position in the multidimensional space. The identification code of artichoke samples (capital letter) is referred to in Figure 2.

The samples were overlapped in the LDA biplot (Figure 3b) wherein sample B was positioned on the right part of the graph, separated from the other capitulum samples. In fact, Figure 3c displays the mean shape of the five artichoke samples and the "*Carciofo di Malegno*" was close to samples C and E while the artichoke B had a completely different form and was more rounded. These results were also confirmed by a MANOVA test that showed significant shape differences between the five artichokes' capitula ($F_{96,145}$ = 15.119, $p < 0.01$).

Figure 3d shows the PCA biplot of the artichokes' bracts of the five artichokes' genotypes where the first two principal components (PCs) explain 72% of total variance (PC1 = 49.2%; PC2 = 22.8%) although a linear discriminant analysis of principal components (LDA) biplot of the artichokes' bracts is shown in Figure 3e. Both of these discriminant analyses showed that only artichoke B had a very diverse bracts shape among all samples. In the bottom left part of the PCA biplot, the bracts become more rounded, while along the second axis the apex becomes spikier.

Figure 3f displays the mean shape of bracts of each artichoke genotype. While the medium shape of samples A, C, D, and E is pointed, that of artichoke B is oval. The results of pairwise MANOVA (Figure 3f) confirmed the previous outcome and demonstrates that there are significant differences between the bracts shape of "*Carciofo di Malegno*" and the other four commercial varieties ($F_{52,145}$ = 64.777, $p < 0.01$).

3.2. Phytochemical Characteristics

Figure 4a displays the chlorogenic acid content in the stems and in the edible and non-edible parts of the heads of the five cultivars included in the analysis. The content of chlorogenic acid resulted higher in the stems than in the edible and non-edible parts of heads for most of the selected artichokes. The stems of the artichoke B had the highest content (1414.0 ± 325.2 mg × 100 g^{-1} DW), followed by the artichoke C (1222.2 ± 196.2 mg × 100 g^{-1} DW) and the "*Carciofo di Malegno*" (A) (1032.9 ± 294.9 mg × 100 g^{-1} DW). Conversely, artichokes D and E showed a content in the stems significantly lower (76.0 ± 3.0 and 445.9 ± 22.6 mg × 100 g^{-1} DW, respectively) than that of the other cultivars. Moreover, the stems of the Sardinian artichoke (D) had a content of this caffeoylquinic acid lower than the edible and non-edible parts of heads, resulting in the sample with the lowest content. The chlorogenic acid content of the edible part of the heads ranged from 321.8 ± 126.0 mg × 100 g^{-1} DW and 814.8 ± 178.8 mg × 100 g^{-1} DW, resulting in higher than that of the non-edible parts of the heads. The heads of the "*Carciofo di Malegno*" (A) having a chlorogenic acid content of 497.2 ± 116.0 mg × 100 g^{-1} DW and 164.2 ± 42.7 mg × 100 g^{-1} DW in the edible and non-edible parts, respectively, were comparable with most of the other cultivars.

Figure 4b displays the cynarine contents of the samples. The heads of almost all the cultivars of artichoke showed a higher content of cynarine than the stems. In particular, this compound is concentrated in the edible parts showing a content ranging from 6.7 ± 0.4 mg × 100 g^{-1} DW to 8.2 ± 1.3 mg × 100 g^{-1} DW. Conversely, the non-edible parts had a lower content of cynarine with values approximatively 2-fold lower than the inner edible parts of the capitula. In artichokes C, D, and E, the stems had a lower cynarine content than the edible parts, while the stems "*Carciofo di Malegno*" (A) showed a similar content (7.4 ± 1.2 mg × 100 g^{-1} DW). The cynarine content of artichoke B stems (12.5 ± 0.7 mg × 100 g^{-1} DW) was higher than that of the capitula, resulting in the richest caffeoylquinic acid sample.

Figure 4c displays the free luteolin content measured after the acidic hydrolysis of sample extracts. The edible parts of the "*Carciofo di Malegno*" (A) resulted in one of the richest samples in free luteolin (9.4 ± 1.5 mg × 100 g^{-1} DW), showing a content similar to that of artichokes C and D (10.9 ± 4.0 and 9.8 ± 1.8 mg × 100 g^{-1} DW, respectively). Conversely, only a trace of luteolin was detected in the non-edible parts of A, while samples of the other cultivars resulted in higher content. In particular, the non-edible parts of artichoke D showed the highest luteolin content recorded (17.7 ± 2.0 mg × 100 g^{-1} DW). The luteolin was less concentrated in the stems than in the heads in most of the cultivars, apart from artichoke B. Artichoke E resulted in the poorest in luteolin, with only traces detected in all the parts.

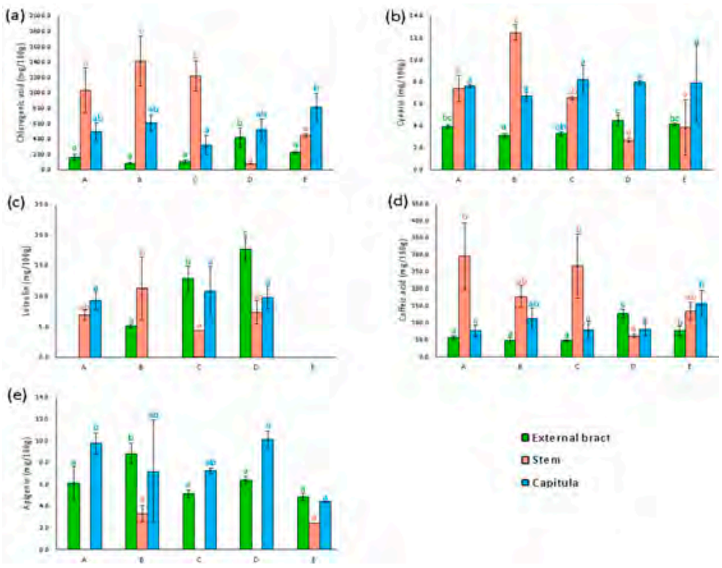


Figure 4. Results of phytochemical assays on external bract, steam, and capitula of artichoke samples. Chlorogenic acid (**a**), cynarin (**b**), luteolin (**c**), caffeic acid (**d**), and apigenin (**e**) content were expressed in mg/100 g dw. The identification code of artichoke samples (capital letter) is referred to in Figure 2.

Figure 4d displays the caffeic acid content measured after the basic hydrolysis of the stems, edible, and non-edible part extracts. The content of this compound in the samples followed the same distribution of the chlorogenic acid.

Figure 4e displays the free apigenin content measured after the acidic hydrolysis of sample extracts. Apigenin was more concentrated in the heads than in the stems for all the cultivars. Indeed, the stems of artichoke B showed a significant amount of free apigenin (3.3 ± 0.8 mg $\times$ 100 g^{-1} DW and 1.9 ± 0.0 mg $\times$ 100 g^{-1} DW, respectively) while in the others this flavonoid was detected only in traces. The capitula of "*Carciofo di Malegno*" had a higher content of apigenin, with results comparable to artichoke D and similar to artichoke C. In these cultivars, the apigenin was more concentrated in the inner part of the heads, while in artichokes B and E the external non-edible parts showed a higher content than the inner bracts and receptacle.

3.3. Current and Future Potential Distribution

Figure 5 shows the maps of the potential distribution of the "*Carciofo di Malegno* " for the current and future scenarios. The predicted AUC value for the future periods was 0.96, indicating excellent predictions. Among the bioclimatic factors, temperature plays an important role in the definition of the ecological niche of "*Carciofo di Malegno*". Isothermality (mean diurnal range/temperature annual range—BIO3) and the mean diurnal range [mean of monthly (max temp–min temp)—BIO2] are the topmost contributing factors accounting for 36% and 33.2% of the total contribution, respectively (Figure S1).

The "*Carciofo di Malegno*" has the potential to thrive in the medium-southern regions of the Camonica Valley, especially in its native zone (Malegno) and nearby areas including the valley floor of Camonica and Valtellina valleys situated at altitudes between 300 and 400 m above sea level. This landrace finds the appropriate, although not excellent, climatic conditions (probability of occurrence: 0.6–0.3) in the hilly areas of the Lombardy Prealps close to the Po Plain.

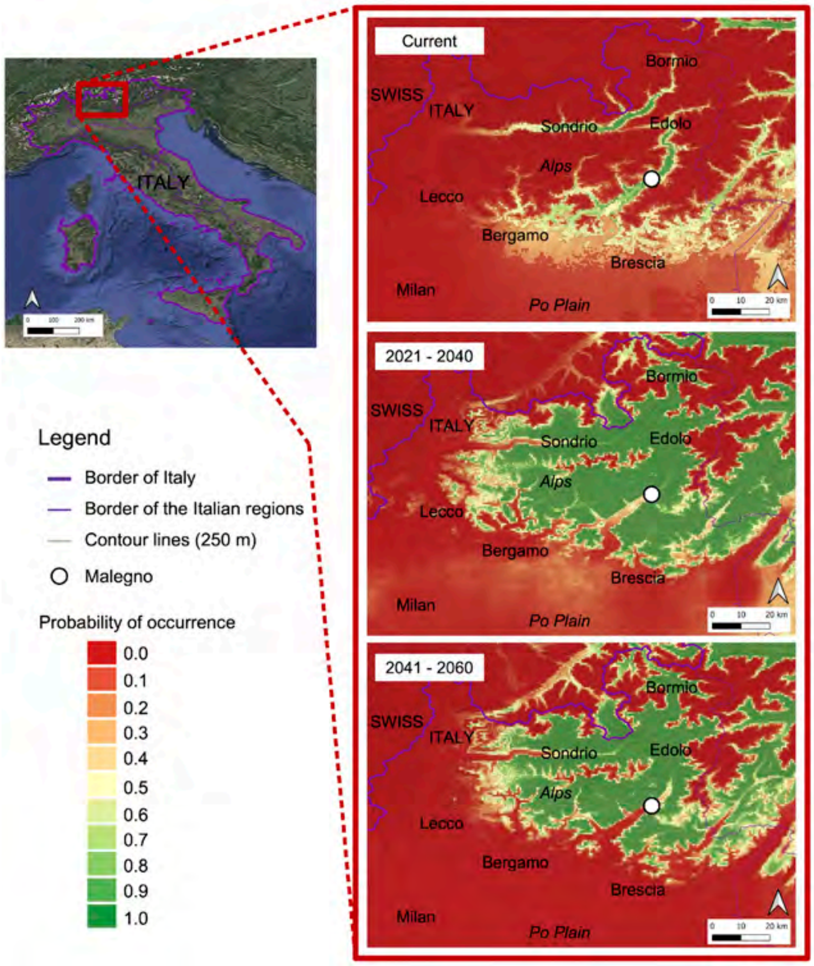


Figure 5. Projections of the spatial distribution of "*Carciofo di Malegno*" according to MaxEnt across the north of Italy at present with occurrence points and at horizon 2021–2040 and 2041–2060.

In the 2021–2040s period, the "*Carciofo di Malegno*" could be cultivated in other areas of the Camonica Valley (not only in Malegno) compared with the potential current distribution. In fact, the territory with a suitable bioclimate was significantly increased including Edolo as other municipalities in the upper part of the Camonica Valley, which are located at higher elevations than Malegno, altitude (600–1000 m asl). In the 2041–2060 scenario, the areas with favorable climatic conditions for the cultivation of the "*Carciofo di Malegno*" extend further north in Lombardy (and in Switzerland), while the probability of occurrence in the original area (Malegno) is low (0.4–0.2), and it is even more so in the hilly areas of the lower Camonica Valley and in the Po Plain (0.2–0.0).

4. Discussion

In this study, the artichoke outline analyses demonstrated that there are differences among the five genotypes assessed, particularly between "*Carciofo di Malegno*" and the commercial varieties.

Morphometric analysis showed that the landrace was different from each other artichoke sample. Due to its spiky shape, this landrace seems to be similar to the samples belonging to the "Spinosi" group (artichoke C and D) while was very different from sample B, which had a rounder profile typical of the "Romaneschi" category [26].

Modern geometric morphometric analyses (GMM), such as outline analysis, are an innovative way to better describe and compare shapes and forms. GMM methods find applications in numerous fields, spanning from plant biology to other disciplines. They enable the examination of the relative locations of landmarks and point sets utilized to approximate curves (outlines) and surfaces, facilitating the measurement of size and shape [29,36]. In recent years, botanists have also applied these types of assays to study the shape of leaves and other plant organisms. For example, Giupponi and Giorgi [27] were able to distinguish leaf shape between two varieties of *Primula albenensis*, while Chitwood and Otoni [37] analyzed more than 3300 leaves from 40 different *Passiflora* species using GMM. In 2020 Giupponi and his coworkers [8] used these methods to compare the shapes of three potato landraces (*Solanum tuberosum* L.) from the *Consorzio della Quarantina* (Genova, Italy) in order to find potential differences between different species and the comparison between populations of the same species for the definition of morphotypes and ecotypes. In particular, as previous work explored, geometric morphometric analyses are useful for the characterization and identification of landraces, and they could replace or supplement the morphological forms of variety description called Distinctness, Uniformity, and Stability (DUS) Testing [27].

GMM are effective and economical, and they allow an objective analysis minimizing human error; they only require plant samples and low-cost equipment like a digital scanner for image acquisition, software for digital analysis, and statistical analysis of data [27] Today, DUS testing redacted by the International Union for the Protection of New Varieties of Plants (UPOV) and the Community Plant Variety Office are mandatory to register cultivars both in the register of conservation varieties and in the register of plant agrobiodiversity. The possibility to have models of cultivar shapes uploaded and shared in an open tool (folder, dataset) to import in a GMM software could improve the comparison among landraces (or plants in general) and characterize new varieties accurately. Nowadays the standards present in DUS forms are drawn, and, in addition, not everyone has the opportunity to grow even the varieties of comparison to make a real contrast between cultivars.

Globe artichoke holds significance not only as a culinary vegetable but also as a medicinal plant and a source of secondary metabolites with health-promoting activities. This plant is indeed known for its abundant phenolic compounds, with caffeoylquinic acids being the primary constituents. Among these compounds, chlorogenic acid stands out as the most abundant derivative. However, it is cynarine that has garnered significant attention due to its beneficial and health-promoting properties [19].

In this study, the capitula of the "*Carciofo di Malegno*" showed a content of chlorogenic acid and cynarine comparable with that of the commercial cultivar. In all the artichokes included in the analysis, the receptacle and the inner fleshy bracts (edible parts) had a content of these two caffeoylquinic derivatives higher than the non-edible parts of the capitula. These observations agree with the literature-reported data [16,38]. The stems of artichokes A, B, and C had a higher content of chlorogenic acid compared to the heads, while, conversely, those of cultivars D and E showed significantly lower content. Possibly, this could be attributed to tissue aging. In fact, the stems of the latter artichokes were discovered to exhibit a greater degree of lignification. It is well established that the overall content of caffeoylquinic acids is closely linked to the physiological condition of the tissues, with a decrease in content observed in correlation with lignification [19,38]. In general, the stems of the analyzed cultivars demonstrated a lower cynarine content compared to the edible portions of the capitula. However, "*Carciofo di Malegno*" and sample C exhibited a similar content, while cultivar B displayed a higher content. These elevated levels signify the potential of these two cultivars in extracting valuable caffeoylquinic acids from artichoke stems, which are typically discarded as waste.

Apart from chlorogenic acid and cynarine, several other caffeoylquinic derivatives are present in the artichokes [19]. Hence, the total caffeic acid content was measured after basic hydrolysis as an indirect measure of the total caffeoylquinic acid. The content of caffeic acid followed a distribution in the different samples similar to that of chlorogenic acid. These results are unsurprising as chlorogenic acid is the most abundant caffeoylquinic derivative in artichoke, and, consequently this compound represents the main source of caffeic acid during the process of hydrolysis [39].

Luteolin and apigenin are the main flavonoids in globe artichoke and are present in the tissues as glycoside derivatives [39]. In this study, to assess the content of these two flavonoids, the extracts of the different samples were subjected to acid hydrolysis to free the aglycone from the saccharide moieties. In the "*Carciofo di Malegno*", luteolin was detected in significant amounts only in the stems and in the edible parts of the capitula.

Conversely, a significative content of apigenin was detected only in the edible and non-edible parts of the capitula. The content of these two flavonoids in the different samples was comparable with that of the other cultivars.

These results suggest that the receptacle and inner bracts of the "*Carciofo di Malegno*" may represent an excellent dietary source of these two flavonoids. Moreover, the stems together with the outer non-edible parts of the capitula, which are typically considered as waste, may be used in herbal fields for the preparation of flavonoid-rich extracts in terms of circular economy.

From a phytochemical perspective, the "*Carciofo di Malegno*" has similar properties to the commercial artichokes and for this reason can be used for food (although this variety is smaller and therefore is not very competitive with others) and herbal use. These bioactive molecules are interesting for human health and can be used for the production of innovative-functional products too.

This information is essential to encourage farmers and land managers to create new local supply chains that allow the sustainable development of mountain areas and, at the same time, the in situ conservation of the landrace. In fact, much crop diversity is now held ex situ in gene banks or breeders' materials rather than on-farm (in situ) [4].

To contrast this trend, the MaxEnt distribution model was combined with R language to predict the potential distribution of "*Carciofo di Malegno*" in the north of Italy under current and future climatic conditions.

Based on the analysis of the ecological niche of the Malegno artichoke, it emerged that it can currently be grown in a fairly restricted area in Lombardy (the valley floor of the Camonica and Valtellina valleys). However, considering climate change, it is probable that in the coming decades the area where this landrace could be cultivated will be much wider, including a large part of the pre-Alps and Alps of Lombardy (Figure 5).

In effect, due to the global warming that will affect the study area in the coming decades, the climatic optimum of the "*Carciofo di Malegno*" could be located at higher altitudes than the current ones. This scenario could make cultivation difficult (in situ conservation) of this landrace in the area where it is and has been cultivated up to now (Malegno), and therefore it is possible that the "*Carciofo di Malegno*" (as well as other landraces) could become a traditional variety of a territory and be cultivated outside that territory.

Any translocation of the "*Carciofo di Malegno*" outside the area where it is traditionally cultivated would create problems for its protection/enhancement through the current regulatory instruments for agrobiodiversity protection. In fact, both the European Register of Conservation Varieties [40] and the Italian Register of Agrobiodiversity protect the landraces and the geographical area where they are traditionally cultivated (and not the areas where they may be cultivated in the future). This problem/paradox could be solved by allowing a ten-year update of the registers and differentiating the area where landraces were traditionally cultivated (in the past) and the area (or areas) where is possible to cultivate them based on the contingent environmental conditions.

It is very probable that in the future the "*Carciofo di Malegno*" will be able to be cultivated at higher altitudes than the current ones (since, according to the model used in this research, they will have the same climatic conditions) while it is less clear if in the future it will not be possible to grow it in the current areas (Malegno and the valley floor of Val Camonica in general) where the climate will be warmer.

Under the SSP145 scenario, horizon 2021–2040, the expansion of "*Carciofo di Malegno*" will increase, reaching other zones in the north of the Lombardy region, like high Camonica Valley, Valtellina, and other areas in the Alps. The tendency of this landrace to encroach hilly and mountain areas is presumably based on the temperature change that among climate factors plays an important role in the distribution of artichokes (Figure S1). However, in the 2041–2060s, the future potential habitat of "*Carciofo di Malegno*" under the SSP145 scenario seems to be less expanded than the previous temporal horizon. In fact, the temperature probably will be too high such that it will be impossible to grow artichokes in the original zone.

In fact, the plasticity (understood as the ability of plants to adjust their phenotype in response to different environmental conditions) [41] of this landrace (as well as that of most landraces) is not known; therefore its ability to grow and produce in different environmental conditions is not known. Ad hoc studies (ecological and/or physiological) could be conducted to clarify the real adaptive capacity of the "*Carciofo di Malegno*" as it would be interesting to understand, through genetic analyses, if the few custodian farmers have different populations of "*Carciofo di Malegno*" or the same genotype. Although the "*Carciofo di Malegno*" is traditionally propagated by agamic means (with the suckers), it is not improbable that there would be a genetic difference (even minimal) among the populations of the local farmers. If this were confirmed, cross-fertilization programs (among populations) and sexual propagation (with seeds) of the "*Carciofo di Malegno*" could be planned in order to minimize genetic erosion, improve genetic mixing, and promote evolution and adaptation of the landrace to environmental changes [42].

For example, Bonasia et al. [28] determined the total polyphenolic concentration and the antioxidant activity in five artichoke varieties of which three were hybrid. The latter showed a lower amount of polyphenols than the traditional cultivars, particularly in leaf waste. Mauromicale and his co-workers [43] carried out a two-year study wherein they shifted the harvest period of seed-grown globe artichokes on a new F1 seed-grown hybrid of artichoke [*Cynara cardunculus* L. var. *scolymus* (L.) Fiori] including "Violetto di Sicilia," traditionally vegetatively propagated, as a comparison. The hybrid sample produced heads with low weight during late autumn—early winter; on the contrary, for "Violetto Siciliano" the effects of the studied factors were less evident.

The "*Carciofo di Malegno*" could also be an interesting genetic resource for commercial variety improvement programs [15,44]. In fact, the ability of the "*Carciofo di Malegno*" to grow (and complete its biological cycle) in an Alpine valley that has decidedly lower temperatures than those of the Mediterranean areas could be enhanced with the production of varieties/hybrids capable of growing in cold areas [16]. The latter could be used to extend the cultivation of artichokes outside the Mediterranean area in countries with cooler climates. For Italy, which is the most important producer of artichokes among European countries (more than 44,000 ha and 406,000 tons per year [39]), it would mean extending production to the mountain areas of the Apennines and the Alps.

5. Conclusions

This research has allowed us to characterize from the morphometric and phytochemical point of view the artichoke of Malegno from which it emerged that this landrace was distinct from any other artichoke sample due to its spiky shape, and it appears to be similar to those belonging to the "Spinosi" group because of its thorny shape. Modern geometric morphometric analyses better describe and compare shapes and forms, and they could integrate the classical morphological description of the varieties. The concentration of chlorogenic acid and cynarine in "*Carciofo di Malegno*" was comparable to that of the commercial cultivars. In "*Carciofo di Malegno*", luteolin was detected in a significant amount only in the stems and in the edible parts of the capitula. In contrast, apigenin was detected only in the edible and non-edible parts of artichokes.

The area where the "*Carciofo di Malegno*" could be cultivated has been explored. GMM analysis has shown that in the coming decades (2040–2060s) it can be grown on a larger area than the current one, reaching another mountain environment (also outside Camonica Valley). The results of the characterization (morphological and phytochemical) and the analysis of the ecological niche of the "*Carciofo di Malegno*" will be useful to begin the registration practices to register it in the Italian Register of Agrobiodiversity, promoting events of dissemination of knowledge on the territory and therefore preserve this landrace in situ (on farm). This work is an example of how researchers and stakeholders should collaborate to encourage landraces in situ conservation, creating positive incentives for farmers and enterpreneurs to support territories that hold unique and little-known agri-food resources to establish a new supply chain (healthy agri-food and/or herbal). The latter could allow sustainable development of mountain areas.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants13050680/s1, Figure S1: Bioclimatic factors' contribution to "*Carciofo di Malegno*" distribution.

Author Contributions: Conceptualization D.P., L.G. and A.G.; methodology, D.P. and M.Z.; validation, M.Z., L.G. and A.G.; formal analysis, D.P., M.Z. and L.G.; investigation, D.P. and M.Z.; resources, D.P. and M.Z.; data curation, D.P., M.Z. and L.G.; writing—original draft preparation, D.P., S.S. and M.Z.; writing—review and editing, D.P., M.Z., S.S. and L.G.; supervision, L.G. and A.G.; project administration, A.G.; funding acquisition, A.G. and L.G. All authors have read and agreed to the published version of the manuscript.

Funding: This study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR)—MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4—D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions; neither the European Union nor the European Commission can be considered responsible for them.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Acknowledgments: We thank Felice Pezzoni and the other custodian farmers and mayor from Malegno for the information provided. We thank Valeria Leoni for her support to the work team in sample collection and historical information research.

Conflicts of Interest: The authors declare no conflicts.

References

1. FAO. *Building on Gender, Agrobiodiversity and Local Knowledge*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2004.
2. FAO. *Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture. Commission on Genetic Resources for Food and Agriculture*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2010.
3. Villa, T.; Maxted, N.; Scholten, M.; Ford-Lloyd, B. Defining and identifying crop landraces. *Plant Genet. Res.* **2005**, *3*, 373–384. [CrossRef]
4. FAO. *Building on Gender, Agrobiodiversity an Local Knowledge—A Training Manual*; FAO: Rome, Italy, 2005.
5. Pedrali, D.; Proserpio, C.; Borgonovi, S.M.; Zuccolo, M.; Leoni, V.; Borgonovo, G.; Bernardi, A.M.; Scarafoni, A.; Pagliarini, E.; Giorgi, A.; et al. Nutritional Characterization and Novel Use of "Copafam" Bean (*Phaseolus coccineus* L.) for the Sustainable Development of Mountains Areas. *Sustainability* **2022**, *14*, 13409. [CrossRef]
6. Giupponi, L.; Leoni, V.; Colombo, F.; Cassani, E.; Hejna, M.; Rossi, L.; Pilu, R. Characterization of "Mais delle Fiorine" (*Zea mays* L.) and nutritional, morphometric and genetic comparison with other maize landraces of Lombardy region (Northern Italy). *Genet. Resour. Crop Evol.* **2021**, *68*, 2075–2091. [CrossRef]

7. Giupponi, L.; Pedrali, D.; Leoni, V.; Rodari, A.; Giorgi, A. The Analysis of Italian Plant Agrobiodiversity Databases Reveals That Hilly and Sub-Mountain Areas Are Hotspots of Herbaceous Landraces. *Diversity* **2021**, *13*, 70. [CrossRef]
8. Giupponi, L.; Leoni, V.; Pedrali, D.; Ceciliani, G.; Bassoli, A.; Borgonovo, G. Morphometric and phytochemical characterization and elevation effect on yield of three potato landraces of the Ligurian Apennines (Northern Italy). *J. Appl. Bot. Food* **2020**, *93*, 234–243. [CrossRef]
9. Giupponi, L.; Borgonovo, G.; Panseri, S.; Giorgi, A. Multidisciplinary study of a little known landrace of Fagopyrum tataricum Gaertn. of Valtellina (Italian Alps). *Genet. Resour. Crop Evol.* **2019**, *66*, 783–796. [CrossRef]
10. EU Commission. EUROPE 2020 a Strategy for Smart, Sustainable and Inclusive Growth. 2010. Available online: https://eurlex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52010DC2020 (accessed on 20 May 2023).
11. National Recovery and Resilience Plan (NRRP). Available online: https://www.governo.it/sites/governo.it/files/PNRR.pdf (accessed on 19 November 2021).
12. United Nations. Transforming Our World: The 2030 Agenda for Sustainable Development. 2015. Available online: https://undocs.org/A/RES/70/1 (accessed on 11 June 2023).
13. De Rossi, S.; Di Marco, G.; D'Agostino, A.; Braglia, R.; Mecca, G.; Canini, A.; Gismondi, A. Influence of environmental conditions on the production of nutraceuticals in Italian edible plant landraces. *Food Res. Int.* **2023**, *165*, 112483. [CrossRef]
14. Giupponi, L.; Pilu, R.; Scarafoni, A.; Giorgi, A. Plant agro-biodiversity needs protection, study and promotion: Results of research conducted in Lombardy region (Northern Italy). *Biodivers. Conserv.* **2020**, *29*, 409–430. [CrossRef]
15. Bonasia, A.; Conversa, G.; Lazzizera, C.; Elia, A. Characterization of targeted phenolic compounds in globe artichoke heads and waste from vegetatively and "seed"-propagation genotype. *Plants* **2023**, *12*, 2579. [CrossRef]
16. Pérez-Esteve, E.; Sałata, A.; Barat, J.M.; Stępniowska, A.; López-Galarza, S.; Nurzyńska-Wierdak, R. Polyphenolic composi-tion of Spanish cultivar of globe artichoke (Cynara cardunculus L. var. scolymus (L.) Fiori). *Acta Sci. Pol. Hortorum Cultus* **2018**, *17*, 177–184. [CrossRef]
17. Pignatti, S.; Guarino, R.; La Rosa, M. *Flora d'Italia*, 2nd ed.; Edagricole: Bologna, Italy, 2017.
18. Enciclopedia Bresciana di Antonio Fappani. Available online: http://www.enciclopediabresciana.it/enciclopedia/index.php?title=MALEGNO_(2) (accessed on 12 June 2023).
19. Lattanzio, V.; Kroon, P.A.; Linsalata, V.; Cardinali, A. Globe artichokes: A functional food and source of nutraceutical ingredients. *J. Funct. Foods* **2009**, *1*, 131–144. [CrossRef]
20. Fratianni, F.; Pepe, R.; Nazzaro, F. Polyphenol composition, antioxidant, antimicrobial and quorum quenching activity of the "Carciofo di Montoro" (*Cynara cardunculus* var. *scolymus*) global artichoke of the Campania region, Southern Italy. *Food Nutr. Sci.* **2014**, *5*, 2053–2062. [CrossRef]
21. Li, A.-N.; Li, S.; Zhang, Y.-J.; Xu, X.-R.; Chen, Y.-M.; Li, H.-B. Resources and biological activities of natural polyphenols. *Nutrients* **2014**, *6*, 6020–6047. [CrossRef]
22. Ganesan, K.; Xu, B. A critical review on polyphenols and health benefits of black soybeans. *Nutrients* **2017**, *9*, 455. [CrossRef]
23. Grosso, G. Effects of polyphenol-rich foods on human health. *Nutrients* **2018**, *10*, 1089. [CrossRef]
24. Gouveia, S.C.; Castilho, P.C. Phenolic composition and antioxidant capacity of cultivated artichoke, Madeira cardoon and artichoke-based dietary supplements. *Food Res. Int.* **2012**, *48*, 712–724. [CrossRef]
25. Gezer, C. Potential health effects of the popular compound of artichoke: Cynarin. *Prog. Nutr.* **2017**, *19*, 5–9.
26. Rau, D.; Attene, G.; Rodriguez, M.; Baghino, L.; Pisanu, A.B.; Sanna, D.; Acquadro, A.; Portis, E.; Comino, C. The population structure of a globe artichoke worldwide collection, as revealed by molecular and phenotypic analyses. *Front. Plant Sci.* **2022**, *13*, 898740. [CrossRef] [PubMed]
27. Giupponi, L.; Giorgi, A. Effectiveness of modern leaf analysis tools for the morpho-ecological study of plants: The case of Primula albenensis. *Nord. J. Bot.* **2019**, *37*, 1–10. [CrossRef]
28. Bonhomme, V.; Picq, S.; Gaucherel, C.; Claude, J. Momocs: Outline Analysis Using R. *J. Stat. Softw.* **2014**, *56*, 1–24. [CrossRef]
29. Claude, J. *Morphometrics with R*, 1st ed.; Springer: New York, NY, USA, 2008; Volume XVIII, p. 317. [CrossRef]
30. R Development Core Team. R: A Language and Environment or Statistical Computing. R Foundation for Statistical Computing. Available online: http://www.r-project.org/ (accessed on 30 September 2023).
31. Jombart, T.; Devillard, S.; Balloux, F. Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. *BMC Genet.* **2010**, *11*, 94. [CrossRef]
32. Nuutila, A.M.; Kammiovirta, K.; Oksman-Caldentey, K.-M. Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. *Food Chem.* **2002**, *76*, 519–525. [CrossRef]
33. Voldoire, A.; Saint-Martin, D.; Sénési, S.; Decharme, B.; Alias, A.; Chevallier, M.; Colin, J.; Guérémy, J.F.; Michou, M.; Moine, M.P.; et al. Evaluation of CMIP6 DECK experiments with CNRM-CM6-1. *J. Adv. Model. Earth Syst.* **2019**, *11*, 2177–2213. [CrossRef]
34. Masson-Delmotte, V.; Zhai, P.; Pirani, A.; Connors, S.L.; Péan, C.; Berger, S.; Caud, N.; Chen, Y.; Goldfarb, L.; Gomis, M.I.; et al. IPCC: Summary for Policymakers. In *Climate Change 2021: The Physical Science Basis, Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*; Cambridge University Press: Cambridge, UK, 2021; *in press*.
35. Phillips, S.J.; Dudík, M.; Schapire, R.E. Maxent Software for Modeling Species Niches and Distributions (Version 3.4.1). Available online: http://biodiversityinformatics.amnh.org/open_source/maxent/ (accessed on 30 September 2023).
36. Jensen, R.J. The Conundrum of Morphometrics. *Taxon* **2003**, *52*, 663–671. [CrossRef]

37. Chitwood, D.H.; Otoni, W.C. Morphometric analysis of Passiflora leaves: The relationship between landmarks of the vasculature and elliptical Fourier descriptors of the blade. *GigaScience* **2017**, *6*, giw008. [CrossRef]
38. Lombardo, S.; Pandino, G.; Mauromicale, G.; Knödler, M.; Carle, R.; Schieber, A. Influence of genotype, harvest time and plant part on polyphenolic composition of globe artichoke [*Cynara cardunculus* L. var. *scolymus* (L.) Fiori]. *Food Chem.* **2010**, *119*, 1175–1181. [CrossRef]
39. De Falco, B.; Incerti, G.; Amato, M.; Lanzotti, V. Artichoke: Botanical, agronomical, phytochemical, and pharmacological overview. *Phytochem. Rev.* **2015**, *14*, 993–1018. [CrossRef]
40. Spataro, G.; Negri, V. The European seed legislation on conservation varieties: Focus, implementation, present and future impact on landrace on farm conservation. *Genet. Resour. Crop Evol.* **2013**, *60*, 2421–2430. [CrossRef]
41. Sultan, S.E. Phenotypic plasticity and plant adaptation. *Acta Bot. Neerl.* **1995**, *44*, 363–383. [CrossRef]
42. Mercer, K.L.; Perales, H.R. Evolutionary response of landraces to climate change in centers of crop diversity. *Evol. Appl.* **2010**, *3*, 480–493. [CrossRef]
43. Mauromicale, G.; Ierna, A. Characteristics of heads of seed-grown globe artichoke [*Cynara cardunculus* L. var. *scolymus* (L.) Fiori] as affected by harvest period, sowing date and gibberellic acid. *Agronomie* **2000**, *20*, 197–204. [CrossRef]
44. Azeez, M.A.; Adubi, A.O.; Durodola, F.A. Landraces and Crop Genetic Improvement. In *Rediscovery of Landraces as a Resource for the Future*; InTechOpen: London, UK, 2018. [CrossRef]

7. Case study III: Grano Siberiano Valtellinese

7.1 *Introduction*

Buckwheat is a dicotyledonous herbaceous plant belonging to the family Polygonaceae, is one such underutilized crop. Although commonly referred to as a pseudo-cereal due to its grain-like use, it is botanically distinct from true cereals. There are two major cultivated species of buckwheat: common (*Fagopyrum esculentum* Moench) and Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) (Bonafaccia & Fabjan, 2003).

In Europe, tartaric buckwheat is currently grown only to a small extent, mainly in the north (because it can withstand harsher climatic conditions), while common buckwheat is grown in the central states (Rufa et al., 2001). Tartary buckwheat has recently gained attention as a functional food ingredient due to its high content of health-promoting compounds. Naturally gluten-free and suitable for celiac individuals, it provides a rich nutritional profile, with high-quality proteins, essential amino acids, and significant amounts dietary fiber (Gullón et al., 2017). Moreover, it is a valuable source of bioactive compounds, particularly polyphenols like rutin and quercetin as well as vitamins and minerals, supporting its use in both food products and dietary supplements (Appiani et al., 2021).

Although the cotyledons and leaves of both contain considerable rutin, concentrations in the seeds of Tartary buckwheat are approximately 100-fold higher than those of common buckwheat (Suzuki et al., 2015; Fabjan et al., 2003). The buckwheat plant contains high levels of rutin (flavonol 3-O-rutinoside) in many organs, including its seeds, cotyledon, leaves, stem, and flowers (Suzuki et al., 2015).

The “Grano Siberiano Valtellinese” is a little-known landrace of buckwheat (*Fagopyrum tataricum* Gaertn) introduced in Italy from the late eighteenth century in the alpine region of Valtellina (Lombardy) and now at risk of extinction as it is grown by an extremely limited number of farmers, mostly hobbyists, and is considered a pest for common buckwheat (Giupponi et al., 2019). The reasons that have led to the abandonment of this crop are mainly due to the abandonment of mountain areas by man and because in these areas for the preparation of typical regional food products common buckwheat is used. In Tartary buckwheat seeds, high levels of rutin content and rutinosidase activity cause strong bitterness, which may effectively protect the seeds from being eaten by animals (Suzuki et al., 2015).

Previous analysis has shown that this landrace is the most suitable for the environmental conditions of the mountain areas of Valtellina as, in these areas, it is able to grow higher and produce more flowers than other genotypes of *F. tataricum* coming from other geographical areas (Giupponi et al., 2019). The analysis of its CSR strategy also showed a particular adaptation of this landrace to the territory in which it has been cultivated. In fact it turned out to be the most stress tolerator among the cultivars considered. In our opinion, the stress to which this landrace would have adapted consists in the low temperatures of the high mountains and the thin soils poor in nutrients and dry.

However, to the best of our knowledge, nothing about nutritional and phytochemical composition of this underutilize landrace was explored.

This study aimed to investigate the nutritional and phytochemical profile of the “Grano Siberiano Valtellinese”, with a particular focus on its antioxidant potential and suitability for valorization in sustainable food systems.

7.2 *Characterization*

The Siberian buckwheat landrace from Valtellina exhibited an interest nutritional profile (Table 6). Its protein content (13.26 ± 0.44%) was in according to Bonafaccia et al (2003) and other authors, confirming its relevance as a protein-rich pseudocereal, with high levels of essential amino acids compared to true cereals (Lițoiu et al., 2025). Lipid content (3.54 ± 0.01%) was higher than the values generally reported in the literature (Bonafaccia et al., 2003; Singh et al., 2024), possibly reflecting an enrichment in polyunsaturated fatty acids (Raina et al., 2025). In contrast, the ash fraction (2.90 ± 0.00%) was consistent with Singh et al. (2024), confirming a relevant mineral contribution. Dietary fiber reached 12.52 ± 0.83 g/100 g, with both insoluble (9.02 ± 0.58 g/100 g) and soluble (3.20 ± 0.41g/100 g) fractions, supporting potential prebiotic functionality and glycemic control effects.

Table 6. nutritional composition of “Grano Siberiano Valtellinese” and its byproduct

	TS	PS
H_2o	9.63 ± 0.33	5.37 ± 0.71
Ash %	2.90 ± 0.00	6.48 ± 0.63
Lipids %	3.54 ± 0.01	1.18 ± 0.23
Protein %	13.26 ± 0.44	2.46 ± 0.09
CHO (g glu/100g)	8.25 ± 0.85	27.78 ± 2.66
TDF (g/100g)	12.52 ± 0.83	61.98 ± 0.52
IDF (g/100g)	9.02 ± 0.58	61.77 ± 0.54
SDF (g/100g)	3.20 ± 0.41	0.25 ± 0.08

TS: “Grano Siberiano Valtellinese” seed; PS: “Grano Siberiano Valtellinese” straw. Key: CHO: carbohydrates; IDF: insoluble dietary fiber; SDF: soluble dietary fiber

In addition to its nutritional composition, this landrace demonstrated a rich polyphenolic profile. The solvent system had a profound impact on the extraction efficiency and profile of phenolic compounds from “Grano Siberiano Valtellinese”. The HPLC analysis revealed the hydroalcoholic extraction yielded a diversified spectrum of phenolics, including hydroxybenzoic and hydroxycinnamic acids (protocatechuic, gallic, chlorogenic, neochlorogenic), flavanols (epicatechin), and flavonol glycosides (rutin and kaempferol rutinoside). Among these, quercetin was by far the dominant phenolic compound, quantified at 20.64 ± 0.57 mg/g, a value in agreement with previous reports describing Tartary buckwheat as one of the richest dietary sources of quercetin aglycone (Zhang et al., 2012). In addition to quercetin, rutin (129.27 ± 6.20 µg/g) and kaempferol rutinoside (26.71± 0.74 µg/g) were also identified, confirming that mixed organic solvents are effective in solubilizing both aglycones and glycosylated flavonoids. The acidic conditions (pH 2) further stabilized these compounds by preventing oxidative degradation and by inhibiting endogenous hydrolytic enzymes such as rutinosidase.

In contrast, the aqueous extraction produced a markedly different phenolic pattern. Rutin and kaempferol rutinoside were not detected, while quercetin was recovered only in trace amounts (200.00 ± 10.00 µg/g). Instead, the aqueous extracts were relatively enriched in more polar phenolic acids, such as protocatechuic (114.77 ± 2.37 µg/g), p-coumaric (8.25 ± 0.36 µg/g), caffeic (2.20 ± 0.07 µg/g), and ferulic acid (1.84 ± 0.17 µg/g). This shift in composition is consistent with the strong activity of rutinosidase, an enzyme highly expressed in Tartary buckwheat seeds, which catalyses the hydrolysis of rutin into quercetin in case of aqueous solvent (Kreft et al., 2006). Although rutin hydrolysis is expected under these conditions, the poor aqueous solubility of quercetin (Srinivas et al., 2010) limits its recovery, as the aglycone readily precipitates or becomes adsorbed to insoluble components of the matrix.

The absence of kaempferol rutinoside in the aqueous extract can be explained by the same mechanisms. Like rutin, it is a glycosylated flavonol whose extraction depends on both solvent polarity and enzymatic stability. Hydroalcoholic solvents efficiently solubilize kaempferol glycosides, whereas in water their extraction is hindered and the compounds are likely degraded or converted during the enzymatic hydrolysis process (Zhang et al., 2012).

Considering the minor phenolic acids, the solvent effect was also evident. In hydroalcoholic extracts, compounds such as gallic acid (9.86 ± 0.55 µg/g), chlorogenic acid (4.38 ± 0.03 µg/g), and quinic acid (14.61 ± 1.46 µg/g) were recovered, whereas they were absent or present in trace amounts in aqueous extracts. Conversely, protocatechuic, caffeic, and ferulic acids were more abundant in the aqueous extract compared to the hydroalcoholic one. This distribution reflects both differences in polarity and compound stability as hydroalcoholic solvents are better suited for extracting less polar and conjugated phenolic acids, while highly polar acids are more readily solubilized in water.

The quantitative differences observed at the compound level were confirmed by total phenolic content and antioxidant assays, reinforcing the functional potential of this landrace. The Folin–Ciocalteu assay revealed low polyphenol content in the straw, with values of 0.68 ± 0.12 g GAE/100 g for the hydroalcoholic extract and 0.10 ± 0.04 g GAE/100 g for the aqueous extract. Similarly, the FRAP assay confirmed that antioxidant capacity was closely dependent on both the amount and type of phenolic compounds, recording 9.75 ± 0.75 g TE/100 g for the hydroalcoholic extract and only 0.26 ± 0.02 g TE/100 g for the aqueous extract.

Table 7. Total phenolic content and antioxidant assessment.

Samples	Total polyphenols (µg/g)	Total phenolic content (g GAE/100g)	FRAP (g TE/100g)	ORAC (µmol TE/g)
TS_ solvent	20873.32 ± 587.89	0.68 ± 0.12	9.75 ± 0.75	914.43 ± 37.57
TS_ H_2O	446.44 ± 174.12	0.10 ± 0.04	0.26 ± 0.02	239.99 ± 22.88

TS: "Grano Siberiano Valtellinese" seed

The hydroalcoholic extract (TS solvent) showed markedly higher Folin and FRAP values due to its richness in flavonols, particularly quercetin, rutin, and kaempferol rutinoside. Conversely, the aqueous extract (TS H_2O), which contained mainly phenolic acids such as protocatechuic, caffeic, and ferulic acid, exhibited substantially lower antioxidant activity
The values observed were consistent with or higher than those reported for other tartary buckwheat accessions, particularly in relation to phenolic acid and flavonoid content (Alvarez-Jubete et al., 2010). Notably, the strong reducing power in FRAP and radical-scavenging activity in ORAC correlated with the abundance of quercetin and phenolic acids, reinforcing the link between phytochemical composition and bioactivity (Zielińska et al., 2012).
These results clearly demonstrate that the choice of solvent system fundamentally alters the phenolic profile recovered from Tartary buckwheat. Hydroalcoholic extraction provides a comprehensive profile, dominated by quercetin and enriched with flavonol glycosides and flavanols, while aqueous extraction favours polar phenolic acids but results in the loss of rutin and quercetin recovery.
Overall, the combination of high-quality proteins, relevant dietary fiber content, and a diverse phenolic profile provides a strong basis to consider "Grano Siberiano Valtellinese" as a promising landrace for both nutritional and functional food applications.

7.3 *Valorization*

The nutraceutical aspect of foods, which in past centuries was not taken into consideration (or indeed not even known), is nowadays highly appreciated by consumers who are increasingly careful about the quality and health properties of the foods they eat (Appiani et al., 2021). Notably, rutin and other functional compounds are not limited to the seeds but is also found in the leaves, flowers, and stems of the plant (Suzuki et al., 2015). However, non-milled plant parts often become waste, despite their potential as sources of natural bioactive and functional molecules. The sustainable recovery and valorization of these compounds could offer promising solutions to current environmental and economic challenges. Traditionally, extraction of such compounds has relied on conventional methods, which are often limited by low selectivity, degradation of heat-sensitive compounds, and the use of hazardous organic solvents. Additionally, a substantial portion of antioxidants exists in the form of insoluble bound phenolic compounds, which are covalently linked to components of the plant cell wall. This makes their extraction more difficult using conventional techniques.

To overcome these limitations, alternative and more sustainable strategies are being explored. Enzyme-assisted extraction allows for more specific and efficient release of antioxidants by targeting structural components of the plant, often requiring fewer steps and enabling the use of the whole plant. Application of high hydrostatic pressure, as previous described for "Copafam" bean, could enhancing the accessibility of the plant cell wall. This may improve the extraction of various bioactive compounds, including soluble dietary fiber, without compromising the nutritional quality of the final product.

Therefore, the second aim of this study was the nutritional and phytochemical characterization of ''Grano Siberiano Valtellinese" by-product (straw). Moreover, the waste was treated by HHP (100, 200 and 400 MPa) either assisted or not by the food-grade enzymes Ultimase® or Ultraflo® to maximize the soluble fiber and phenolic extraction, comparing both enzymes activity.

The "Grano Siberiano Valtellinese" residue was characterized by a nutritional profile dominated by dietary fiber (61.98 ± 0.52 g/100 g dry weight), almost entirely in the insoluble fraction (61.77 ± 0.54 g/100 g), while the soluble fiber content was negligible (0.25 ± 0.08 g/100 g) (Table 6). This composition reflects the structural nature of the matrix, largely composed of cellulose, hemicelluloses, and lignin, which are typical components of plant litters (Dai et al., 2025).

The fiber content and the available carbohydrates (27.78 ± 2.66 g/100 g) represents the major components and is mainly associated with structural polysaccharides, as starch is virtually absent in the field residues.

The protein content (2.46 ± 0.09 %) was markedly lower than that typically reported for buckwheat grains (11–14%) (Lițoiu et al., 2025) confirming that proteins are mostly concentrated in seeds and leaves, while residues remain poor in nitrogenous compounds.

The lipid content (1.18 ± 0.23%) was also modest, as expected for structural plant tissues, but while quantitatively limited, these lipids may contain bioactive molecules such as phytosterols (Dziedzic et al., 2015) and there is a prevalence of unsaturated fatty acids (Bonafaccia & Fabjan, 2003).

The ash content (6.48 ± 0.63%) was relatively high compared to cereal straw that usually were compose by about 3–5% of this nutritional component (Stolarski et al., 2024), suggesting a notable mineral contribution, in agreement with the known ability of buckwheat to accumulate macro- and micro-elements, including potassium, magnesium, and iron, in vegetative tissues (Kreft et al., 2006).

The nutritional characterization confirms that “Grano Siberiano Valtellinese” straw represents a fiber-rich matrix, even if the strong predominance of insoluble fraction suggests limited functional benefits in their raw form, as insoluble fractions are mainly associated with bulking effects rather than prebiotic activity (Slavin, 2013). For this reason, processing strategies such as high hydrostatic pressure and enzymatic hydrolysis become crucial to increase the proportion of soluble oligosaccharides, thereby improving the nutritional and functional value of plant by-product (De La Peña-Armada et al., 2020, 2021).

Hence, data analysis from “Grano Siberiano Valtellinese” byproduct aqueous extract performed at atmospheric pressure were presented in table 8.

The chromatographic profile of the aqueous extraction, conceded control, was characterized by a predominance of the HMWC fraction, consistent with the structural role of cellulose, hemicellulose, and pectic polymers in buckwheat cell walls. The limited presence of LMWC fractions and only trace levels of low-degree polymerization oligosaccharides suggest that in the untreated state, the plant residue is mainly composed of insoluble, high-molecular-weight fibers with low bioaccessibility.

The application of HHP treatments at 100, 200, and 400 MPa, either alone or in combination with enzymatic preparations (Ultimase ®, Ultraflo ®), significantly influenced the release of soluble carbohydrate fractions and altered the monomeric composition of the fiber.

Specifically, pressure-induced disruption of the cell wall matrix enhanced the accessibility of hemicellulosic and pectic polymers, facilitating their hydrolysis into shorter, more soluble oligosaccharides.

Table 8. HPLC-RID analysis of water-soluble carbohydrates released from "Grano Siberiano Valtellinese" by-product after HHP treatment assisted by Ultimase ® and Ultraflo ®.

Peak n°		Control	100 Mpa			200 Mpa			400 Mpa		
			No enzyme	Ultimase	Ultraflo	No enzyme	Ultimase	Ultraflo	No enzyme	Ultimase	Ultraflo
1	HMWC a	48.33 ± 3.71^{a}	-	-	-	-	-	-	-	-	-
2	LMWC a	-	42.74 ± 1.65^{a}	42.97 ± 1.26^{a}	43.05 ± 0.73^{a}	41.82 ± 0.95^{a}	44.48 ± 1.13^{a}	41.55 ± 1.19^{a}	42.22 ± 0.53^{a}	43.91 ± 1.30^{a}	41.99 ± 1.78^{a}
3	LMWC b	-	15.7 ± 0.86^{a}	16.3 ± 0.83^{a}	15.82 ± 0.68^{a}	15.91 ± 1.01^{a}	15.51 ± 0.89^{a}	14.99 ± 0.91^{a}	16.42 ± 0.87^{a}	15.76 ± 0.91^{a}	16.01 ± 0.61^{a}
4	LMWC c	-	2.84 ± 0.21^{ab}	3.6 ± 0.51^{ab}	6.09 ± 0.14^{ab}	3.07 ± 0.22^{b}	3.13 ± 0.24^{ab}	6.02 ± 0.47^{a}	2.98 ± 0.11^{ab}	5.07 ± 0.27^{ab}	7.04 ± 0.32^{ab}
5	LMWC d	4.78 ± 0.41^{a}	2.5 ± 0.25^{a}	2.21 ± 0.11^{a}	2.46 ± 0.19^{c}	2.46 ± 0.05^{a}	2.75 ± 0.07^{a}	2.19 ± 0.28^{c}	2.26 ± 0.14^{a}	2.60 ± 0.03^{b}	2.43 ± 0.15^{d}
6	O-5	2.17 ± 0.18^{a}	-	-	-	-	-	-	-	-	-
7	Cellotriose	1.91 ± 0.36^{a}	-	-	-	-	-	-	-	-	-
8	O-3	0.59 ± 0.57^{a}	3.48 ± 0.19^{b}	3.06 ± 0.43^{b}	3.00 ± 0.43^{b}	2.64 ± 0.10^{b}	3.57 ± 0.41^{b}	2.81 ± 0.57^{b}	2.42 ± 0.14^{b}	3.29 ± 0.51^{b}	3.14 ± 0.58^{b}
9	Cellobiose	8.02 ± 0.12^{b}	3.25 ± 0.11^{a}	3.28 ± 0.33^{a}	3.40 ± 0.17^{a}	3.26 ± 0.04^{a}	3.79 ± 0.10^{a}	3.24 ± 0.24^{a}	3.15 ± 0.10^{a}	3.49 ± 0.04^{a}	3.27 ± 0.25^{a}
10	Disaccharides	4.68 ± 0.84^{a}	7.09 ± 0.21^{b}	6.61 ± 0.27^{bc}	6.71 ± 0.25^{bc}	6.70 ± 0.26^{bc}	7.67 ± 0.42^{c}	6.74 ± 0.27^{bc}	6.58 ± 0.13^{b}	6.67 ± 0.04^{b}	6.67 ± 0.23^{b}
11	Glucose	37.30 ± 1.44^{a}	42.94 ± 0.10^{b}	45.35 ± 0.74^{d}	43.09 ± 0.23^{bc}	42.96 ± 0.13^{b}	45.05 ± 0.14^{d}	42.67 ± 0.27^{b}	42.26 ± 0.35^{b}	44.35 ± 0.20^{cd}	42.77 ± 0.33^{b}
12	Fructose	41.18 ± 2.62^{a}	53.8 ± 0.37^{b}	57.99 ± 0.67^{cde}	59.78 ± 0.65^{cd}	53.50 ± 0.09^{b}	58.04 ± 0.56^{c}	61.22 ± 1.08^{e}	52.60 ± 0.67^{b}	58.89 ± 0.16^{cd}	61.15 ± 0.49^{de}
13	Arabinose	3.14 ± 0.41^{a}	5.84 ± 0.64^{b}	5.44 ± 0.39^{ab}	10.38 ± 0.76^{c}	4.55 ± 0.26^{ab}	4.90 ± 0.47^{ab}	9.91 ± 0.31^{c}	4.53 ± 0.53^{ab}	5.77 ± 0.42^{b}	11.94 ± 2.19^{c}

Data are expressed as g/100 g dry material. Mean values ± standard deviation (n = 3). Different superscript letters in each raw denote significant differences. Regression equations used: Peak 1 was estimated as polysaccharides or high molecular weight carbohydrates (HMWC); Peaks 2–5 were estimated as oligosaccharides and simple sugars or low molecular weight carbohydrates (LMWC); Peak 6 was estimated as verbascose; Peak 8 was estimated as raffinose.

At 100 MPa (Figure 8), only modest modifications were detected, with a slight reduction in HMWC and the initial appearance of LMWC peaks. This suggests that at relatively low pressures, structural disruption of cell wall polysaccharides is limited and only partial solubilization occurs.

In contrast, at 200 MPa, the chromatographic profile showed a clear decrease of HMWC and a concomitant increase of LMWC, indicating pressure-induced depolymerization of hemicellulose and pectin-like domains. These compounds are known prebiotic oligosaccharides, selectively stimulating the growth of Bifidobacterium species (Broekaert et al., 2011).

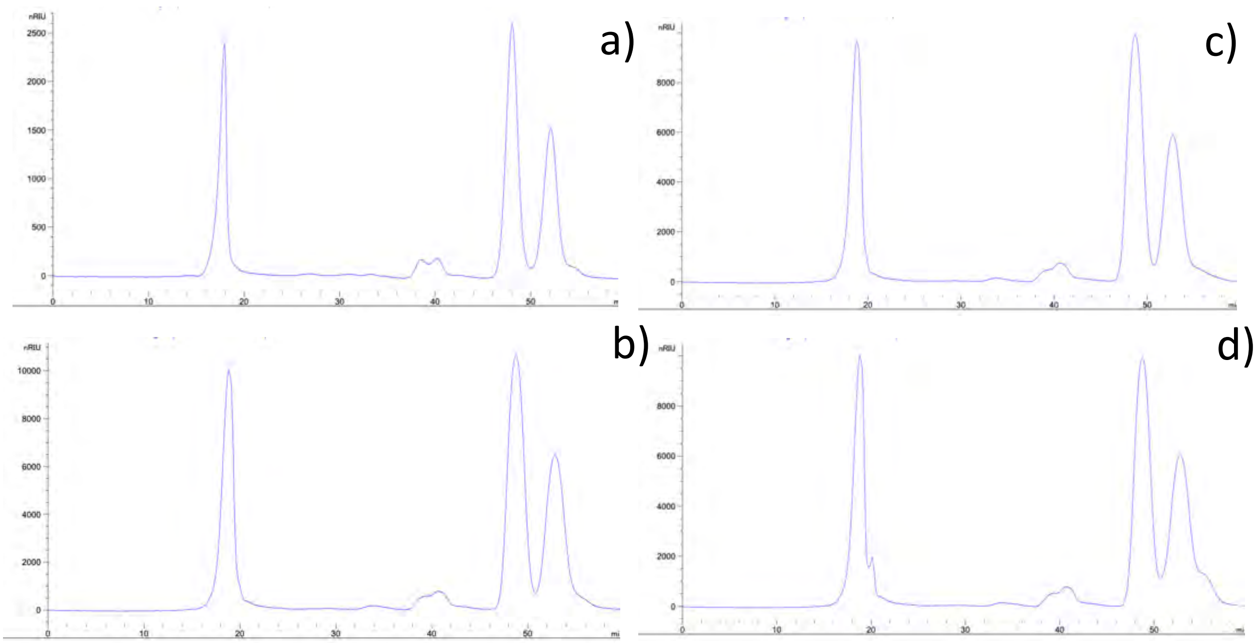


Figure 8. HPLC-RID analysis of water-soluble carbohydrates released from "Grano Siberiano Valtellinese" by-product after HHP treatment assisted by Ultiase ® and Ultraflo ®. Key: (a) control; (b) 100Mpa; (c) 100Mpa+ Ultimase ®; (d) 100mPa + Ultraflo ®.

The effect of enzymatic supplementation was dependent on the enzyme type. Ultimase ®, rich in cellulase and hemicellulase activities, enhanced the release of cellobiose and small oligosaccharides, confirming its capacity to hydrolyze the cellulose backbone and partially degrade hemicelluloses. Ultraflo ®, mainly composed of xylanase and β-glucanase activities, promoted the formation of arabino- and xylo-oligosaccharides, evidenced by the increased intensity of signals in the LMWC region and the higher release of RFOs at both 200 and 400 MPa. These oligosaccharides are considered emerging prebiotic fibers with well-documented effects on gut microbiota composition and short-chain fatty acid (SCFA) production (Broekaert et al., 2011; Slavin, 2013).

Overall, the combination of HHP and enzymatic treatments was effective in the conversion of a lignocellulosic, poorly fermentable matrix into a source of potentially fermentable dietary components represents a valuable strategy for upcycling agricultural by-products into functional food ingredients.

However, numerous authors have reported the synergistic effect of high pressure and enzyme activity for bioactive compounds extraction (Gligor et al., 2019; Kitrytė et al., 2017), achieving, when applying both methodologies together.

The untreated aqueous extraction from buckwheat residues was taken as the reference control, yielding relatively low phenolic content (0.48 ± 0.11 g GAE/100g), reducing power (FRAP 0.66 ± 0.13 g TE/100g), and radical scavenging activity (ORAC 355.74 ± 25.55 μmol TE/g) (Table 9).

Table 9. Polyphenols content and antioxidant activity of "Grano Siberiano Valtellinese" byproduct after HHP treatment.

Samples	Total polyphenols (μg/g)	Total phenolic content (g GAE/100g)	FRAP (g TE/100g)	ORAC (μmol TE/g)
Control	388.87 ± 32.32	0.48 ± 0.11^{e}	0.66 ± 0.13^{a}	355.74 ± 25.55^{c}
PS100	256.54 ± 27.52	0.33 ± 0.05^{abc}	1.88 ± 0.09^{de}	277.05 ± 11.24^{b}
PS100 UT	242.75 ± 0.56	0.28 ± 0.05^{ab}	1.87 ± 0.13^{de}	485.95 ± 35.65^{d}
PS100 UF	278.70 ± 4.10	0.31 ± 0.09^{abc}	1.87 ± 0.13^{de}	264.11 ± 10.71^{a}
PS200	254.89 ± 6.58	0.25 ± 0.04^{a}	1.60 ± 0.06^{c}	546.33 ± 46.29^{e}
PS200 UT	253.32 ± 1.17	0.32 ± 0.07^{abc}	2.09 ± 0.35^{e}	639.81 ± 4.91^{f}
PS200 UF	224.76 ± 3.07	0.34 ± 0.09^{bc}	1.66 ± 0.18^{cd}	1081.47 ± 76.83^{h}
PS400	274.98 ± 6.55	0.38 ± 0.04^{cd}	1.10 ± 0.05^{b}	704.93 ± 50.11^{g}
PS400 UT	267.92 ± 1.54	0.31 ± 0.05^{abc}	1.01 ± 0.08^{b}	1081.80 ± 85.30^{h}
PS400 UF	299.05 ± 4.10	0.44 ± 0.08^{de}	0.92 ± 0.1^{b}	281.00 ± 15.67^{b}

Key: UT: Ultimase ®; UF: Ultraflo ®

Compared to the control, HHP treatment did not improve total phenolic content (Folin assay), either with or without enzymes; instead of antioxidant capacity (FRAP and ORAC methods). At 200 MPa, ORAC increased to 546.33 ± 46.29 μmol TE/g with pressure alone, and further to 639.81 ± 4.91 μmol TE/g when combined with Ultimase ®. The most remarkable effect was obtained at 200 MPa + Ultraflo ® (PS200 UF), which reached the highest activity across all assays, with ORAC more than tripling the value of the reference extract (1081.47 ± 76.83 μmol TE/g). At 400 MPa, HHP alone improved phenolic recovery compared to the simply aqueous extract (control), but the addition of enzymes did not provide further benefits and, in the case of Ultimase ®, resulted in a decrease of activity.

These findings indicate that moderate pressure (200 MPa) combined with Ultraflo ® was the most effective condition, suggesting a synergistic effect between cell wall disruption by HHP and enzymatic hydrolysis of polysaccharides, which facilitated the release of bound phenolic acids. At higher pressures (400 MPa), enzyme inactivation or phenolic degradation may have limited the extraction efficiency.

Considering the individual phenolics compound, the untreated aqueous extract exhibited the highest concentration, including protocatechuic acid (169.28 ± 3.21 μg/g), gallic acid (12.27±

0.21µg/g), and quercetin (103.62 ± 26.39 µg/g), indicating that simple aqueous extraction was particularly effective for recovering free, soluble phenolic acids and flavonols.

In general, HHP treatments at 100–400 MPa caused a reduction in the recovery of free phenolic acids compared to the control. Protocatechuic acid, for example, decreased from 169.28 ± 3.21 µg/g in untreated straw to 81.97 ± 1.00 µg/g in PS200_UF. Similarly, epicatequin, which was abundant in the control, was not detected in most HHP-treated samples, except for very low amounts at 400 MPa (0.29 ± 0.02 – 0.75 ± 0.01µg/g). This loss is consistent with the known instability of quercetin under pressure and aqueous conditions, which can lead to degradation or transformation into glycosylated derivatives (Corrales et al., 2009).

On the other hand, HHP treatments enhanced the release of bound phenolic acids, such as chlorogenic acid, neochlorogenic acid, and cryptochlorogenic acid. At 400 MPa, for instance, chlorogenic acid increased up to 14.50 ± 0.42 µg/g (PS400_UF) compared to 2.00 ± 0.03 µg/g in control, while neochlorogenic acid rose from 33.16 ± 0.12 µg/g in untreated extract to over 50 µg/g in PS400 and PS400_UF samples. These trends suggest that cell wall disruption at high pressure facilitated the release of esterified hydroxycinnamic acids. The synergistic action of Ultraflo ® at 400 MPa further enhanced this effect, particularly for chlorogenic derivatives.

The enzyme-assisted treatments at 100 and 200 MPa (Ultimase ® and Ultraflo®) did not substantially increase the concentration of phenolics compared to pressure alone. In fact, at 200 MPa the levels of most compounds, including protocatechuic and caffeic acids, were lower than the control. This may indicate that partial enzyme inactivation or phenolic–protein/phenolic–polysaccharide interactions limited the efficiency of extraction under these conditions (Puri et al., 2012). By contrast, the combination of 400 MPa with Ultraflo ® (PS400_UF) resulted in one of the richest profiles of hydroxycinnamic acids, suggesting that at higher pressure the enzyme was still partially active and able to hydrolyze glycosidic linkages, releasing bound compounds.

Overall, the data suggest a trade-off between free flavonols (quercetin) and bound hydroxycinnamic acids: simple aqueous extraction favors free flavonoids, whereas HHP and enzymatic treatments favor the release of bound phenolic acids. This duality highlights the importance of optimizing extraction strategies depending on the target compounds.

Interestingly, rutin, which is typically reported as one of the major phenolics in buckwheat leaves and flowers (Fabjan et al., 2003), was not detected in the dried plant residues analyzed in this study. This discrepancy can be explained by rutin distribution within buckwheat plants is tissue- and stage-dependent, with the highest concentrations occurring in seeds and young leaves, while senescent biomass generally contains lower levels. In addition, rutin is prone to degradation during drying and prolonged exposure to oxygen and light, which may lead to its hydrolysis into quercetin or other derivatives (Kreft et al., 2006). Finally, the extraction method used in this study (aqueous extraction) favors the recovery of phenolic acids and flavonol aglycones, whereas rutin, being a glycosylated flavonoid, is more efficiently recovered using organic solvents (Kreft et al., 2006).

Overall, the results demonstrate that HHP-assisted enzymatic extraction can substantially enhance the antioxidant potential of buckwheat residues, offering a promising green technology for the valorization of this agricultural by-product.

7.4 Conclusion and future activities

The “Grano Siberiano valtellinese” demonstrated a distinctive nutritional and phytochemical profile that supports its potential as a high-value genetic resource. The seeds were characterized by a relevant protein content (13.3%), and a notable dietary fiber fraction (12.5%), Antioxidant assays confirmed a strong bioactive potential, consistent with the richness in phenolic compounds, especially quercetin and phenolic acids.

The processing by-product emerged as a promising matrix for valorization. High-pressure and enzyme-assisted extractions enhanced the release of soluble fiber fractions with prebiotic potential, highlighting opportunities to transform its waste into high-value ingredients for the food, feed sectors and to make herbal teas or cosmetics (*F. tataricum* is included in the European Cosmetic Ingredient database.

By integrating both seeds and by-products into the food supply chain, this landrace can serve as a model for circular economy strategies in mountain agriculture. Valorizing ‘‘Grano Siberiano Valtellinese’’ and the products that derive from it, it could be a strategic action that, in addition to contributing to the protection of agro-biodiversity, could lead to the creation of niche, high-quality food chain in order to create income for the farmers and restaurateurs of Lombardy mountain territories, but not only.

7.5 *Bibliography*

Appiani, M., Rabitti, N. S., Proserpio, C., Pagliarini, E., & Laureati, M. (2021). Tartary Buckwheat: A New Plant-Based Ingredient to Enrich Corn-Based Gluten-Free Formulations. Foods, 10(11), 2613. https://doi.org/10.3390/foods10112613

Bonafaccia, G., & Fabjan, N. (2003). Nutritional comparison of tartary buckwheat with common buckwheat and minor cereals. Acta Agriculturae Slovenica, 81(2). https://doi.org/10.14720/aas.2003.81.2.15303

Broekaert, W. F., Courtin, C. M., Verbeke, K., Van De Wiele, T., Verstraete, W., & Delcour, J. A. (2011). Prebiotic and Other Health-Related Effects of Cereal-Derived Arabinoxylans, Arabinoxylan-Oligosaccharides, and Xylooligosaccharides. Critical Reviews in Food Science and Nutrition, 51(2), 178–194. https://doi.org/10.1080/10408390903044768

Dai, W., Xiao, R., Wei, C., & Yang, F. (2025). Plant litter traits control the accumulation of mineral-associated organic carbon by influencing its molecular composition and diversity. Soil and Tillage Research, 253, 106667. https://doi.org/10.1016/j.still.2025.106667

De La Peña-Armada, R., Villanueva-Suárez, M. J., Molina-García, A. D., Rupérez, P., & Mateos-Aparicio, I. (2021). Novel rich-in-soluble dietary fiber apple ingredient obtained from the synergistic effect of high hydrostatic pressure aided by Celluclast®. LWT, 146, 111421. https://doi.org/10.1016/j.lwt.2021.111421

De La Peña-Armada, R., Villanueva-Suárez, M. J., Rupérez, P., & Mateos-Aparicio, I. (2020). High Hydrostatic Pressure Assisted by Celluclast® Releases Oligosaccharides from Apple By-Product. Foods, 9(8), 1058. https://doi.org/10.3390/foods9081058

Dziedzic, K., Górecka, D., Marques, A., Rudzińska, M., & Podolska, G. (2015). Content of phytosterols in raw and roasted buckwheat groats and by-products. Czech Journal of Food Sciences, 33(5), 424–430. https://doi.org/10.17221/121/2015-CJFS

Fabjan, N., Rode, J., Košir, I. J., Wang, Z., Zhang, Z., & Kreft, I. (2003). Tartary Buckwheat (Fagopyrum tataricum Gaertn.) as a Source of Dietary Rutin and Quercitrin. Journal of

Agricultural and Food Chemistry, 51(22), 6452–6455. https://doi.org/10.1021/jf034543e

Giupponi, L., Borgonovo, G., Panseri, S., & Giorgi, A. (2019). Multidisciplinary study of a little known landrace of Fagopyrum tataricum Gaertn. Of Valtellina (Italian Alps). Genetic Resources and Crop Evolution, 66(4), 783–796. https://doi.org/10.1007/s10722-019-00755-z

Gullón, B., Lú-Chau, T. A., Moreira, M. T., Lema, J. M., & Eibes, G. (2017). Rutin: A review on extraction, identification and purification methods, biological activities and approaches to enhance its bioavailability. Trends in Food Science & Technology, 67, 220–235. https://doi.org/10.1016/j.tifs.2017.07.008

Kreft, I., Fabjan, N., & Yasumoto, K. (2006a). Rutin content in buckwheat (Fagopyrum esculentum Moench) food materials and products. Food Chemistry, 98(3), 508–512. https://doi.org/10.1016/j.foodchem.2005.05.081

Kreft, I., Fabjan, N., & Yasumoto, K. (2006b). Rutin content in buckwheat (Fagopyrum esculentum Moench) food materials and products. Food Chemistry, 98(3), 508–512. https://doi.org/10.1016/j.foodchem.2005.05.081

Lițoiu, A. A., Păucean, A., Lung, C., Zmuncilă, A., & Chiș, M. S. (2025). An Overview of Buckwheat—A Superfood with Applicability in Human Health and Food Packaging. *Plants*, *14*(14), 2200. https://doi.org/10.3390/plants14142200

Puri, M., Sharma, D., & Barrow, C. J. (2012). Enzyme-assisted extraction of bioactives from plants. Trends in Biotechnology, 30(1), 37–44. https://doi.org/10.1016/j.tibtech.2011.06.014

Raina, K., & Chaudhary, A. (2025). Distribution of nutritional components in grains, shoot and flowers of common and tartary buckwheat landrace variety collected from Chamba district of Himachal Pradesh. *South African Journal of Botany*, *183*, 158–166. https://doi.org/10.1016/j.sajb.2025.05.017

Rufa, L., Yunning, Z., Rui, W., & Jianying, L. (s.d.). A Study on the Extract of Tartary Buckwheat I. Toxicological Safety of the Extract of Tartary Buckwheat.

Singh, B., Oberoi, S., & Kaur, A. (2024). Phenolic composition, antioxidant activity and health benefits of Tartary (Fagopyrum tataricum Gaerth) and common (F. esculentum Moench) buckwheat grains: A review. Food Chemistry Advances, 5, 100820. https://doi.org/10.1016/j.focha.2024.100820

Slavin, J. (2013). Fiber and Prebiotics: Mechanisms and Health Benefits. Nutrients, 5(4), 1417–1435. https://doi.org/10.3390/nu5041417

Srinivas, K., King, J. W., Howard, L. R., & Monrad, J. K. (2010). Solubility and solution thermodynamic properties of quercetin and quercetin dihydrate in subcritical water. Journal of Food Engineering, 100(2), 208–218. https://doi.org/10.1016/j.jfoodeng.2010.04.001

Stolarski, M. J., Welenc, M., Krzyżaniak, M., Olba-Zięty, E., Stolarski, J., & Wierzbicki, S. (2024). Characteristics and Changes in the Properties of Cereal and Rapeseed Straw Used as Energy Feedstock. Energies, 17(5), 1243. https://doi.org/10.3390/en17051243

Suzuki, T., Morishita, T., Kim, S.-J., Park, S.-U., Woo, S., Noda, T., & Takigawa, S. (2015). Physiological Roles of Rutin in the Buckwheat Plant. Japan Agricultural Research Quarterly: JARQ, 49(1), 37–43. https://doi.org/10.6090/jarq.49.37

Zhang, Z.-L., Zhou, M.-L., Tang, Y., Li, F.-L., Tang, Y.-X., Shao, J.-R., Xue, W.-T., & Wu, Y.-M. (2012). Bioactive compounds in functional buckwheat food. Food Research International, 49(1), 389–395. https://doi.org/10.1016/j.foodres.2012.07.035

Zielińska, D., Szawara-Nowak, D., & Michalska, A. (2007). Antioxidant capacity of thermally-treated buckwheat. 57, 465–470.

Zielinska, D., Szawara-Nowak, D., & Zielinski, H. (2013). Antioxidative and Anti-Glycation Activity of Buckwheat Hull Tea Infusion. International Journal of Food Properties, 16(1), 228–239. https://doi.org/10.1080/10942912.2010.551308

8. Case study IV: Italian Saffron

8.1 *Introduction*

Crocus sativus L., commonly known as saffron crocus, is a perennial (geophyte) male-sterile triploid species ($2n = 3\times = 24$) of the Iridaceae family cherished for its stigmas which are used to produce saffron, the world's most expansive spice (Winterhalter et al., 2000).

While it is primarily used in the food industry, it also finds applications in the textile and cosmetic sectors. The leading producers are Iran (>150,000 kg/year) and India (>15,000 kg/year), but saffron is also cultivated in several European countries, mainly Greece, Spain, Portugal, France and Italy. In Italy, saffron cultivation covers approximately 35 hectares, with the largest production areas located in Sardinia (about 25 hectares) and in Abruzzo (about 6 hectares) (Gresta et al., 2008). Despite its limited volume (annual production ranges between 450 and 600 kg; OEC, 2019), Italian saffron is cultivated by hundreds of small-scale farms, scattered throughout the national territory, from the southern regions and islands (Cardone et al., 2019; Gresta et al., 2008) to the Alps (Caser et al., 2018; Giorgi et al., 2017; Manzo et al., 2015) but mainly in hilly and sub-mountain areas (Giupponi et al., 2023).

Italian saffron is recognized for its exceptional quality, consistently meeting the highest standards in international classifications. Beyond its traditional use as a high-value spice, *Crocus sativus* L. has increasingly attracted attention as a functional food and herbal product, due to the presence of bioactive compounds such as crocin, picrocrocin, and safranal, which are known for their antioxidant, neuroprotective, and mood-enhancing properties. This multifunctional profile has expanded the relevance of saffron beyond gastronomy and cultural heritage to include potential applications in nutraceuticals and health-promoting products.

In marginal and mountainous regions, such as those found in the Italian Alps, saffron cultivation represents a strategic opportunity for rural diversification and socio-economic regeneration. As reported by Giorgi et al. (2017), saffron cultivation can represent an effective strategy to increase the income of multifunctional farms in Alpine areas, contributing to the sustainable revitalization and development of mountain territories. Its high adaptability to low-input and organic farming systems is particularly relevant in these contexts. In many production areas, saffron is grown without the use of irrigation, synthetic fertilizers, or herbicides, making it a highly suitable crop for farms operating under organic regimes (Giupponi et al., 2019).

Moreover, Italian saffron commands a premium price in the market (approximately € 23/g) and is produced using environmentally sustainable and artisanal techniques. Recent data show that only 1% of Italian saffron growers use agrochemicals, fewer than 10% irrigate their fields, and only 40% of farms are mechanized (Giupponi et al., 2023). These production characteristics, combined with saffron's high product quality, strong cultural significance, and broad pedoclimatic adaptability across the Italian territory, underline its importance as a strategic agri-food and herbal resource.

Considering the increasing interest in saffron production and need to preserve and valorize its heritage, the need was to investigate the quality of Italian saffron according to the ISO 3632 1,2:2010-2011 standards during 2022-2025 seasons. Furthermore, the goal was to build a strong and dynamic knowledge-sharing network involving all the stakeholders, to raise their awareness about the sector.

8.2 *Characterization*

Since 2015, UNIMONT has played a central role in building a national network aimed at enhancing the value of Italian saffron. In collaboration with the VAL.TE.MO. association (https://www.valtemo.it), it provides a saffron quality analysis service available to farmers across the country.

During these three years, a total of 482 saffron samples were analyzed. Results showed that more than 93% of Italian saffron was classified as Category I (the highest quality) according to ISO 3632 standards, while 4% fell into Category II, 2% into Category III, and only 1% was deemed out of category. These results confirm the excellent quality of Italian saffron and highlights the importance of enhancing this valuable supply chain to support the sustainable development of marginal and mountainous areas. Each producer who submits a sample received a detailed report indicating the quality category along with specific values for aroma, bitterness, and color strength and becomes a part of the network.

An interactive map hosted on the UNIMONT website (https://www.unimontagna.it/servizi/analisi-zafferano/) displays 900 Italian saffron producers, the outcome of over ten years of data collection and collaboration (Figure 9). This freely accessible tool helps to visualize the national distribution of saffron cultivation and promotes dialogue, cooperation, and transparency among the supply chain actors. It supports the creation of organized, traceable, and high-quality production systems, reinforcing the value of networking and collective action in this niche sector.

Figure 9. Italian saffron producers map

Beyond conventional UV-Vis spectrophotometric techniques mandated by ISO 3632, the study investigated the applicability of Fourier-transform Near-Infrared (FT-NIR) spectroscopy as a rapid, non-destructive, and environmentally friendly tool for saffron quality evaluation. Chemometric modelling based on FT-NIR spectral data yielded promising results. The Linear Discriminant Analysis (LDA) model achieved high classification accuracy (92.7% in calibration, 86.4% in cross-validation, and 87.0% in prediction) with minimal misclassification and particularly strong specificity for the premium class. Soft Independent Modelling of Class Analogy (SIMCA), although slightly less performant in terms of predictive accuracy, was particularly effective in avoiding incorrect classification of premium samples as high-quality, indicating a higher degree of selectivity in the model.

These findings align with previous work in saffron authentication and composition analysis, confirming that FT-NIR can detect subtle differences in chemical profiles that correlate with quality attributes. The main advantage of FT-NIR lies in its potential for implementation in routine quality control, offering a fast and reagent-free method that does not require destruction of the sample. This could be particularly beneficial for small-scale producers and consortia interested in batch authentication, origin certification, or fraud detection. Moreover, it supports broader sustainability goals by reducing the analytical footprint of saffron quality assessment.

In addition, the current and projected future climatic suitability for the cultivation of *Crocus sativus* L. in Italy, was analyzed. Given its vegetative propagation and limited adaptive capacity to rapid environmental shifts, understanding the ecological niche of saffron is important.

A dataset of 721 georeferenced points corresponding to high-quality saffron production sites (as defined by ISO 3632) was compiled from the Val.Te.Mo. association database. Bioclimatic variables (n=19) were sourced from the WorldClim database and used to construct predictive models using two algorithms: Bioclim and MaxEnt.Species Distribution Models (SDMs) revealed that while a large proportion of the Italian territory is presently suitable for saffron cultivation, particularly the central Apennines and parts of the Po Valley (Figure 10).

However, all models predict a progressive contraction of suitable habitats due to climate change. By 2060, only certain mountain regions in the Central Apennines and the Central-Western Alps will maintain high habitat suitability. The modelled future contraction of saffron's suitable habitat aligns with projections of Mediterranean climate change under the SSP2-4.5 scenario, characterized by moderate warming and altered precipitation patterns. Notably, suitability shifts toward higher altitudes reflect the broader ecological phenomenon of "elevational migration" driven by climate warming. In the Alps, future suitability is concentrated in the central and western ranges, while in the Apennines, potential persists primarily in the central regions (e.g., Abruzzo, Umbria, and Lazio), particularly at elevations above 500–800 m a.s.l.

This work provides valuable insights to support the resilience and suitability of the Italian saffron supply chain in the context of climate chance.

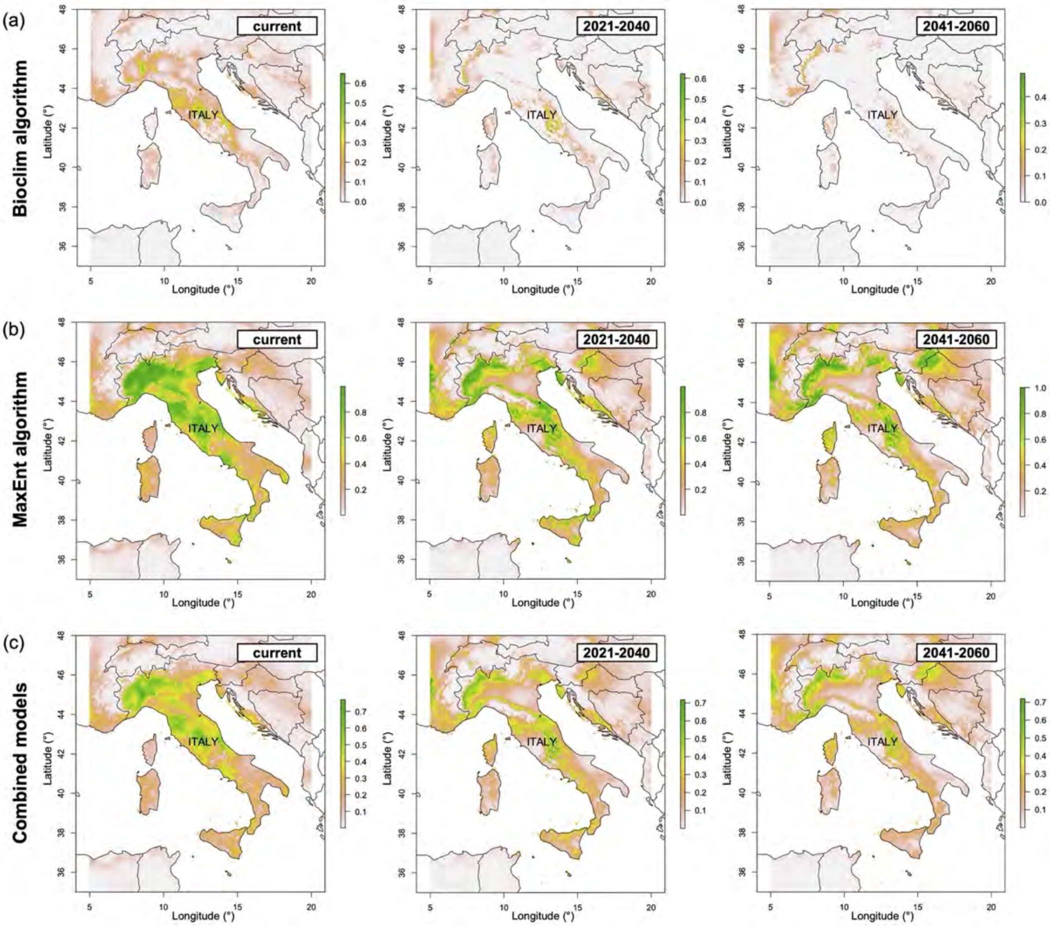


Figure 10. Habitat suitability maps of c. Sativus generated by different models (a, using the bioclim algorithm; b, using the maxent algorithm; c, combining models a and b) and referred to the periods 2016-2020 (current), 2021-2040, and 2041-2060. The color scale indicates the probability of occurrence.

8.3 *Valorization*

High moisture content and low crocin and safranal levels were identified as the main factors contributing to the downgrading of saffron samples. The pigment profile of saffron, particularly crocin, which is primarily responsible for its coloring strength (Giupponi, Ceciliani, et al., 2019).

In this regard, to further enhance the saffron product quality, a new optional subclassification system within the ISO 3632 first category was proposed. This system divides first- category saffron into "premium", "superior" and "high-quality" subcategories, based on coloring strength and bittering power (Figure 11).

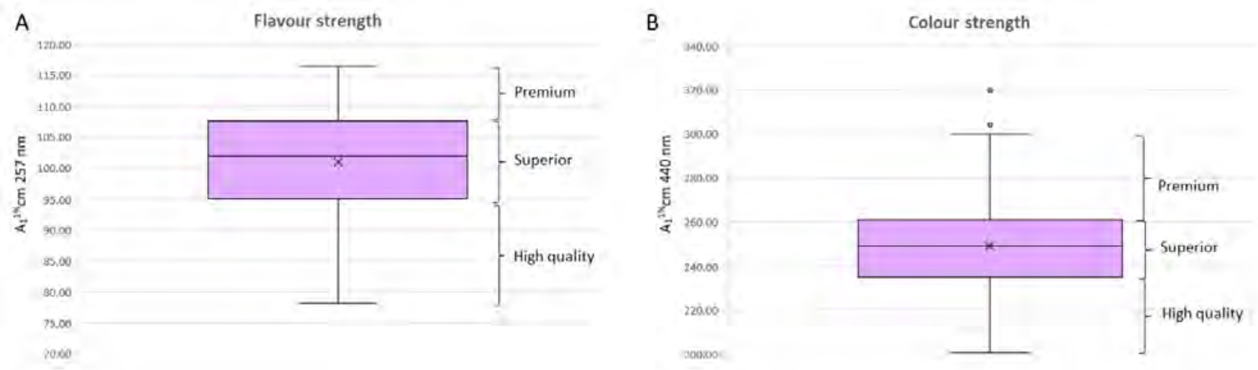


Figure 11: boxplots relating to flavor strength (a) and color strength (b) showing the method of quartiles, used to divide the samples into four subcategories.

It provides a more precise tool to recognize and reward top-quality saffron, opening new opportunities for market differentiation. This new system was be tested in collaboration with farmers and other stakeholders to ensure its effectiveness and promote adoption, encouraging continuous improvement not only in Italy but also globally.

This initiative was supported by a national survey conducted over a two-year period, which collected 48 responses, 58.3% of which came from farmers cultivating saffron for more than three years. The producers provided valuable feedback: 72.9% rated the map as very useful, and 97.9% assigned a satisfaction score of 4 or 5 out of 5 on a 5-point scale. The enhancement of Italian saffron goes beyond laboratory analysis; it relies on building a collaborative and informed community. To effectively promote and safeguard this niche, high-quality product, collaboration among farmers, associations, and researchers is essential. UNIMONT play an active role in disseminating knowledge regarding Italian saffron. One of its core strategies has been to strengthening relationships with local producers. Through technical support, scientific article publications and direct engagement, UNIMONT assists farmers in enhancing their knowledge of cultivation practices and understanding the economic and environmental potential of *Crocus sativus*, even in mountainous areas.

UNIMONT also carries out a wide range of dissemination activities, including the organization of seminars, conferences, technical round tables, and workshops designed to disseminate the latest research and best practices to farmers, citizen and other stakeholders. These resources provide essential cultivation guidelines in a clear and accessible format, helping both new and experienced growers improve their practices.

Social networks also play a key role in community building and knowledge sharing. A Facebook group specifically dedicated to Italian saffron growers (https://www.facebook.com/groups/zafferanoUNIMONT; more than 3,000 members) has become a dynamic platform for dialogue, peer learning, and the exchange of experiences. It facilitates real-time interaction among stakeholders, demonstrating how digital tools and social media can effectively support innovation and collaboration in rural contexts.

The network extends to education and gastronomy. UNIMONT has involved culinary high schools in the development of new saffron-based products, thus linking agriculture with gastronomy and training the next generation of professionals to appreciate and use this precious ingredient.

8.4 *Conclusion and future activities*

Enhancing a high-quality yet niche product like Italian saffron requires strong collaboration between farmers, associations and researchers. Building a network through social media, technical seminars, and updated quality assessment methods is essential to support and promote this sector. Continuing knowledge-sharing efforts that strengthen the connections among researchers and all the actors involved will be crucial to ensuring the sustainable growth and valorization of the Italian saffron value chain.

As highlighted by Giupponi et al. (2019), saffron cultivation plays a strategic role in the development of local agro-food chains, contributing significantly to sustainable agriculture, the conservation of agrobiodiversity, and the implementation of circular economy approaches at both national and European levels.

Further steps could include:

- Support innovation in quality control by implementing the proposed subcategories in collaboration with producers, researchers, and certifying bodies. Promote the adoption of FT-NIR spectroscopy not only for quality assessment but also for detecting adulteration and tracing the geographical origin of saffron.
- Expanding the use of digital tools, including mobile applications for producers, and targeted communication strategies, to enhance outreach, both nationally and internationally.
- Guide cultivation toward climate-resilient areas by providing technical support based on future suitability analyses.

In addition, from the perspective of a circular economy, saffron could be a resource because tepals of *Crocus sativus* (the main waste product during the production phases of saffron) can be used not only as a fertilizer for the soil or as ornamentation of dishes/products based on saffron, but also as a raw material for the cosmetic and pharmaceutical industry since saffron petals have substances (flavonoids and anthocyanins) with antioxidant, anti-inflammatory and antidepressant activity.

8.5 *Bibliography*

Cardone, L., Castronuovo, D., Perniola, M., Cicco, N., & Candido, V. (2019). Evaluation of corm origin and climatic conditions on saffron (Crocus sativus L.) yield and quality. Journal of the Science of Food and Agriculture, 99(13), 5858–5869. https://doi.org/10.1002/jsfa.9860

Caser, M., Victorino, Í. M. M., Demasi, S., Berruti, A., Donno, D., Lumini, E., Bianciotto, V., & Scariot, V. (2018). Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds. Agronomy, 9(1), 12. https://doi.org/10.3390/agronomy9010012

Giorgi, A., Pentimalli, D., Giupponi, L., & Panseri, S. (2017). Quality traits of saffron (Crocus sativus L.) produced in the Italian Alps. Open Agriculture, 2(1), 52–57. https://doi.org/10.1515/opag-2017-0005

Giupponi, L., Ceciliani, G., Leoni, V., Panseri, S., Pavlovic, R., Lingua, G., Di Filippo, A., & Giorgi, A. (2019). Quality traits of saffron produced in Italy: Geographical area effect and good practices. Journal of Applied Botany and Food Quality, 336-342 Pages. https://doi.org/10.5073/JABFQ.2019.092.045

Giupponi, L., Leoni, V., Sala, S., Giorgi, A., & Bertoni, D. (2023). Saffron growing in Italy: A sustainable secondary activity for farms in hilly and sub-mountain areas. International Journal of Agricultural Sustainability, 21(1), 2270263. https://doi.org/10.1080/14735903.2023.2270263

Gresta, F., Lombardo, G. M., Siracusa, L., & Ruberto, G. (2008). Saffron, an alternative crop for sustainable agricultural systems. A review. Agronomy for Sustainable Development, 28(1), 95–112. https://doi.org/10.1051/agro:2007030

Manzo, A., Panseri, S., Bertoni, D., & Giorgi, A. (2015). Economic and qualitative traits of Italian Alps saffron. Journal of Mountain Science, 12(6), 1542–1550. https://doi.org/10.1007/s11629-015-3448-3

OEC, 2019: Saffron trade. Retrieved 18th September 2023, from https://atlas.media.mit.edu/en/profile/hs92/091020/

Winterhalter, P., Straubinger, M., 2000: Saffron: Renewed interest in an ancient spice. Food Rev. Int. 16, 39-59. DOI: 10.1081/FRI-100100281

Article 5:

www.nature.com/scientificreports

scientific reports

Check for updates

OPEN

Progress in quality assessment of Italian saffron

Irene Locatelli[1], Davide Pedrali[2], Silvia Grassi[1]✉, Susanna Buratti[1], Annamaria Giorgi[2,3] & Luca Giupponi[2,3]

Saffron (*Crocus sativus* L.) is the most expensive spice in the World, and Italy is among the major European producers. The study aims to improve value and recognition of high quality saffron by proposing a subdivision within the first quality category, according to ISO 3632 standards. The analysis of 125 saffron samples, collected from different Italian regions in 2021–2022 harvesting seasons, revealed that 95% of the samples met the first quality criteria, following ISO guidelines. Consequently, for the first quality samples, a differentiation into "premium", "superior" and "high-quality" subcategories was proposed. Along with traditional methods, FT-NIR spectroscopy combined with chemometrics was employed for a comprehensive quality assessment. Discriminant and class-modelling approaches were developed to predict saffron quality subcategory. Best results were obtained by linear discriminant analysis models with accuracy in calibration (92.7%), cross-validation (86.4%) and prediction (87.0%). However, SIMCA modelling resulted more appropriate for class-modelling, confirming that none of the "premium" samples were misclassified as "high-quality" and vice-versa. The results support the inclusion of subcategories within ISO 3632 standards, thus refining the classification of saffron quality. Furthermore, the study emphasises the effectiveness of FT-NIR spectroscopy as a valuable tool for saffron quality assessment, with potential implications for industry standards and practices.

Keywords Saffron, ISO 3632, Quality assessment, Subcategories, FT-NIR spectroscopy, Chemometrics

Globally, the spice market is certainly growing, with a value of 7.4 billion USD in 2021 and a growth expectation of about 8.3 billion USD in 2026[1]. Among them, saffron, which is obtained from the dried stigma of *Crocus sativus* L., has the highest economic value[2] and it plays an important role in the culinary culture of various regions of the World due to its glycosidic constituents that impart colouring and flavouring properties[3]. Indeed, saffron quality is determined by its colour, aroma, and flavour, which are related to the levels of crocin and crocetins, safranal volatiles, and picrocrocin[4].

Until today, saffron quality characteristics are only evaluated on a voluntary basis, according to ISO 3632 1,2:2010–2011[5,6]. This standard requires the use of target methods for the quantification of a specific marker, indicative of a particular property, to obtain saffron classification against the established limits. The ISO 3632[5,6] defines three quality categories (I, II and III) according to the content of crocin, safranal, picrocrocin and humidity (Table 1).

The ISO standard establishes that for moisture and aroma strength the values assigned are the same for all three quality categories, whereas the values for colouring and flavour strength are the only parameters that discriminate among the three quality categories (Table 1)[5,6].

In particular, Giupponi et al.[7] demonstrated that saffron produced in Italy is mostly classified in the first ISO class[5,6], with 84–93% falling into this category. This trend was confirmed by Leoni[8] who studied how some good practices adopted by Italian saffron farmers, as flowers being harvested before light exposure and dark conservation, could affect the quality of the spice. In particular, they showed that the quality of saffron seems to be strongly linked to the changes in its pigments, which are responsible for its colouring strength.

The trend leads to the willingness for stricter quality standards able to valorise products which highly exceed the limits to be considered in I category according to the ISO standard, in particular saffron reaching flavour strength above 90 $A_{1cm}^{1\%}$ $(257\ nm)$ and colouring strength above 240 $A_{1cm}^{1\%}$ $(440\ nm)$.

[1]Department of Food, Environmental, and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Via G. Celoria 2, 20133 Milan, Italy. [2]Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy. [3] Department of Agricultural and Environmental Sciences-Production, Landscape, Agroenergy, Università degli Studi di Milano, Milano, Italia. ✉email: silvia.grassi@unimi.it

Characteristics	Specifications categories		
	I	II	III
Moisture and volatile matter content (%)	≤ 12	≤ 12	≤ 12
Flavour strength (picrocrocin)	≥ 70	≥ 55	≥ 40
Aroma strength (safranal)	20–50	20–50	20–50
Colouring strength (crocin)	≥ 200	≥ 170	≥ 120

Table 1. Classification of saffron according to ISO 3632 standard.

Among the stakeholders need there is also the possibility of testing saffron quality by alternative approaches to ISO standards. From this side several methods have been applied for saffron quality assessment, including traditional identification (e.g., crocin, picrocrocin and safranal determination, moisture content) using UV-vis spectrophotometer[9], thermostatic heater as expected by ISO 3632, and HPLC[10]. Traditional identification methods are sensitive but have some disadvantages which can be overcome by non-target techniques, such as NIR, that are inexpensive, timesaving, non-destructive, reagents-saving, and not requiring skilled operators. Additionally, they provide a fingerprint of the analysed food products in a holistic way[11].

In recent years, the interest and the motivation to establish rapid and non-destructive methods for the evaluation of saffron have increased[12] and NIR approaches have been developed to face adulteration and origin issues[13–16].

However, those methods could be also useful to address the stakeholder need for the valorisation of premium products, and, thus, stimulate farmers to continuously improve the produce quality.

In this context, the present study aimed to address two main goals. Firstly, it aims at defining a subdivision within the first quality category based on ISO standard, thus responding to the need of producers to recognize premium products. Secondly, a method based on FT-NIR spectroscopy, combined with chemometric methods, is proposed for the assessment of the new quality sub-classes discrimination.

Materials and methods

Saffron collection and quality analysis

One hundred twenty-five Italian saffron samples were collected from different Italian regions: 5 Calabria, 3 Campania, 23 Emilia-Romagna, 4 Friuli Venezia Giulia, 8 Lazio, 39 Lombardy, 3 Marche, 7 Piedmont, 3 Apulia, 3 Sardinia, 6 Tuscany, 6 Trentino Alto Adige, 15 Veneto. Each sample was analysed in duplicate. Saffron harvested in 2021 and 2022 was stored in specially sealed vials to minimize degradation, in the dark and at room temperature.

Analysis of saffron followed the procedures outlined in ISO 3632 (ISO 3632 1,2:2010–2011)[5,6]. Initially, 500 mg of saffron sample was weighed, dried for 16 h in an oven at 105 °C and reweighed to determine moisture and volatile matter content. Subsequently, 125 mg of powdered saffron was weighed and transferred into a 250 mL volumetric flask. The flask was filled to volume with Millipore water and stirred at room temperature for 1 h using a magnetic stirrer bar (1000 r/min). After stirring, an aliquot of the solution was filtered into a volumetric cylinder, discarding the initial 40 ml and retaining the subsequent 20 ml. Finally, 10 ml of solution was transferred to a 100 ml volumetric flask, filled to volume and stirred for a few minutes.

Moisture and volatile matter content, W%, expressed as a percentage of the initial sample, was calculated according to formula (1):

$$W\% \frac{m_0 - m_f}{m_0} * 100\% \quad (1)$$

where m_0 represents the initial mass (g) of the test sample, and m_f represents the mass (g) of the dry residue.

The resulting solution was analysed using a UV-vis spectrophotometer (Varian Cary 50). Absorbance measurements were taken at 257 nm, 330 nm and 440 nm, corresponding to picrocrocin, safranal and crocin molecules, respectively by zeroing with Millipore water and using quartz cuvettes.

The value of A1cm1% was calculated with the following formulae (2, 3, 4):

$$\text{Colouring strength} : A^{1\%}_{1cm}\,(440\ nm) = \frac{A440 * 2000}{W\%} \quad (2)$$

$$\text{Flavour strength} : A^{1\%}_{1cm}\,(257\ nm) = \frac{A257 * 2000}{W\%} \quad (3)$$

$$\text{Aroma strength} : A^{1\%}_{1cm}\,(330\ nm) = \frac{A330 * 2000}{W\%} \quad (4)$$

The results obtained from the UV-Vis spectrophotometer allowed the samples to be classified according to ISO 3632 standard[5,6], as can be shown in the table (Table 1).

FT-NIR analysis

The MPA FT-NIR spectrophotometer (Bruker Optics, Milano, Italy), managed by the software Opus™ (v. 6.5, Bruker Optics, Milano, Italy), was used for saffron powder analysis. Measurements were performed in reflection, using an integrating sphere, and spectra collection was in the range of 12,500–3800 cm^{-1}, with a resolution of 8 cm^{-1}, and with each measurement consisting of 32 scans for both samples and background[13]. To carry out the analyses, special vials with 0.5 ± 0.01 g of saffron powder were used; two vials were filled for each sample and each of them was analysed in duplicate.

Statistical data analysis

Data exploration was performed in the MATLAB environment (v. 2017b, Mathworks, Inc., Natick, MA, USA) using the PLS toolbox (v. 8.5, Eigenvector Research, Inc., Aeattle, WA, USA). Classification models and variable selection were performed by the V-Parvus package[17].

- Principal component analysis.

Principal component analysis (PCA) was applied to explore sample distribution based on the data collected according to ISO analytical procedures and FT-NIR spectra. As an unsupervised exploratory procedure, PCA allows visualization, in a reduced space, of relationships between items (via the score plot), variables (via the load plot) and their relationship (via the bi-plot)[18].

PCA was applied to ISO results to identify sample grouping, which were further classified into (i.e., "premium", "superior", "high-quality") to be used as a priori information in classification model development.

For FT-NIR data, PCA was applied after spectral range reduction and pre-treatments. Specifically, the NIR spectral range was reduced to the region between 9000 and 3800 cm^{-1}, and different spectral pretreatments, such as Standard Normal Variate (SNV) and first derivate (Savitzky–Golay filter) were applied alone or in combination. Samples were colour-coded based on the quality subcategories identified after the PCA on the ISO results.

PCA was also used to identify outliers by calculating the Euclidian distance of each sample from the centre of the N-dimensional principal component space.

- Classification model development.

The development of classification models for saffron quality prediction involved the associating the FT-NIR spectra with an a priori information. Subcategories, previously identified by the characterization of saffron using ISO methods, were used to define the classes ("premium", "superior", "high-quality").

The classification models were developed by Linear Discriminant Analysis (LDA) and Soft Independent Modelling of Class Analogy (SIMCA).

As LDA, a supervised pattern recognition method, utilizes discriminant canonicals to calculate the centre of matrix covariance, requires the number of samples exceeding the number of variables a feature selection approach (fifteen wavenumbers) was implemented using the SELECT algorithm[17,19].

SIMCA, a supervised classification method developed by Wold and Sjöström[20], followed the same feature selection approach as LDA, then an independent PCA on the spectral variables of calibration sets for the considered subcategories ("premium", "superior", "high-quality") is performed. In the PCA-reduced space, SIMCA constructs a multidimensional space for classifying external test set samples based on the distance between each sample and the models. SIMCA creates models equal to the number of classes, leading to sample assignments as follows: (1) exclusive assignment to one class; (2) no assignment to any class; (3) fitting two or more classes.

Supervised classification models were validated through both internal cross-validation, by 5 cancellation groups, and prediction, by an independent random external test set containing around 30% of the total data.

Model performance was evaluated in terms of sensitivity (TP/(TP + FN)), specificity (TN/(TN + FP)), and correct classification percentage, a.k.a. the proportion of samples correctly accepted in the external test set belonging to the modelled class.

Results and discussion

Qualitative characterization

Out of the one hundred and twenty-five samples analysed, 95% ($n = 117$) belong to the first category of quality, whereas five samples (4%) belong to the second category and none to the third category according to the ISO standard[5,6]. Two saffron samples were not classified (nc) due to their high humidity content (> 12%) (Fig. 1); these results are consistent with previous literature findings[7].

The standard deviation values of the whole dataset are higher for colour strength (246.25 ± 25.03 $A_{1cm}^{1\%}\ (440\ nm)$) and flavour strength (99.33 ± 9.09 $A_{1cm}^{1\%}\ (257\ nm)$) than for the other parameters, i.e. aroma strength (28.87 ± 3.89 $A_{1cm}^{1\%}\ (330\ nm)$ and moisture (6.92 ± 1.81%); these findings aligned with previous works[7,8]. Therefore, the division of first category into three subcategories was primarily focused on colour and flavour strength, as illustrated in Fig. 2. The Box and Whisker representation of the flavour strength (Fig. 2A) allowed to identify that most of the samples (interquartile range, IQR) are characterised by 95–107 $A_{1cm}^{1\%}\ (257\ nm)$, this range was preliminary identified as "superior" subcategory.

The range included in the upper whisker, i.e. 107–117 $A_{1cm}^{1\%}\ (257\ nm)$, was preliminary identified as "premium" subcategory; whereas range included in the lower whisker, i.e. 95–78 $A_{1cm}^{1\%}\ (257\ nm)$, was preliminary identified as "high-quality" subcategory. Similarly for the Box and Whisker representation of the colour strength (Fig. 2B) it was identified a "superior" subcategory included in the IQR (235–260 $A_{1cm}^{1\%}\ (440\ nm)$, a "premium"

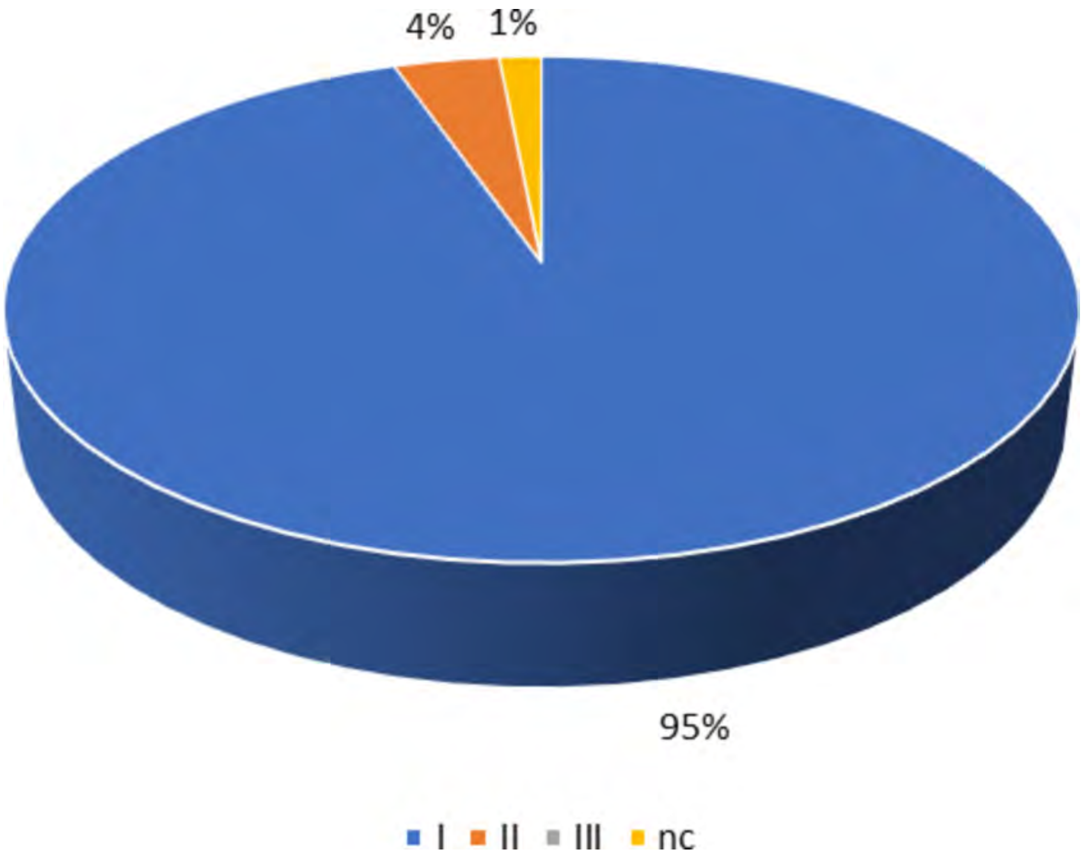


Fig. 1. Result of ISO 3632 for all 125 saffron samples.

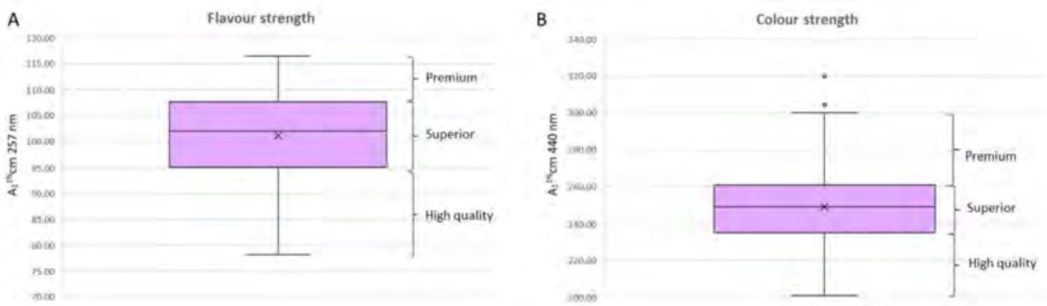


Fig. 2. Boxplots relating to flavour strength (**A**) and colour strength (**B**) showing the method of quartiles, used to divide the samples into four subcategories.

subcategory in the upper whisker, i.e. 260–300 $A_{1cm}^{1\%}$ $(440\ nm)$, and a "high-quality" subcategory included in the lower whisker, i.e. 235–201 $A_{1cm}^{1\%}$ $(440\ nm)$. Samples belonging to the same subcategory for both properties were assigned to that subcategory.

However, some samples could not be unambiguous assigned to a subcategory, so a multivariate exploration of ISO results, by PCA, was used to solve the lack of assignation.

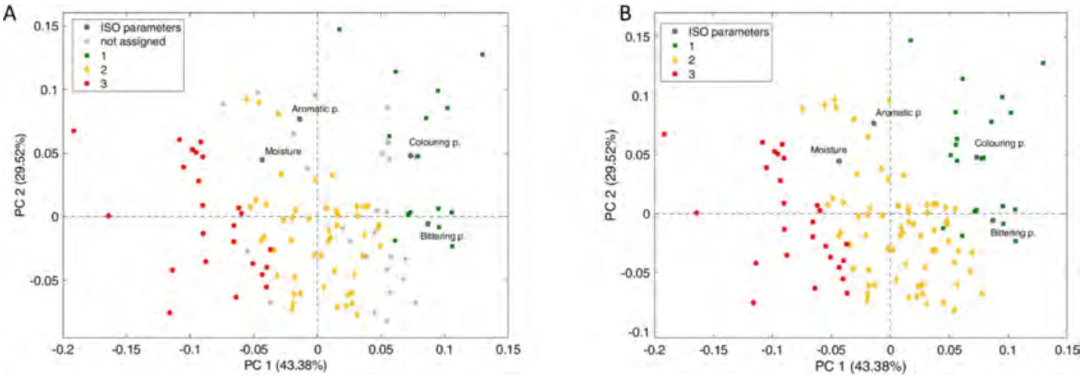


Fig. 3. PCA biplot of ISO data for saffron samples divided into 3 quality subcategories.

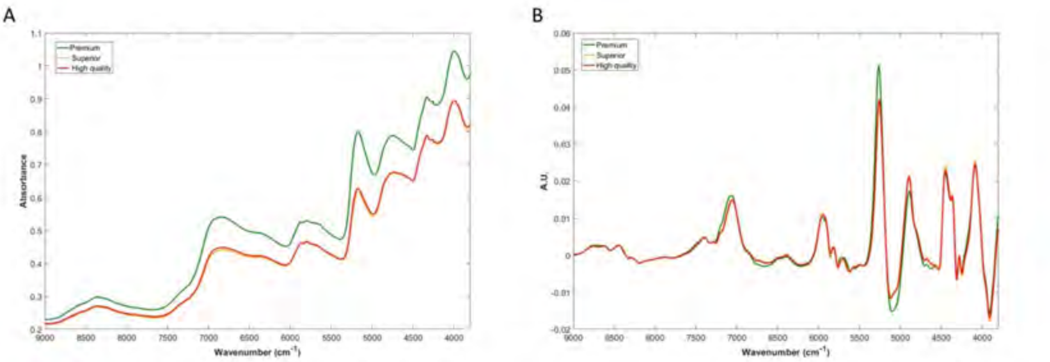


Fig. 4. NIR spectra (9000 and 3800 cm^{-1}) of three samples representing the 3 qualitative subcategories: (**A**) raw NIR spectra, (**B**) spectra after SNV and first derivative transformation.

On the resulting PC1 (43.38% of explained variance) vs. PC2 (29.52% of explained variance) bi-plot (Fig. 3A), samples with unambiguous subcategory assignation, i.e. sample resulting in the same subcategory for both flavour and colour strength, were coloured according to the specific subcategory, whereas samples with ambiguous quality category were marked with a star (*).

Focusing on the distribution along the principal component axis (PC1) for the ISO data, a general trend can be observed: "premium" samples (green squares), align with positive values, "high-quality" samples (red circles) with negative values, and "superior" samples (yellow diamonds) occupy central positions along PC1.

The distribution is explained by the variable position in the bi-plot: aroma strength (a.k.a. aromatic power) as almost no influence on PC1 distribution, having values proximate to 0; moisture confirms to have a negative effect on quality distribution, having negative PC1 values; both flavour strength (a.k.a bittering p.) and colouring strength (a.k.a. colouring p.) contributed to positive positioning of "premium" samples along PC1.

Consequently, to assign the ambiguous samples (marked with a star, *) to a subcategory, the biplot plane of PC1 vs. PC2 (Fig. 3A) was divided into three sagittal portions. Samples within each section were then appropriately assigned (Fig. 3B).

This approach effectively grouped "premium", "superior" and "high-quality" saffron samples, highlighting the importance of a multivariate approach in data exploration when more than two variables are considered.

FT-NIR

- Spectra inspection.

The FT-NIR spectra obtained from the analysis of saffron samples belonging to the first ISO category ($n = 117$) presented similar trends (Fig. 4). Consequently, directly distinguishing sample quality or quality subcategories proved challenging.

In any case, before conducting chemometric analysis on FT-NIR spectra, the spectral features of saffron were examined to gain insight into the functional groups responsible for the observed characteristics. The absorption bands within the FT-NIR spectra (Fig. 4A) can be described as follows[13,16]: (i) at approximately 8300 cm^{-1} there is an absorption associated with the second overtone of C–H stretching; (ii) an absorption band related to the first overtone of O–H stretching or N–H stretching is visible in the range of 7100–6000 cm^{-1}; (iii) the first overtone of C–H stretching is discernible within the range of 6000–5400 cm^{-1}; (iv) around 5170 cm^{-1} a combination of O–H stretching and the first overtone of C–O deformation is observed; (v) at 4750 cm^{-1} a band corresponds to the combination of C–O stretching and O–H deformation; and (iv) at 4320 cm^{-1} there is an absorption associated with the combination of C–C and C–H stretching.

This spectral region (9000 and 3800 cm^{-1}) was utilized for subsequent analysis.

In conclusion, the analysis of FT-NIR spectra provided valuable insights into the chemical composition of saffron samples, guiding subsequent chemometric analysis for quality assessment.

- PCA analysis.

By examining the components PC1 (76.34% of explained variance) and PC2 (11.14% of explained variance) of the FT-NIR spectra, no discernible distribution of samples based on qualitative subcategories was revealed (data not shown). However, through an exploration of PC3 (4.81% of total variance) and PC4 (2.26% of total variance), a distribution of samples according to the previously identified subcategories was evident (Fig. 5A–C).

This became particularly clear plotting PC4 against PC3, as shown in the scores plot in Fig. 5C, samples positioned in the IV quadrant were mainly of "premium" quality, whereas intermediate scores were typical for "superior" samples; "high-quality" samples were positioned in the II quadrant. The region most influencing the shift of samples along PC4 (Fig. 5D, PC4-black line) lay between 4700 and 4000 cm^{-1}, which was also identified as the most insightful region in a study by Castro et al.[21]. Specifically, this region is attributed to the combination band category, characterized by vibrations involving C–H paired with C–C, C–H paired with C–H, and N–H paired with C–H bonds. Chemical compounds frequently associated to these bond vibrations include carbohydrates and proteins[18].

- Quality subcategories identification.

To discriminate the samples according to the three different quality subcategories, the FT-NIR spectra (selected range 9000 and 3800 cm^{-1}) were used to create LDA and SIMCA models. Different spectra pre-treatments were evaluated. Approximately 70% of the samples belonging to the first quality class according to the ISO (79 samples, 13 in premium, 46 in superior, 20 in high quality classes) were allocated for model validation and cross-validation, whereas the remaining 30% (38 samples, 7 in premium, 23 in superior, 8 in high quality classes) were used for predictive performance evaluation. The fifteen variables were selected from the calibration dataset using the SELECT algorithm (Fig. 6).

The best performance was observed for the models after pretreatment with SNV in combination with 1th derivative. Tables 2 and 3 present the results obtained by LDA and SIMCA models, respectively. A highly accurate weighted correct classification rate was achieved with the LDA model (Table 2): 92.75% in calibration, 86.44% in cross-validation, and 87.02% in prediction.

Weighted specificity and sensitivity in calibration were both higher than 90% (specificity of 94.60% and sensitivity of 91.14%) with prediction values ranging between 82.36 and 95.49%, for sensitivity and specificity, respectively. Notably, the discriminant model demonstrated a significant quality, in that no "premium" samples were misclassified as "high-quality" and vice versa. This finding aligns with the researched conducted by Zalacain et al.[14], which underscored the capabilities of NIR spectroscopy for determining the chemical composition of saffron according to ISO 3632 parameters. Indeed, the models developed by Zalacain et al.[14] exhibited an error of 20.40 $A^{1\%}_{1cm}$ for the colour strength and 6.48 $A^{1\%}_{1cm}$ for flavour strength, comparable to the distance between the "premium" and the "high-quality" subcategories, characterised by errors of 26.10 $A^{1\%}_{1cm}$ and 12. 29 $A^{1\%}_{1cm}$, respectively. This also explain why some samples from "premium" and "high-quality" subcategories are confused with the "superior" ones.

For SIMCA model (Table 3), the correct classification rate was 88.59% in calibration. However, this value exhibited a decline during cross-validation and prediction, reaching 69.62% and 61.55%, respectively.

The developed model achieved a weighted specificity of 90.80% in calibration and 82.76% in prediction, while sensitivity in calibration reached 88.61% and 76.69% in prediction. Thus, the model appears to be overfitted on the calibration data, suggesting the need for better sampling considering highest variability to develop a class-modelling approach. In SIMCA model, being based on a class-modelling approach, there may be samples that are not assigned to any class or assigned to multiple classes, thus generating an ambiguous assignment. The graphical representation by Coomans' plot highlights these cases. In Fig. 7 the Cooman's plot of the prediction phase is represented, considering two classes at a time.

In Fig. 7A the axes represent the distances of samples from the models of class 1 (premium) and class 2 (superior), respectively. The two dashed lines correspond to the critical acceptance thresholds for each model at the specified confidence level (in this case 95%). Samples from both classes are plotted as scatter points, with their coordinates reflecting their relative similarity to the two models, and the plot is divided into four sectors. In sector 1 (S1), samples are accepted solely by class 1 (premium), only samples belonging to "premium" class are in this section. In sector 2, samples are accepted only by class 2 (superior), here are also located two samples belonging to "high-quality" class. In sector 3 (S3), samples that are accepted by both models are located. This overlap occurs because the models for each class are built independently, and their class spaces may intersect; here one sample belonging to "Premium" class is present, together with 5 samples belonging to "Superior"

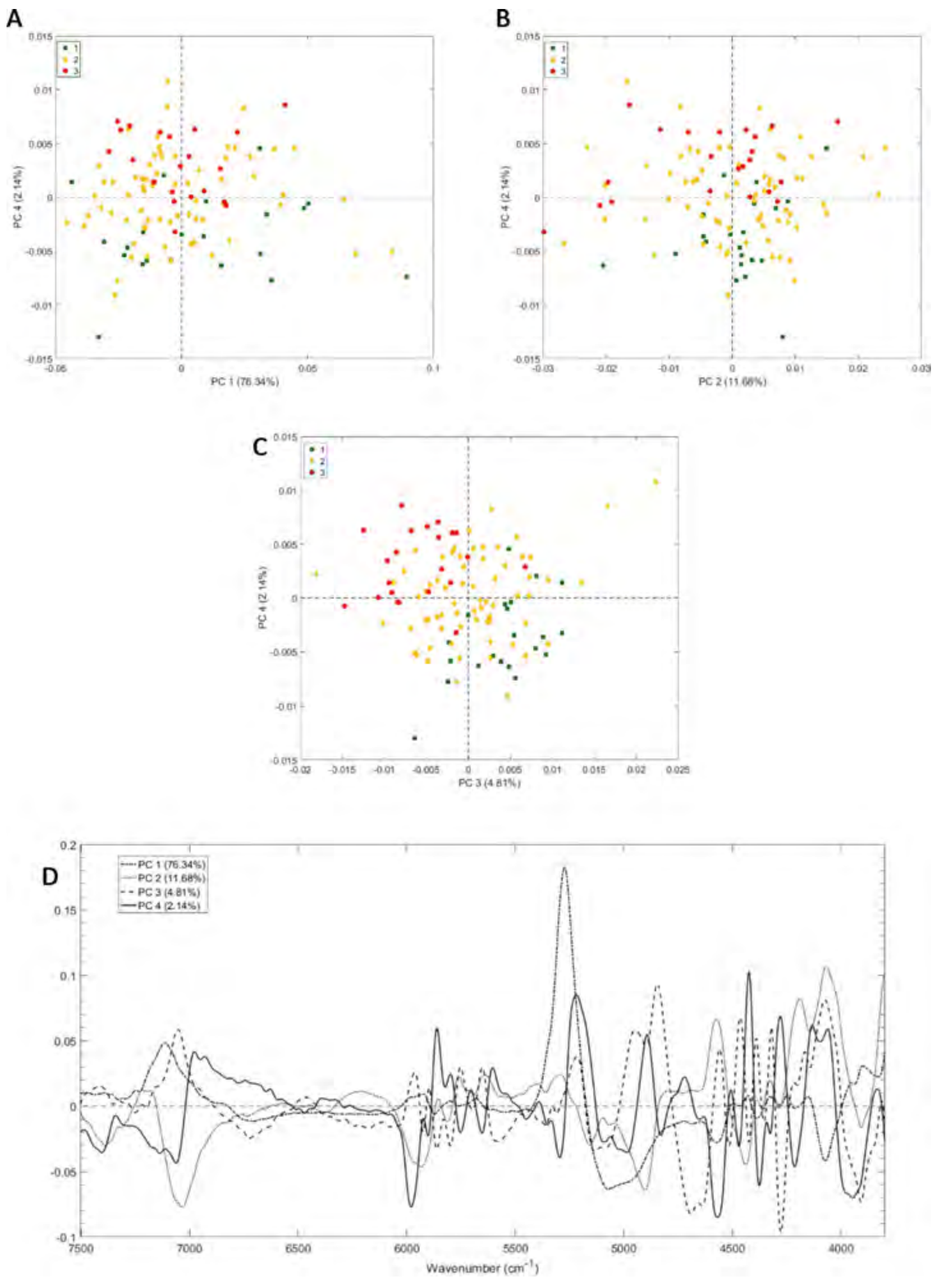


Fig. 5. Score plot PC1 vs. PC4 (**A**), PC2 vs. PC4 (**B**) and PC3 vs. PC4 (**C**) coloured according to the 3 quality subcategories and loading plot (**D**). The plots are obtained from PCA applied on the NIR spectra of saffron samples, pretreated by SNV and first derivate.

class. Finally, in sector 4 (S4) are located six "high-quality" samples that are correctly rejected by both models. Furthermore, some "superior" samples are in this sector, these samples indicate that the variables used do not fully define the class boundaries, preventing forced (and potentially incorrect) classifications that might instead arise in discriminant analysis approaches. Similar consideration could be retrieved from Fig. 7B,C, where it is

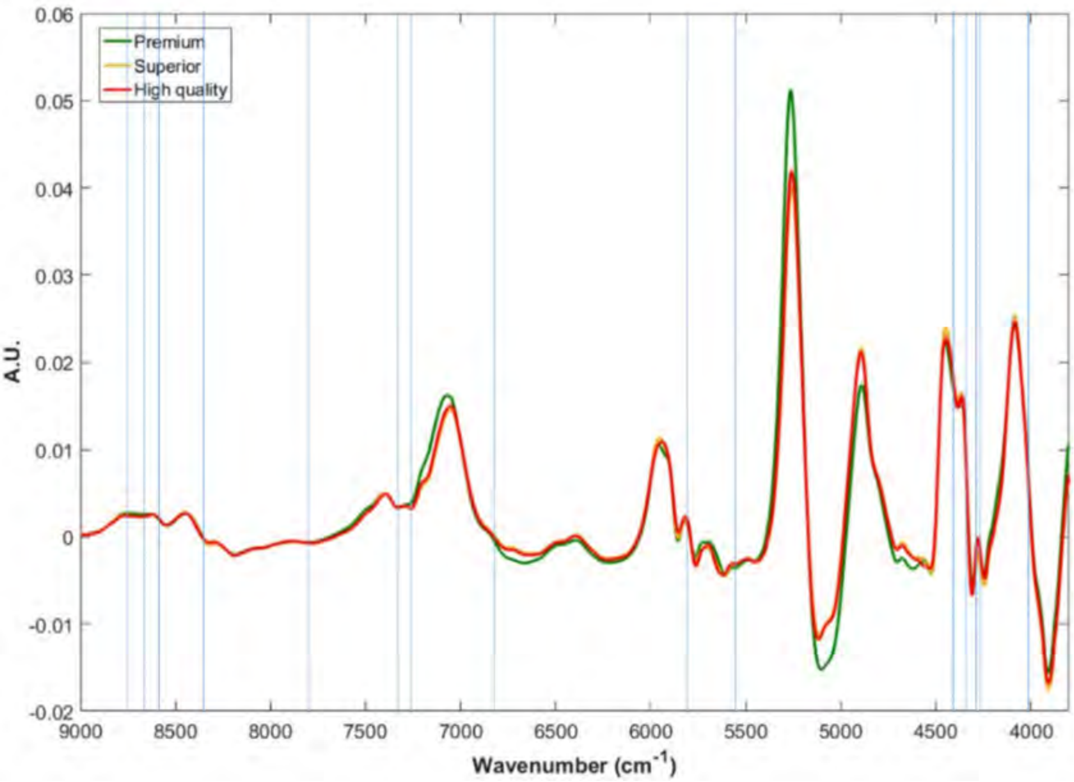


Fig. 6. NIR spectra of three saffron samples, representing the identified quality subcategories, pre-treated with SNV derived first. The vertical lines identify the 15 variables, with greater weight in the classification, used in the models.

	Calibration			Cross-validation			Prediction		
	True class			True class			True class		
	1 Premium	2 Superior	3 High	1 Premium	2 Superior	3 High	1 Premium	2 Superior	3 High
Assigned									
1 Premium	13/13 100%	2/46 4.35%	0/20 0%	12/13 92.31%	3/46 6.52%	0/20 0%	5/7 71.4%	0/23 0%	0/8 0%
2 Superior	0/13 0%	41/46 89.13%	2/20 10.0%	1/13 7.70%	40/46 86.95%	6/20 30.0%	2/7 28.6%	21/23 91.30%	2/8 25.0%
3 High	0/13 0%	3/46 6.52%	18/20 90.0%	0/13 0%	3/46 6.52%	14/20 70.0%	0/7 0%	2/23 8.70%	6/8 75.0%

Table 2. Results of Linear discriminant analysis for saffron qualitative subcategories discrimination: confusion matrix based on the 15 most informative variables selected for FT-NIR techniques.

possible to notice that none of samples of "Premium" class is confused, not even with multiple assignments, with "high – quality" class.

Overall, LDA and SIMCA modelling of the FT-NIR spectra proved to be effective in discriminating between different subcategories of saffron quality. LDA demonstrates high accuracy and specificity, however the assignation to any quality subcategory is always forced. On the other hand the soft approach proposed by SIMCA modelling allowed for perfect discrimination between "premium" and "high-quality" saffron, even if some samples resulted unassigned or multiple assigned.

	Calibration			Cross-validation			Prediction		
	True class			True class			True class		
	1 Premium	2 Superior	3 High	1 Premium	2 Superior	3 High	1 Premium	2 Superior	3 High
Assigned class									
1 Premium	13/13 100%	0/46 0%	0/20 0%	11/13 84.6%	5/46 10.9%	0/20 0%	6/7 85.71%	6/23 26.1%	0/8 0%
2 Superior	0/13 0%	41/46 89.1%	4/20 20.0%	2/13 15.4%	31/46 67.4%	7/20 35.0%	1/7 14.29%	12/23 52.2%	2/8 25.0%
3 High	0/13 0%	5/46 10.9%	16/20 80.0%	0/13 0%	10/46 21.7%	13/20 65.0%	0/7 0%	5/23 21.7%	6/8 75.0%

Table 3. Results of soft independent modelling of class analogy for saffron qualitative subcategories discrimination: confusion matrix based on the 15 most informative variables selected for FT-NIR techniques.

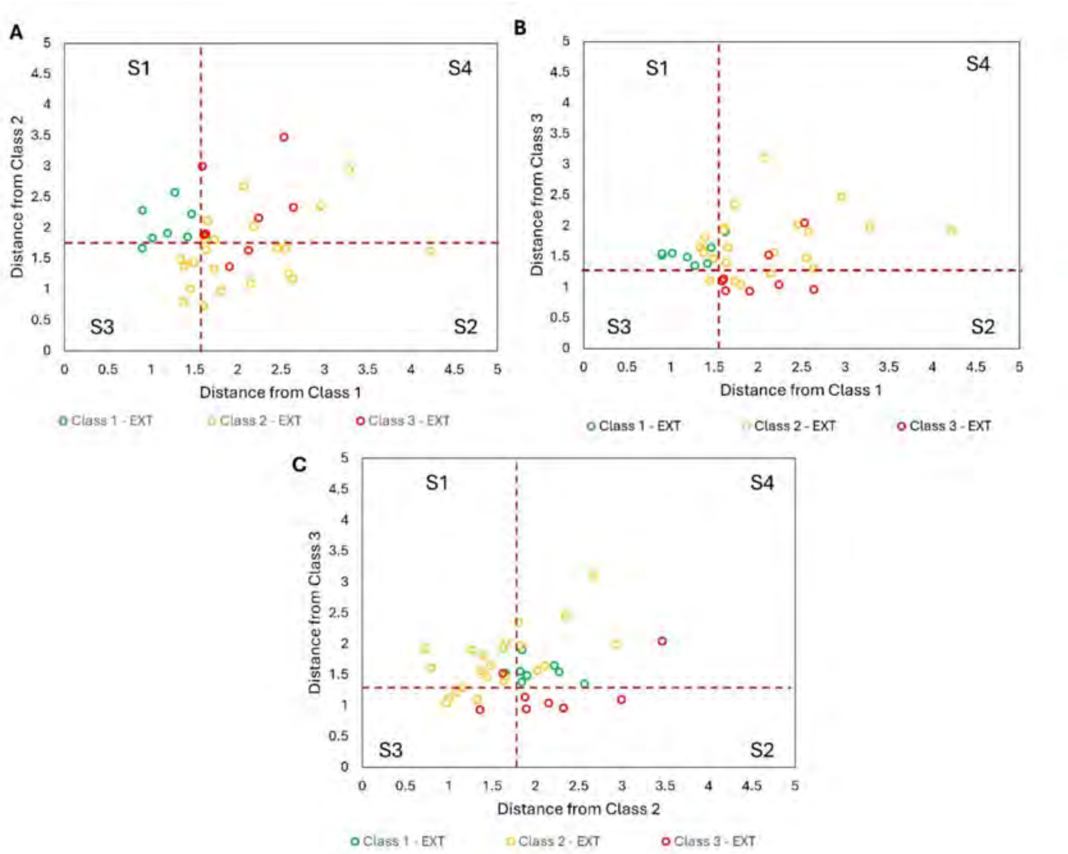


Fig. 7. Cooman's plots resulting from the prediction phase of SIMCA modelling: Class 1 vs. Class 2 (**A**), Class 1 vs. Class 3 (**B**), Class 2 vs. Class 3 (**C**).

Conclusion

This work has confirmed the high quality of Italian saffron, with 95% ($n = 117$) of the samples belonging to the first ISO 3632 quality category. The outcome highlights the success of past efforts in promoting good agricultural practices and remarks the need of recognition of restrictive quality standard.

Thus, to encourage the quality differentiation and the continuous improvement among farmers, this study proposed a subdivision of the first ISO 3632 quality category into "premium", "superior" and "high – quality" based on flavour and colour strength. This subdivision promises to improve the quality recognition of saffron not only in Italy but also on a global scale.

Additionally, this study has successfully developed a FT-NIR spectroscopy approach to address the need of the control bodies for a cost-effective and environmentally friendly method to assess saffron quality. The LDA classification method achieved high predictive accuracy (87.0%) in discriminating between subcategories. The SIMCA model allowed the discrimination between "premium" and "high-quality" saffron. In light of these findings, it would be reasonable to consider the integration in the future analytical standards of the subdivision of the first quality category into subcategories (premium, superior and high-quality), as well as the implementation of qualitative analysis of saffron by NIR spectroscopy.

Data availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

Received: 13 May 2024; Accepted: 10 January 2025
Published online: 16 January 2025

References

1. https://www.businesscoot.com/en/study/the-spice-market-italy (2023).
2. Kiani, S., Minaei, S. & Ghasemi-Varnamkhasti, M. Instrumental approaches and innovative systems for saffron quality assessment. *J. Food Eng.* **216**, 1–10. https://doi.org/10.1016/j.jfoodeng.2017.06.022 (2018).
3. Bagur, M. et al. Saffron: an old medicinal plant and a potential novel functional food. *Molecules* **23** (1), 30. https://doi.org/10.3390/molecules23010030 (2017).
4. Heidarbeigi, K. et al. Detection of adulteration in saffron samples using electronic nose. *Int. J. Food Prop.* **18** (7), 1391–1401. https://doi.org/10.1080/10942912.2014.915850 (2015).
5. ISO 3632-1:2011. Spices:Saffron (Crocus sativus L.)—Part 1: Specification.
6. ISO 3632-2.:2010. Spices: Saffron (Crocus sativus L.)–Part 2: Test methods.
7. Giupponi, L. et al. Quality traits of saffron produced in Italy: Geographical area effect and good practices. *J. Appl. Bot. Food Qual.* **92**, 336–342. https://doi.org/10.5073/jabfq.2019.092.045 (2019).
8. Leoni, V. et al. How harvest, cleaning and conservation good practices affect the quality of saffron: results of a research conducted in Italy. *J. Appl. Bot. Food Qual.* **113**, 105–113. https://doi.org/10.5073/JABFQ.2022.095.014 (2022).
9. Maggi, L. et al. Rapid determination of safranal in the quality control of saffron spice (Crocus sativus L). *Food Chem.* **127** (1), 369–373. https://doi.org/10.1016/j.foodchem.2011.01.028 (2011).
10. Valle García-Rodríguez, M. et al. Determination of saffron quality by high-performance liquid chromatography. *J. Agric. Food Chem.* **62** (32), 8068–8074. https://doi.org/10.1021/jf5019356 (2014).
11. Grassi, S. & Alamprese, C. Spectroscopic non-targeted techniques in combination with linear discriminant analysis for wine vinegar authentication. *Food Bioprocess. Technol.* **17** (2), 479–488 https://doi.org/10.1007/s11947-023-03143-9 (2023).
12. Kiani, S., Minaei, S. & Ghasemi-Varnamkhasti, M. Instrumental approaches and innovative systems for saffron quality assessment. *J. Food Eng.* **216**, 1–10. https://doi.org/10.1016/j.jfoodeng.2017.06.022 (2018).
13. Li, S. et al. Rapid determination of crocins in saffron by near-infrared spectroscopy combined with chemometric techniques. *Spectrochim Acta A: Mol. Biomol. Spectrosc.* **190**, 283–289. https://doi.org/10.1016/j.saa.2017.09.030 (2018).
14. Zalacain, A. et al. Near-infrared spectroscopy in saffron quality control: Determination of chemical composition and geographical origin. *J. Agric. Food Chem.* **53** (24), 9337–9341. https://doi.org/10.1021/jf050846s (2005).
15. Shawky, E., El-Khair, R. M. A. & Selim, D. A. NIR spectroscopy-multivariate analysis for rapid authentication, detection and quantification of common plant adulterants in saffron (Crocus sativus L.) stigmas. *LWT* **122**, 109032. https://doi.org/10.1016/j.lwt.2020.109032 (2020).
16. Amirvaresi, A., Nikounezhad, N., Amirahmadi, M., Daraei, B. & Parastar, H. Comparison of near-infrared (NIR) and mid-infrared (MIR) spectroscopy based on chemometrics for saffron authentication and adulteration detection. *Food Chem.* **344**, 128647. https://doi.org/10.1016/j.foodchem.2020.128647 (2021).
17. Forina, M. et al. PARVUS: An extendable package of programs for data exploration, classification and correlation. *J. Chemometr.* **4** (2), 191–193 (1988). https://hdl.handle.net/11581/110109
18. Wold, S., Esbensen, K. & Geladi, P. Principal component analysis. *Chemometri. Intell. Lab. Sys.* **2** (1–3), 37–52. https://doi.org/10.1016/0169-7439(87)80084-9 (1987).
19. Kowalski, B. R. & Bender, C. F. An orthogonal feature selection method. *Patt. Recognit.* **8** (1), 1–4. https://doi.org/10.1016/0031-3203(76)90023-6 (1976).
20. Wold, S. & Sjöström, M. SIMCA: a method for analyzing chemical data in terms of similarity and analogy. (1977). https://doi.org/10.1021/bk-1977-0052.ch012
21. Castro, R. C., Ribeiro, D. S., Santos, J. L. & Pascoa, R. N. Near infrared spectroscopy coupled to MCR-ALS for the identification and quantification of saffron adulterants: Application to complex mixtures. *Food Control.* **123**, 107776. https://doi.org/10.1016/j.foodcont.2020.107776 (2021).

Acknowledgements

This study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Author contributions

I. L.: Formal analysis; Data curation; Visualization; Writing - original draft.D. P.: Formal analysis; Visualization; Writing - original draft.S. G.: Conceptualization; Investigation; Methodology; Data curation; Visualization; Writing - review & editing. S. B.: Conceptualization; Writing - original draft.A. G.: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing - review & editing.L. G.: Conceptualization; Investigation; Methodology; Writing - original draft; Writing - review & editing.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to S.G.

Reprints and permissions information is available at www.nature.com/reprints.

Article 6: *under review in plants 2025*

plants

MDPI

Article

The species distribution models reveal that mountain areas will be the most suitable for *Crocus sativus* L. in Italy

Luca Giupponi[1,2], Davide Pedrali[1*], Annamaria Giorgi[1,2]

[1] Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas (CRC Ge.S.Di.Mont.), University of Milan, Edolo (BS), Italy
[2] Department of Agricultural and Environmental Sciences - Production, Landscape and Agroenergy (DiSAA), University of Milan, Milan, Italy.
* Correspondence: davide.pedrali@unimi.it

Abstract

Italy is one of the main European producers of saffron, the most expensive spice in the world made from the stigmas of *Crocus sativus* L. Most Italian saffron is of excellent quality and is produced by small-sized farms located in marginal areas, using sustainable and low-input agricultural techniques. This research aimed to identify the current and future (2021-2040 and 2041-2060) areas in Italy where the climatic conditions are suitable for the growth/cultivation of *C. sativus*, using species distribution models. Predictive maps of the current and future distribution of *C. sativus* in Italy were generated using two types of algorithms (Bioclim and MaxEnt), 19 bioclimatic variables, and more than 700 occurrences. Currently, much of the Italian territory has suitable climatic conditions for saffron cultivation, but in the future, all models have highlighted a gradual compression of the *C. sativus* habitat, which will only be able to be cultivated in some mountain areas of the Apennines and the Alps. The MaxEnt model proved to be the most performant (AUC = 0.732) and allowed the identification of the main climatic variables for predicting *C. sativus* distribution. The results of this research will be useful for promoting and enhancing the Italian saffron supply chain.

Keywords: *Crocus sativus*, saffron, MaxEnt, mountain areas, climate change, suitable habitat

1. Introduction

Crocus sativus L., commonly known as saffron crocus, is a perennial (geophyte) male-sterile triploid species (2n = 3× = 24) of the Iridaceae family cherished for its stigmas which are used to produce saffron, the world's most valuable spice [1]. The saffron crocus is native to Greece, specifically to the Attica region [2,3], and its cultivation dates back more than 3,000 years [4]. This species has been cultivated throughout the Mediterranean basin, including ancient Greece and Persia, but also outside of this area, such as in Iran and India [5] because its distinctive flavour, aroma, and colour have led it to occupy a significant place in culinary traditions, medicinal practices, and cultural rituals worldwide, encompassing many cultures, continents, and civilizations.

Currently, the main saffron-producing countries are Iran (>150,000 kg per year) and India (>15,000 kg per year) [6], but *C. sativus* is also cultivated in some European countries (mainly Greece, Spain, Portugal, France and Italy) whose spice productions are significantly lower compared to those of the two aforementioned Asian countries.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date

Citation: To be added by editorial staff during production.

In Italy, saffron has been cultivated since Roman times [5] and today this country is one of the main producers among those of the Mediterranean basin (450-600 kg of spice per year). In Italy, *C. sativus* is cultivated by hundreds of small farms scattered throughout the national territory, from the southern regions and islands [7,8] to the Alps [9–11] but mainly in hilly and sub-mountain areas [12]. Recent studies have shown that the majority (>80%) of the saffron produced by Italian farms is of excellent quality [13,14] according to the standards defined by ISO 3632 which classifies the spice based on flavour strength, aroma strength, colouring strength and moisture content. Furthermore, Italian saffron is sold at a medium-high price (around 23.7 euros per gram) and is produced using sustainable and low-input agricultural techniques: only 1% of Italian farmers use agrochemicals, less than 10% irrigate fields, and only 40% of the farms are mechanized [12].

The high quality, economic (and historical-cultural) value, environmental sustainability of the production chain, and the possibility of cultivation throughout much of the national territory, make saffron a strategically important agri-food and herbal resource for Italy and the European Union (EU). The production of saffron (as done in Italy) can indeed contribute to achieving the goals of the European "Farm to Fork" strategy, including making the European food system more sustainable, primarily by reducing the use of pesticides in agriculture, increasing organic farming, and promoting crop diversification and biodiversity conservation [15]. Furthermore, the saffron supply chain can contribute to the sustainable development of Italy's marginal and mountainous territories following the National Recovery and Resilience Plan (NRRP) launched by the Italian government to address the economic and social challenges arising from the COVID-19 pandemic and to promote economic recovery, innovation, and sustainability [16]. Within the framework of the NRRP, Italy has funded various research and development projects, including "Agritech - National Center for the Development of New Agricultural Technologies" (https://agritechcenter.it/it/), of which the activities of Spoke 7 (where this research falls) focus on the development of marginal areas by promoting multifunctional and sustainable production systems.

One of the yet unaddressed themes of fundamental importance to initiate actions (investments) aimed at supporting the Italian saffron supply chain concerns the analysis of the most suitable environmental conditions for producing a high-quality spice and, above all, identifying the geographical areas where such conditions may emerge in the coming decades within the context of the ongoing climate change [17]. Indeed, although *C. sativus* is a hardy species [8] with high genetic variability [18], its propagation occurring exclusively through vegetative/asexual means (corm duplication) [19] prevents the evolution of populations, hence their ability to adapt to rapid climate change.

This research aims to predict the current and future geographic distribution of *C. sativus* in Italy using Species Distribution Models (SDMs), which are analytical tools used in ecology, biogeography, and conservation biology to forecast the spatial distribution of species based on environmental variables [20,21]. Specifically, based on the occurrence data of saffron crocus (which allows the production of high-quality spice) in Italian territory and the climatic characteristics of the areas where it is present/cultivated, maps of the potential distribution (habitat suitability) of this species in Italy will be produced, thereby providing useful information for its in situ conservation/cultivation and the management and valorization of this agri-food and herbal resource and their respective supply chains.

2. Materials and Methods

2.1. Occurrence data source

The geographical coordinates of 721 areas (occurrence points) where Italian saffron is produced were collected in March 2023 from the database of the "Val.Te.Mo." association (https://www.valtemo.it). This association, which aims to enhance the resources of mountain territories of Italy, has been conducting qualitative analyses (according to the ISO 3632 1,2:2010-2011 standard) of saffron produced in Italy in collaboration with the University of Milan since 2015. The database of the Val.Te.Mo. association is the most comprehensive and up-to-date database regarding saffron produced in Italy (both by farmers and hobbyists), and it was possible to access it following an agreement.

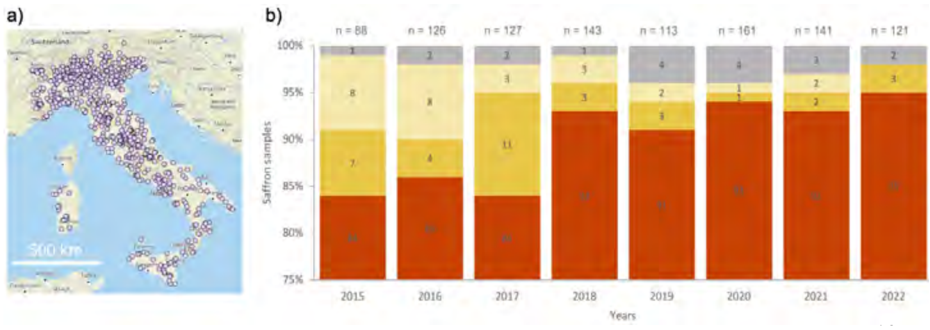


Figure 1. Sites (purple dots) where saffron was cultivated in Italy from 2015 to 2022 (a) and the quality of the spice produced each year (b). Key: I category, saffron of first quality category; II category, saffron of second quality category; III category, saffron of third quality category; n.c., saffron not classifiable according to ISO 3632. Data source: 2015-2018, Giupponi et al. (2019); 2019-2022, database of Val.Te.Mo. association (original data).

Figure 1 displays the sites of Italy where saffron has been produced (from 2015 to 2022) and the quality of the spice across different years. This research only considered the geographical coordinates of areas where top-quality saffron (first-category saffron as defined by ISO 3632 standards) has been produced for more than one year. In detail, top-quality saffron must have the following characteristics: flavour strength (picrocrocin) ≥70; aroma strength (safranal): 20-50; colouring strength (crocin) ≥200; moisture content ≤12% (ISO 3632). The coordinates of the occurrence points were arranged in a table, which was then imported into the R software [22] to carry out statistical analysis aimed at identifying the suitable habitat of *C. sativus* and predicting its possible future distribution.

2.2. Spatial distribution prediction

Species distribution models (SDMs) were adopted to predict the spatial distribution (current and future) of *C. sativus* in Italy based on bioclimatic data. In particular, 19 bioclimatic variables were considered (Table 1) in addition to the 721 occurrence data (presence only) of this species. The bioclimatic layers were obtained from the WorldClim 2.1 data website (http://worldclim.org) at a spatial resolution of 2.5 arc-minutes (20.25 km2). All the bioclimatic variables were used to establish the distribution model of *C. sativus* in Italy under current climatic conditions (2016-2020) and for two future periods: 2021-2040 and 2041-2060.

Table 1. Bioclimatic variables used for modelling the distribution of *C. sativus* in Italy.

Code (unit)	**Bioclimatic variable**
BIO1 (°C)	Annual Mean Temperature
BIO2 (°C)	Mean Diurnal Range (Mean of monthly (max temp - min temp)
BIO3 (-)	Isothermality (BIO2/BIO7 × 100)
BIO4 (°C)	Temperature Seasonality (standard deviation × 100)
BIO5 (°C)	Max Temperature of Warmest Month
BIO6 (°C)	Min Temperature of Coldest Month
BIO7 (°C)	Temperature Annual Range (BIO5-BIO6)
BIO8 (°C)	Mean Temperature of Wettest Quarter
BIO9 (°C)	Mean Temperature of Driest Quarter
BIO10 (°C)	Mean Temperature of Warmest Quarter
BIO11 (°C)	Mean Temperature of Coldest Quarter
BIO12 (mm)	Annual Precipitation
BIO13 (mm)	Precipitation of Wettest Month
BIO14 (mm)	Precipitation of Driest Month
BIO15 (-)	Precipitation Seasonality (Coefficient of Variation)
BIO16 (mm)	Precipitation of Wettest Quarter
BIO17 (mm)	Precipitation of Driest Quarter
BIO18 (mm)	Precipitation of Warmest Quarter
BIO19 (mm)	Precipitation of Coldest Quarter

The Shared Socio-economic Pathway (SSP) 2-4.5 future scenario ("Middle of the road scenario") was considered in this research because it represents a plausible future pathway characterized by medium challenges to mitigation and adaptation efforts [23]. This SSP2-4.5 assumes moderate global population growth, intermediate economic development, and gradual shifts towards sustainable practices and technologies, leading to a relatively moderate level of climate change impacts (CO2 emissions will begin to decline around mid-century and temperatures will increase by 2.7°C by the end of the century) compared to other SSPs [23]. The CNRM-CM6-1 global climate model [24], utilized for evaluating and forecasting the impact of the SSPs scenarios on future climate, was acquired from the WorldClim data website. This model, representing the latest fully coupled atmosphere-ocean general circulation model of the sixth generation, was utilized to project the effects of the SSP2-4.5 scenario onto the climate of the periods 2021-2040 and 2041-2060.

In this research, the distribution models of *C. sativus* were generated in the R environment using the “dismo” package [25] and two different algorithms: Bioclim [26] and MaxEnt [27]. The Bioclim algorithm is a classic method widely used in species distribution modelling. It assesses location suitability by comparing environmental variables to a percentile distribution of known occurrence sites, with locations closer to the median considered more suitable [26]. The MaxEnt (Maximum Entropy) algorithm, instead, is a modelling technique used for making predictions of species distribution, particularly well-suited for applications involving presence-only data (occurrence data). It allows you to infer a probability distribution that has maximum entropy while still satisfying the constraints imposed by the available environmental information [27].

The relative contribution of each bioclimatic variable generated by the MaxEnt model was extracted and the accuracy of the resulting models was evaluated by computing the Area Under the Curve (AUC) of the Receiver Operating characteristic Curve (ROC), a widely used and robust approach of model evaluation. The AUC values range from 0 to 1 and the higher the value of AUC, the better the performance of the model. The response curves for each of the environmental variables were generated.

The final product of the distribution models of *C. sativus* is a georeferenced map indicating the probability of the species occurrence (0 for low probability; 1 for high probability). In this research, nine model-generated habitat suitability maps of *C. sativus* in Italy were created: 3 maps produced by applying the Bioclim algorithm, considering the climatic conditions of three time periods (2016-2020, 2021-2040, and 2041-2060); 3 maps produced by applying the MaxEnt algorithm, considering the climatic conditions of the same periods; and 3 maps resulting from the combination of the prediction models produced with the two algorithms for the three periods.

3. Results

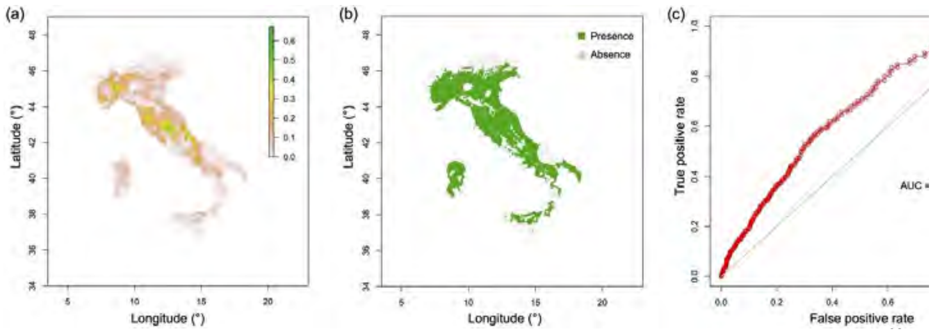


Figure 2. Probability of occurrence (a) and presence/absence (b) of *C. sativus* in Italy as predicted by the model generated using the Bioclim algorithm, and ROC plot (AUC value was reported in the plot) (c). To transform the probability of occurrence (a) into a binary score (presence or absence) (b), the threshold of 0.07 was used, at which the sum of sensitivity (true positive rate) and specificity (true negative rate) is maximized.

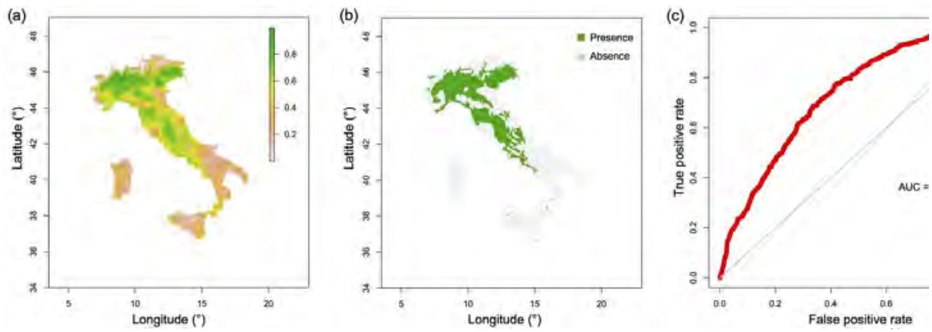


Figure 2. Probability of occurrence (a) and presence/absence (b) of *C. sativus* in Italy as predicted by the model generated using the MaxEnt algorithm, and ROC plot (AUC value was reported in the

plot) (c). To transform the probability of occurrence (a) into a binary score (presence or absence) (b), the threshold of 0.51 was used, at which the sum of sensitivity (true positive rate) and specificity (true negative rate) is maximized.

Figure 2 and Figure 3 show the results of the analysis of the current potential distribution of saffron in Italy returned by using the Bioclim and MaxEnt algorithms, respectively. The maps generated by both models show that the areas with the most suitable climatic conditions for *C. sativus* are located in central and northern Italy, particularly along the central Apennines (in the Emilia-Romagna, Tuscany, Umbria, Abruzzo, and Lazio regions) and in the Po Valley. The model returned by applying the BioClim algorithm produced a presence/absence map (Fig. 2b) in which the ecological niche of *C. sativus* extends to parts of southern Italy and the two major islands (Sicily and Sardinia). However, this model exhibits a lower probability of occurrence and performance (AUC = 0.652) (Fig. 2c) compared to the one obtained with MaxEnt (AUC = 0.732) (Fig. 3c). Among the 19 bioclimatic variables, the contribution of the first 4, including temperature annual range (bio7, 34.7% contribution), precipitation of warmest quarter (bio18, 11.9% contribution), annual precipitation (bio12, 9.3% contribution) and annual mean temperature (bio1, 8.9% contribution), accounted for almost 64.6% of the MaxEnt model prediction (Fig. 4). The response curves generated by the two models (Supplementary Material 1) indicate that *C. sativus* likely prefers areas with a good annual temperature range (25-30 °C), low precipitation of the warmest quarter (15-30 mm), and an average annual temperature between 10-15 °C. According to the response curves of the MaxEnt model, this species would prefer areas with a more humid climate (annual precipitation: >1000 mm) compared to those of the Bioclim model (annual precipitation: 800-1000 mm).

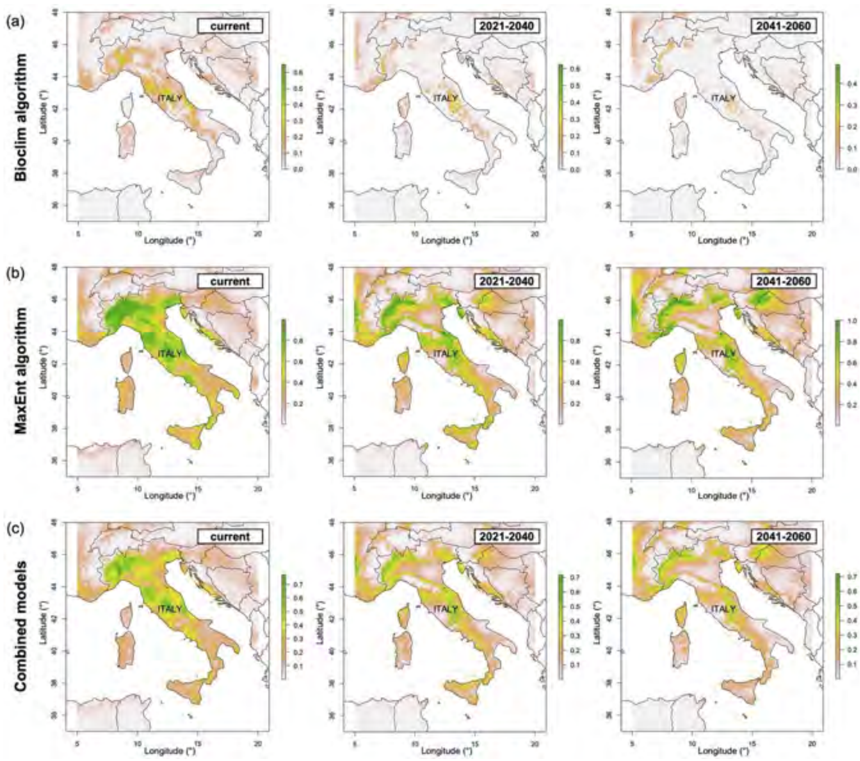


Figure 4. Habitat suitability maps of *C. sativus* generated by different models (a, using the Bioclim algorithm; b, using the MaxEnt algorithm; c, combining models a and b) and referred to the periods 2016-2020 (current), 2021-2040, and 2041-2060. The color scale indicates the probability of occurrence.

Figure 4 displays the maps of the future potential distribution of *C. sativus* in Italy as predicted by the models (Bioclim, MaxEnt and combined models). Generally, the occurrence probabilities returned by the Bioclim model are considerably lower than those of the MaxEnt model; thus, the maps generated by the combined model offer a useful weighted representation of the suitable habitat. All models (Bioclim in particular) show that in Italy, in the coming decades, there will be a loss of areas with climatic conditions suitable for *C. sativus*. Moreover, areas with occurrence probability >0.5 will mainly affect mountain areas of the Apennines and the Alps. As for the Alps, the most suitable areas (from a climatic point of view) for saffron cultivation will mainly be those located in the Central Alps (Lombardy Prealps, Lepontine Alps and Pennine Alps) and the Western Alps (Graian Alps, Cottian Alps and Maritime Alps), excluding the Aosta Valley, the north-eastern Rhaetian Alps and other territories located at high elevation (>1600 m a.s.l.) bordering France and Switzerland. In the Eastern Alps, the models (especially MaxEnt) show that in the coming decades, there will be suitable habitat only in the Venetian Prealps and surrounding mountainous-hilly territories. Many hilly and plain areas of the Po Valley (Northern Italy), currently suitable for saffron cultivation, will be less suitable in the future, especially those more eastern ones.

In Central and Southern Italy as well, model predictions indicate a loss of habitat of *C. sativus* (Figure 5). By 2060, numerous coastal and hilly areas in the regions of Tuscany, Lazio, Campania, Marche and Abruzzo will have climatic conditions unsuitable for saffron crocus, while the most suitable sites will predominantly be located in the inner areas of the Central Apennines (in Abruzzo, Marche, Umbria and Lazio) and at higher elevation (>500 m a.s.l.). Based on the future scenarios depicted in Figure 5, by 2060, Southern Italy and the major islands (Sicily and Sardinia) are projected to predominantly feature areas with climatic conditions not very suitable for the cultivation of *C. sativus* (probability of occurrence <0.6)

4. Discussion

According to the results obtained from the application of species distribution models, today the suitable climatic conditions for *C. sativus* are found in a large part of the Italian territory, while future predictions indicate a compression of its habitat. The application of the two algorithms (Bioclim and MaxEnt) has yielded similar maps regarding the identification of the main occurrence hotspots of the species, but the occurrence probabilities of the maps generated with Bioclim are much lower compared to those produced with MaxEnt. This is because Bioclim and MaxEnt are two different tools for modelling species distribution, although both are useful for understanding species habitats and supporting biodiversity conservation efforts.

The Bioclim algorithm assesses location similarity by comparing environmental variable values with a percentile distribution from known occurrence locations, considering locations closer to the median as the most suitable. In the R implementation used in this research [25], the values returned by the Bioclim algorithm are transformed into a range from 0 to 1 (probability of occurrence) to make the results more similar/comparable to those of other modelling methods (such as MaxEnt). The reason why occurrence probabilities (current and future) obtained in this research are low is justified by the approach that this algorithm uses; a value of 1 is rare because it requires median values for all variables in the occurrence data, while 0 is very common as it is assigned to all areas with a value of an environmental variable that is outside the percentile distribution (the range of the occurrence data) for at least one of the variables.

MaxEnt, on the other hand, is based on the maximum entropy theory proposed in 1957 [27] that relies on the principle that, in the absence of contrary information, the probability distribution of environmental variables that maximizes entropy is the most accurate or least biased. This approach has made MaxEnt one of the most powerful and widely used tools for predicting species distribution in a wide range of ecological contexts [28–31]. Moreover, MaxEnt includes a variable selection process that determines which environmental variables are most important in predicting species distribution, aiding in identifying key factors influencing species distribution.

In this research, the most important variable in predicting the distribution of *C. sativus* is the annual temperature range (bio7) (Figure 5), which must be >25 °C, typical conditions of continental climates. Climates characterized by high temperature fluctuations would be optimal for the saffron crocus, which is a species that tolerates low winter temperatures down to -15 (-20) °C [32], requires 23-27 °C in summer for optimal flower development, and blooms when autumn temperatures drop to the range of 15-17(20) °C [32,33] without excessive rain [7]. A recent study conducted in Iran highlighted how low temperatures at the beginning of autumn are important for stimulating flowering and increasing saffron yields [34], conditions that are found in areas of Iran where *C. sativus* is cultivated at high elevation (over 1,300 m a.s.l.). These climatic conditions are also more common in mountain areas in Italy, and it is likely that, due to climate change, they will occur at increasingly higher elevations in the future, as shown by the results of this research.

In addition to the annual temperature range (bio7), the precipitation of the warmest quarter (bio18), the annual precipitation (bio12), and the annual mean temperature (bio1) were found to be important variables for predicting suitable climatic conditions for *C. sativus* (Figure 5). The analysis of the data (response curves) of these variables confirms the requirements of *C. sativus* reported in the literature. Indeed, the ideal conditions for this species are found in areas with a temperate or semi-arid climate, where the rainfall range is between 420 mm and 1,370 mm per year, and the annual mean temperature varies from 5.9 °C to 18.6 °C [35,36].

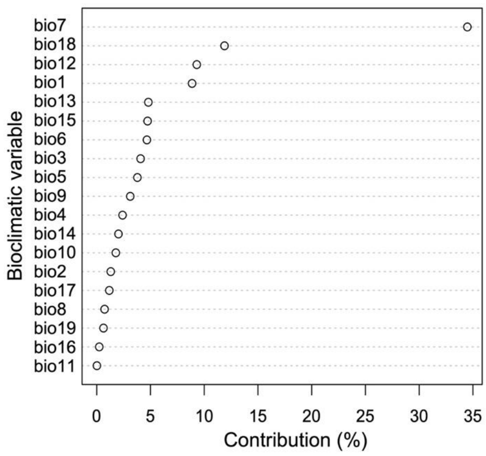


Figure 5. Estimates of contribution importance of environmental variables in MaxEnt modelling of *C. sativus* distribution in Italy. The codes of the bioclimatic variables are the same as those used in Table 1.

Although this study assessed the potential distribution (current and future) of *C. sativus* in Italy considering only climatic variables, which nonetheless represent the primary environmental variables influencing the growth/cultivation of *C. sativus* [37], other environmental factors (e.g., soil chemical and physical characteristics, land use, etc.) should be considered to identify the actual areas where the saffron crocus can or will be cultivated. Some mountain areas of Italy indeed feature steep slopes that do not allow for the cultivation of *C. sativus* (and other crops) unless they undergo costly hydraulic-agrarian arrangements (e.g., terracing). Similarly, some mountain areas are inaccessible to agricultural vehicles due to the lack of adequate road infrastructure. Furthermore, some soils in the mountainous areas highlighted by the maps in Figure 4 may have chemical and physical characteristics that are not ideal for the saffron crocus. Although it is a hardy species, saffron crocus prefers soils that do not promote water stagnation and have a neutral to basic pH to allow for a high yield of saffron [8,38,39].

The analysis of land use in the mountainous areas of Italy is also of fundamental importance for identifying territories where it will be possible to cultivate the saffron crocus and produce the spice. The surfaces of such areas, if the land use does not drastically change in the coming decades, will be much less extensive compared to those of areas where there are climatic (as indicated in the maps of Figure 4) and pedological conditions suitable for *C. sativus*. Indeed, today, a large portion of the mountainous areas of the Alps and the Apennines are covered by forests, which represent habitats unsuitable for saffron cultivation: more than 60% of the Italian forested area is located in mountain municipalities [40], and this area has increased by over 570,000 hectares in the last 30 years [39].

The increase in the extension of forests in the mountain areas of Italy at the expense of meadows, pastures, and fields is primarily due to socio-economic factors that, for over half a century, have been the main cause of the abandonment of such territories [41,42]. In Italy, various instruments have been implemented in recent decades to counteract the abandonment of mountain and marginal areas and currently, several NRRP funds have been committed to projects aimed at enhancing the valuable agri-food resources of these territories (such as saffron) through the establishment or strengthening of traditional and/or innovative supply chains that can promote the sustainable development of these areas. The results of this research could be useful for land managers and policymakers, enabling them to promptly identify the mountain areas in Italy where saffron production could be possible in the coming decades and thus initiate targeted policies and actions in these territories. This research thus represents an example of how researchers can produce tools useful to support actions aimed at safeguarding (agro-)biodiversity and promoting local agri-food excellences, of which Italian mountain areas are particularly rich [43,44]. For this study to be replicated considering other crops and/or geographical areas, however, it requires some important prerequisites upon which the accuracy of the result depends, the most important being the availability of exhaustive occurrence datasets. Indeed, Kumar et al. (2022)[37] used only 20 geographic points located in five countries (Spain, Morocco, Italy, Iran, and India) to identify with MaxEnt new areas in India suitable for saffron cultivation.

The collection and sharing of data on species distribution is therefore a fundamental action for the development of predictive maps that are as reliable as possible. In recent years, various open-source databases have been developed from which georeferenced points (occurrence records) related to the presence of a particular animal or plant species in a specific territory can be obtained. One of these is the Global Biodiversity Information Facility (GBIF) database (https://www.gbif.org), which is an international network and data infrastructure funded by the world's governments and aimed at providing, anyone and anywhere, open access data about all types of life on Earth [45,46]. Although GBIF is the largest source of open scientific data on the planet's biodiversity (2.6 billion occurrence records in 2023), there is still much work to be done to integrate missing data regarding the distribution of many species. Consider that by the end of 2023, GBIF reported only 445 occurrences of *C. sativus* across 32 countries, with just 3 georeferenced points for Iran (and 121 for Italy), the world's leading saffron producer.

In the future, it would be desirable for researchers worldwide to make a greater effort to enrich species occurrence databases, using the citizen science approach, which can play an increasingly important role in biodiversity monitoring [47], provided that citizens have adequate experience/knowledge regarding species identification and methods for collecting their occurrence data [48]. The creation of a sufficiently comprehensive dataset of *C. sativus* occurrences worldwide could allow for the replication of this study in other countries and a better understanding of the environmental requirements of this species. In addition, the collection of data on the average annual yield (as well as quality) of saffron produced in each geographical area could allow for the identification of the most productive areas, whose geographic coordinates could be used to develop maps predicting "optimal/high productivity" (current and future) areas for *C. sativus.*

Finally, concerning the identification of areas that will present suitable climatic conditions for saffron cultivation in the future, it is necessary to consider/monitor the actual SSP scenario that humanity will face, thus the measures that will be adopted in the coming years to mitigate climate change and their effectiveness. Based on the real SSP (which could be more or less favourable compared to that considered in this research) and the development of increasingly reliable global climate models [49], it would be advisable to regularly update the distribution maps of *C. sativus* in Italy, which could undergo more or less significant variations. However, this research represents the first attempt to predict the areas where there are and will be suitable climatic conditions for *C. sativus* in Italy, based on the use of the most comprehensive set of occurrences available to date and employing reliable global climate models and modern species distribution analysis tools.

5. Conclusions

This research identified the geographical areas of Italy where the climatic conditions suitable for the growth/cultivation of *C. sativus* currently persist, as well as those that will be suitable in the coming decades (up to 2060), through the use of a vast dataset of this species occurrence in Italy and the application of species distribution models. Currently, many Italian territories (especially those in central and northern Italy) present climatic conditions suitable for *C. sativus*, but in the next 40 years, models predict a significant loss of habitat. By 2060, in many plains, coastal areas, and hills of Italy, it is likely that the climatic conditions suitable for saffron crocus will not exist, while they will be present in more inland areas and at higher altitudes (compared to current ones) in the Alps (mainly in the central and western Alps) and the Apennines (mainly in the central Apennines). The results obtained in this research could be useful not only to farmers who currently cultivate or will cultivate *C. sativus* but also to land managers and politicians, who will be able to decide with greater awareness and scientific data how and where to allocate investments aimed at promoting and enhancing the chain of the Italian saffron.

Supplementary Materials: Table S1: Response curves for the 19 predictors (bioclimatic variables) used in the Bioclim (a) and MaxEnt (b) distribution models. The codes of the bioclimatic variables are the same as those used in Table 1.

Author Contributions: L.G. and A.G. conceived and designed the study and interpreted the results. L.G. collected the data and carried out the floristic, ecological, and statistical analyses. L.G., and D.P. analyzed the data and wrote the manuscript. L.G. and D.P. created figures and tables. L.G. and A.G.

are the project administrators. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Agritech National Research Centre and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RE- SILIENZA (PNRR)—MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4—D.D. 1032 17/06/2022, CN00000022) and from the Horizon Project "Accelerating transformative climate adaptation for higher resilience in European Mountain regions" (MountResilience) project n◦: 101112876.

Data Availability Statement: The raw data supporting the conclusions of this manuscript will be made available by the corresponding author, without undue reservation, to any qualified researcher.

Acknowledgments: We thank the Val.Te.Mo. APS association, in particular Mrs. Stella Pedrazzi and Dr. Sonia Visioli, for providing the data on the occurrences of *C. sativus* in Italy and the quality of Italian saffron.

Conflicts of Interest: No potential conflict of interest was reported by the authors.

References

1. Winterhalter, P.; Straubinger, M. Saffron—Renewed Interest In An Ancient Spice. *Food Reviews International* **2000**, *16*, 39–59, doi:10.1081/FRI-100100281.
2. Nemati, Z.; Harpke, D.; Gemicioglu, A.; Kerndorff, H.; Blattner, F.R. Saffron (Crocus Sativus) Is an Autotriploid That Evolved in Attica (Greece) from Wild Crocus Cartwrightianus. *Molecular Phylogenetics and Evolution* **2019**, *136*, 14–20, doi:10.1016/j.ympev.2019.03.022.
3. Kazemi-Shahandashti, S.-S.; Mann, L.; El-nagish, A.; Harpke, D.; Nemati, Z.; Usadel, B.; Heitkam, T. Ancient Artworks and Crocus Genetics Both Support Saffron's Origin in Early Greece. *Front. Plant Sci.* **2022**, *13*, 834416, doi:10.3389/fpls.2022.834416.
4. Shokrpour, M. Saffron (Crocus Sativus L.) Breeding: Opportunities and Challenges. In *Advances in Plant Breeding Strategies: Industrial and Food Crops: Volume 6*; Al-Khayri, J.M., Jain, S.M., Johnson, D.V., Eds.; Springer International Publishing: Cham, 2019; pp. 675–706 ISBN 978-3-030-23265-8.
5. Cardone, L.; Castronuovo, D.; Perniola, M.; Cicco, N.; Candido, V. Saffron (Crocus Sativus L.), the King of Spices: An Overview. *Scientia Horticulturae* **2020**, *272*, 109560, doi:10.1016/j.scienta.2020.109560.
6. The Observatory of Economic Complexity Available online: https://oec.world/en (accessed on 10 November 2025).
7. Cardone, L.; Castronuovo, D.; Perniola, M.; Cicco, N.; Candido, V. Evaluation of Corm Origin and Climatic Conditions on Saffron (*Crocus Sativus* L.) Yield and Quality. *J Sci Food Agric* **2019**, *99*, 5858–5869, doi:10.1002/jsfa.9860.
8. Gresta, F.; Lombardo, G.M.; Siracusa, L.; Ruberto, G. Saffron, an Alternative Crop for Sustainable Agricultural Systems. A Review. *Agron. Sustain. Dev.* **2008**, *28*, 95–112, doi:10.1051/agro:2007030.
9. Giorgi, A.; Pentimalli, D.; Giupponi, L.; Panseri, S. Quality Traits of Saffron (Crocus Sativus L.) Produced in the Italian Alps. *Open Agriculture* **2017**, *2*, 52–57, doi:10.1515/opag-2017-0005.
10. Manzo, A.; Panseri, S.; Bertoni, D.; Giorgi, A. Economic and Qualitative Traits of Italian Alps Saffron. *J. Mt. Sci.* **2015**, *12*, 1542–1550, doi:10.1007/s11629-015-3448-3.
11. Caser, M.; Victorino, Í.M.M.; Demasi, S.; Berruti, A.; Donno, D.; Lumini, E.; Bianciotto, V.; Scariot, V. Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds. *Agronomy* **2018**, *9*, 12, doi:10.3390/agronomy9010012.
12. Giupponi, L.; Leoni, V.; Sala, S.; Giorgi, A.; Bertoni, D. Saffron Growing in Italy: A Sustainable Secondary Activity for Farms in Hilly and Sub-Mountain Areas. *International Journal of Agricultural Sustainability* **2023**, *21*, 2270263, doi:10.1080/14735903.2023.2270263.
13. Giupponi, L.; Ceciliani, G.; Leoni, V.; Panseri, S.; Pavlovic, R.; Lingua, G.; Di Filippo, A.; Giorgi, A. Quality Traits of Saffron Produced in Italy: Geographical Area Effect and Good Practices. *Journal of Applied Botany and Food Quality* **2019**, 336-342 Pages, doi:10.5073/JABFQ.2019.092.045.
14. Locatelli, I.; Pedrali, D.; Grassi, S.; Buratti, S.; Giorgi, A.; Giupponi, L. Progress in Quality Assessment of Italian Saffron. *Sci Rep* **2025**, *15*, 2175, doi:10.1038/s41598-025-86440-x.
15. EU COMMISSION, 2020: A Farm to Fork strategy for a fair, healthy and environmentally-friendly food system. COM/2020/381 final, Brussels. Retrieved 20 February 2024, from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52020DC0381
16. Fabbrini, F. Italy's National Recovery and Resilience Plan: Context, Content and Challenges. *Journal of Modern Italian Studies* **2022**, *27*, 658–676, doi:10.1080/1354571X.2022.2124672.
17. Calvin, K.; Dasgupta, D.; Krinner, G.; Mukherji, A.; Thorne, P.W.; Trisos, C.; Romero, J.; Aldunce, P.; Barrett, K.; Blanco, G.; et al. *IPCC, 2023: Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* [Core Writing Team, H. Lee and J. Romero (Eds.)]. IPCC, Pubblischer: Geneva, Switzerland.; 1st ed.; Intergovernmental Panel on Climate Change (IPCC), 2023;
18. Busconi, M.; Wischnitzki, E.; Del Corvo, M.; Colli, L.; Soffritti, G.; Stagnati, L.; Fluch, S.; Sehr, E.M.; de los Mozos Pascual, M.; Fernández, J.A. Epigenetic Variability Among Saffron Crocus (Crocus Sativus L.) Accessions Characterized by Different Phenotypes. *Frontiers in Plant Science* **2021**, *Volume 12-2021*.

19. Fernández, J.A. Biology, Biotechnology and Biomedicine of Saffron. In; Research Signpost: Trivandrum, 2004; pp. 127–159 ISBN 81-7736-239-9.
20. Guisan, A.; Thuiller, W.; Zimmermann, N.E. *Habitat Suitability and Distribution Models: With Applications in R*; Ecology, Biodiversity and Conservation; Cambridge University Press: Cambridge, 2017; ISBN 978-0-521-76513-8.
21. Elith, J.; Leathwick, J.R. Species Distribution Models: Ecological Explanation and Prediction Across Space and Time. *Annual Review of Ecology, Evolution, and Systematics* 2009, *40*, 677–697.
22. R: The R Project for Statistical Computing Available online: https://www.r-project.org/ (accessed on 10 November 2025).
23. IPCC 2021: Summary for Policymakers. In *Climate Change 2021 – The Physical Science Basis: Working Group I Contribution to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*; Intergovernmental Panel on Climate Change (IPCC), Ed.; Cambridge University Press: Cambridge, 2023; pp. 3–32 ISBN 978-1-009-15788-9.
24. Voldoire, A.; Saint-Martin, D.; Sénési, S.; Decharme, B.; Alias, A.; Chevallier, M.; Colin, J.; Guérémy, J.-F.; Michou, M.; Moine, M.-P.; et al. Evaluation of CMIP6 DECK Experiments With CNRM-CM6-1. *Journal of Advances in Modeling Earth Systems* **2019**, *11*, 2177–2213, doi:10.1029/2019MS001683.
25. Hijmans, R.J., Phillips, S., Leathwick, J., Elith, J., Species Distribution Modeling. R Package Version 1.3-14. 2023.
26. Busby, J.R., 1991: Bioclim, a Bioclimatic Analysis and Prediction System. In: Margules, C.R., Austin, M.P. (eds.), Nature Conservation: Cost Effective Biological Surveys and Data Analysis. Canberra: CSIRO, pp. 64-68.
27. Phillips, S.J.; Anderson, R.P.; Schapire, R.E. Maximum Entropy Modeling of Species Geographic Distributions. *Ecological Modelling* **2006**, *190*, 231–259, doi:10.1016/j.ecolmodel.2005.03.026.
28. Fois, M.; Cuena-Lombraña, A.; Fenu, G.; Bacchetta, G. Using Species Distribution Models at Local Scale to Guide the Search of Poorly Known Species: Review, Methodological Issues and Future Directions. *Ecological Modelling* **2018**, *385*, 124–132, doi:10.1016/j.ecolmodel.2018.07.018.
29. Pedrali, D.; Zuccolo, M.; Giupponi, L.; Sala, S.; Giorgi, A. Characterization and Future Distribution Prospects of "Carciofo Di Malegno" Landrace for Its In Situ Conservation. *Plants* **2024**, *13*, 680, doi:10.3390/plants13050680.
30. Fitzgibbon, A.; Pisut, D.; Fleisher, D. Evaluation of Maximum Entropy (Maxent) Machine Learning Model to Assess Relationships between Climate and Corn Suitability. *Land* **2022**, *11*, doi:10.3390/land11091382.
31. Tang, X.; Yuan, Y.; Li, X.; Zhang, J. Maximum Entropy Modeling to Predict the Impact of Climate Change on Pine Wilt Disease in China. *Frontiers in Plant Science* **2021**, *Volume 12-2021*.
32. Molina, R.V.; Valero, M.; Navarro, Y.; Guardiola, J.L.; García-Luis, A. Temperature Effects on Flower Formation in Saffron (Crocus Sativus L.). *Scientia Horticulturae* **2005**, *103*, 361–379, doi:10.1016/j.scienta.2004.06.005.
33. Wang, Z.; Li, X.; Xu, J.; Yang, Z.; Zhang, Y. Effects of Ambient Temperature on Flower Initiation and Flowering in Saffron (Crocus Sativus L.). *Scientia Horticulturae* **2021**, *279*, 109859, doi:10.1016/j.scienta.2020.109859.
34. Pirasteh-Anosheh, H.; Babaie-Zarch, M.J.; Nasrabadi, M.; Parnian, A.; Alavi-Siney, S.M.; Beyrami, H.; Kaveh, H.; Hashemi, S.E.; Durrer, U.; McDonald, K.; et al. Climate and Management Factors Influence Saffron Yield in Different Environments. *Agrosystems, Geosciences & Environment* **2023**, *6*, e20418, doi:10.1002/agg2.20418.
35. Duke, J.A. Ecosystematic Data on Economic Plants. *Quarterly Journal of Crude Drug Research* **1979**, *17*, 91–109, doi:10.3109/13880207909065158.
36. Kumar, R.; Singh, V.; Devi, K.; Sharma, M.; Singh, M.K.; Ahuja, P.S. State of Art of Saffron (Crocus Sativus L.) Agronomy: A Comprehensive Review. *Food Reviews International* **2008**, *25*, 44–85, doi:10.1080/87559120802458503.
37. Kumar, A.; Devi, M.; Kumar, R.; Kumar, S. Introduction of High-Value Crocus Sativus (Saffron) Cultivation in Non-Traditional Regions of India through Ecological Modelling. *Scientific Reports* **2022**, *12*, 11925, doi:10.1038/s41598-022-15907-y.
38. Shinde, D.A.; Talib, A.R.; Gorantiwar, S.M. Composition and Classification of Some Typical Soils of Saffron Growing Areas of Jammu and Kashmir. *Journal of the Indian Society of Soil Science* **1984**, *32*, 473–477.
39. Giorgi, A.; Bertoni, D.; Manzo, A.; Panseri, S. *L'oro Rosso Delle Alpi - Manuale Tecnico-Scientifico Di Produzione Dello Zafferano* 1st ed.; Publisher: Milano, Italy; 2015.

40. Statistiche INFC2015 – Inventario Nazionale Delle Foreste e Dei Serbatoi Forestali Di Carbonio – INFC Available online: https://www.inventarioforestale.org/it/statistiche_infc/ (accessed on 10 November 2025).
41. Cislaghi, A.; Giupponi, L.; Tamburini, A.; Giorgi, A.; Bischetti, G.B. The Effects of Mountain Grazing Abandonment on Plant Community, Forage Value and Soil Properties: Observations and Field Measurements in an Alpine Area. *CATENA* **2019**, *181*, 104086, doi:10.1016/j.catena.2019.104086.
42. Giupponi, L.; Leoni, V. VegeT: An Easy Tool to Classify and Facilitate the Management of Seminatural Grasslands and Dynamically Connected Vegetation of the Alps. *Land* **2020**, *9*, 473, doi:10.3390/land9120473.
43. Giupponi, L.; Pilu, R.; Scarafoni, A.; Giorgi, A. Plant Agro-Biodiversity Needs Protection, Study and Promotion: Results of Research Conducted in Lombardy Region (Northern Italy). *Biodivers Conserv* **2020**, *29*, 409–430, doi:10.1007/s10531-019-01889-3.
44. Giupponi, L.; Pedrali, D.; Leoni, V.; Rodari, A.; Giorgi, A. The Analysis of Italian Plant Agrobiodiversity Databases Reveals That Hilly and Sub-Mountain Areas Are Hotspots of Herbaceous Landraces. *Diversity* **2021**, *13*, 70, doi:10.3390/d13020070.
45. Flemons, P.; Guralnick, R.; Krieger, J.; Ranipeta, A.; Neufeld, D. A Web-Based GIS Tool for Exploring the World's Biodiversity: The Global Biodiversity Information Facility Mapping and Analysis Portal Application (GBIF-MAPA). *Ecological Informatics* **2007**, *2*, 49–60, doi:10.1016/j.ecoinf.2007.03.004.
46. Ivanova, N.V.; Shashkov, M.P. The Possibilities of GBIF Data Use in Ecological Research. *Russian Journal of Ecology* **2021**, *52*, 1–8, doi:10.1134/S1067413621010069.
47. Chandler, M.; See, L.; Copas, K.; Bonde, A.M.Z.; López, B.C.; Danielsen, F.; Legind, J.K.; Masinde, S.; Miller-Rushing, A.J.; Newman, G.; et al. Contribution of Citizen Science towards International Biodiversity Monitoring. *Biological Conservation* **2017**, *213*, 280–294, doi:10.1016/j.biocon.2016.09.004.
48. Bowler, D.E.; Bhandari, N.; Repke, L.; Beuthner, C.; Callaghan, C.T.; Eichenberg, D.; Henle, K.; Klenke, R.; Richter, A.; Jansen, F.; et al. Decision-Making of Citizen Scientists When Recording Species Observations. *Scientific Reports* **2022**, *12*, 11069, doi:10.1038/s41598-022-15218-2.
49. Hartmann, D.L. Chapter 11 - Global Climate Models. In *Global Physical Climatology (Second Edition)*; Hartmann, D.L., Ed.; Elsevier: Boston, 2016; pp. 325–360 ISBN 978-0-12-328531-7.

9. Conclusion

The research presented in this thesis highlights how the conservation and valorisation of mountain agrobiodiversity require an integrated and multidisciplinary approach that connects scientific innovation, traditional knowledge, and territorial development. The characterization of underutilized plant genetic resources, such as landraces and officinal plants, revealed their distinctive nutritional, agronomic, and phytochemical traits, demonstrating their untapped potential as strategic resources for functional food production, climate adaptation, and new value chains. By linking agrobiodiversity with territorial identity, these resources provide opportunities not only for ecological resilience and food innovation but also for the sustainable development of landscapes and communities.

Landraces are the result of a long process of co-evolution between plants, local environments, and farming communities. Unlike commercial cultivars, they have adapted over centuries to specific pedoclimatic conditions, acquiring unique morphological, agronomic, and phytochemical features. This strong bond with their environment ensures resilience to local stresses while giving rise to products whose sensory and nutritional qualities are closely tied to their territories of origin. As such, landraces can be considered direct expressions of their landscapes, acting as bio-indicators of ecosystems and cultural symbols rooted in traditional practices.

Through the four case studies, this thesis has shown that conservation cannot be effective without valorisation; traditional varieties must be embedded in sustainable and economically viable production systems, enabling farmers to benefit directly from their cultivation. In this context, mountain areas play a dual role, as agrobiodiversity hotspots and as living laboratories where innovative practices can be tested while preserving cultural traditions.

For the “Copafam” bean (case study I), the characterization confirmed its high dietary fiber, rich phenolic content, strong antioxidant activity, and low levels of antinutritional factors, positioning it as a promising functional ingredient. Beyond the crop itself, by-products such as pods and cooking water were valorised: high hydrostatic pressure and enzymatic hydrolysis promoted the release of soluble fiber and prebiotic oligosaccharides from pods, while cooking water proved to be a promising source of phenolics. In enriched encapsulation systems, up to 85% of polyphenols were retained and 97% of rutin was successfully encapsulated, confirming its potential for functional hydrogel development. The “Carciofo di Malegno” (case study II) combined morphological and phytochemical characterization with participatory valorisation activities. Geometric morphometrics confirmed its distinctiveness, while phytochemical profiling highlighted valuable bioactive compounds even in discarded tissues. Ecological niche modelling projected an upward shift in suitable cultivation areas under climate change, raising new challenges for its *in situ* conservation. Valorisation initiatives, such as its submission to the National Register of Agrobiodiversity and the development of innovative leaf-based spirit prototypes, reinforced its conservation and market potential. The "Grano Siberiano Valtellinese" (case study III) demonstrated a distinctive nutritional profile rich in protein, fiber, and polyphenols, while its straw was effectively valorised using green technologies. High hydrostatic pressure combined with enzymatic hydrolysis enhanced the release of soluble fiber, prebiotic oligosaccharides, and bound phenolic acids, significantly improving antioxidant potential. These findings confirmed its potential within circular economy models, where agricultural residues are transformed into functional ingredients.

Finally, the saffron case study (case study IV) demonstrated that over 90% of Italian saffron was classified as Category I quality (ISO 3632), confirming its excellence as a niche product. Its adaptability to low-input systems highlights saffron as a strategic resource for mountain territories. Moreover, ecological niche modelling projected that future cultivation will be increasingly confined to mountain areas, making saffron an exclusive high-value crop in these environments. Advanced tools such as NIR spectroscopy for rapid quality assessment, together with the strengthening of producer networks, can further support its competitiveness and resilience.

In Italy, mountain and hilly areas are home to a rich heritage of landraces, many of which remain under-documented and at risk of genetic erosion. This shortcoming highlights the urgent need for systematic characterization and registration to ensure both legal protection and opportunities for valorisation. The results of this thesis provide concrete examples of how this process can be implemented by generating robust scientific data that demonstrate the peculiarities and uniqueness of landraces. These varieties, having evolved and grown within specific environments that shaped their traits, can thus be formally recognized and included in national and European registers. Such recognition is essential to protect them from extinction, secure their link with territories of origin, and open new prospects for their valorisation in sustainable agri-food chains. The re-evaluation of traditional cultivars is already reflected in the increase of their on-farm cultivation areas, driven by the creation of short supply chains of unique traditional and innovative products, supported by national and international conservation and development programs. A concrete example of this pathway is the recovery of the "Nero Spinoso" maize landrace, which was initially cultivated on only 100 m^2 by a single farmer and thus at great risk of extinction. Following its genetic and phytochemical characterization, which revealed a flavonoid-rich profile, it was included in the European Register of Conservation Varieties. Today it is cultivated on about 30,000 m^2 in the Camonica Valley (Brescia, Italy), supported by a farmers' consortium that ensures its preservation, production, and transformation. This case demonstrates how the integration of scientific research, registration tools, and stakeholder engagement can effectively reverse genetic erosion.

The four case studies presented in this thesis move in the same direction. They illustrate how analytical study, such as ecological niche modelling, nutritional and phytochemical profiling, and sensory evaluation, combined with participatory approaches involving custodian farmers, local associations, and seed networks, can bridge scientific outcomes with tangible community benefits. Such integration supports the development of resilient agri-food systems, capable of addressing global challenges ranging from dietary diversity to climate change, while counteracting the abandonment of marginal rural areas.

Ultimately, this thesis demonstrates that mountain landraces are more than genetic resources: they are cultural and economic assets whose protection and valorisation strengthen the link between biodiversity, sustainability, and rural development. The four case studies are only examples, but they show the pathway that should be pursued to protect Europe’s plant genetic heritage: systematic characterization, registration in official agrobiodiversity registers, and valorisation through sustainable and innovative agri-food chains. By embedding agrobiodiversity into innovation pathways, it is possible to promote resilient environments, support local economies, and preserve the heritage of mountain territories for future generations.

Supplementary materials

Additional articles

- **Plant cover is related to vegetation and soil features in limestone screes colonization: A case study in the Italian Alps**
  Published in: *Plant Soil* 2022, 483: 495–513. https://doi.org/10.1007/s11104-022-05760-3
  Authors: Luca Giupponi[1], Valeria Leoni[1], **Davide Pedrali** [1], Marco Zuccolo[1] Alessio Cislaghi [1]
  1 Department of Agricultural and Environmental Sciences-Production, Landscape, Agroenergy, Università degli Studi di Milano, Milano, Italia.

- **Ecology, floristic-vegetational features and future perspectives of spruce forests affected by Ips typographus: insight from the Southern Alps**
  Published in: *Plants* 2025, 14,1681. https://doi.org/ 10.3390/plants14111681
  Authors: Luca Giupponi[1], Riccardo Panza[1], **Davide Pedrali** [1], Stefano Sala[1], Annamaria Giorgi[1]
  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.
  2 Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, 20133 Milan, Italy

- **Restoration of Vegetation Greenness and Possible Changes in Mature Forest Communities in Two Forests Damaged by the Vaia Storm in Northern Italy**
  Published in: *Plants* 2023, 12: 1369. https://doi.org/10.3390/plants12061369
  Authors: Luca Giupponi[1,2], Valeria Leoni[1], **Davide Pedrali** [1], Anna Giorgi [1,2]
  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.
  2 Department of Agricultural and Environmental Sciences-Production, Landscape, Agroenergy, Università degli Studi di Milano, Milano, Italia.
  Correspondence: luca.giupponi@unimi.it

Participation in congress

Year 2025:

- ***Ips typographus* outbreaks in the Southern Alps: what about vegetation and biodiversity?**
  Contribution to Congress: 3rd Conference for Young Botanists (CYBO), June 16-20, 2025, Siena.
  Poster presentation
  Authors: Riccardo Panza[1], Luca Giupponi[1], **Davide Pedrali** [1,2], Annamaria Giorgi[1,2]

1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.
2 Department of Agricultural and Environmental Sciences - Production, Landscape and Agroenergy, University of Milan, Via Celoria 2, 20133 Milan, Italy.

- **Promoting sustainable agriculture in mountain regions: the case of "Carciofo di Malegno" (*Cynara cardunculus* subsp. *scolymus* L. Hayek)**
  Contribution to Congress: 3rd Conference for Young Botanists (CYBO), February 5-7, 2025, Genova.
  Oral presentation
  Authors: Alex Alberto[1], **Davide Pedrali[1]**, Marco Zuccolo[1], Riccardo Panza[1], Beatrice Bisaglia[1], Luca Giupponi[1,2], Annamaria Giorgi[1,2]
  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.
  2 Department of Agricultural and Environmental Sciences - Production, Landscape and Agroenergy, University of Milan, Via Celoria 2, 20133 Milan, Italy.

- **Characterization and future distribution prospects of "Carciofo di Malegno" landrace (*Cynara cardunculus* subsp. *scolymus* l. Hayek) for its *in situ* conservation**
  Contribution to Congress: 120th Congresso della Società Botanica Italiana, September 3-6, 2025, Gorizia. **Poster presentation**
  Authors: Davide Pedrali[1], Alex Alberto[1], Luca Giupponi [1,2], Beatrice Bisaglia[1], Riccardo Panza[1], Annamaria Giorgi [1,2]
  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

- **The importance of genetic resources (landraces) on agriculture, food production and socioeconomic contexts in mountain areas: the beans of Lombardy**
  Contribution to Congress: International Mountain Conference (IMC), September 14-18, 2025, Innsbruck, Austria.
  Oral presentation
  Authors: **Davide Pedrali [1]**, Luca Giupponi[1,2], Alex Alberto[1], Riccardo Panza[1], Beatrice Bisaglia[1], Annamaria Giorgi[1,2]
  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.
  2 Department of Agricultural and Environmental Sciences - Production, Landscape and Agroenergy, University of Milan, Via Celoria 2, 20133 Milan, Italy.

Year 2024:

- **The importance of genetic resources (landraces) on agriculture, food production and socioeconomic contexts in mountain areas: the beans of Lombardy**
  Contribution to Congress: 119th Congresso della Società Botanica Italiana, September 11-13, 2024, Teramo. **Poster presentation**
  Authors: **Davide Pedrali** [1], Luca Giupponi[1], Annamaria Giorgi[1]
  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

- **Plant agro-biodiversity can be resource for the sustainable development of mountain areas: the case of "Copafam" landrace (*Phaseolus coccineus* L.)**
  Contribution to Congress: XX International Botanical Congress (IBC), July 21-27, 2024, Madrid. **Poster presentation**
  Authors: **Davide Pedrali** [1], Luca Giupponi[1], Annamaria Giorgi[1]
  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

- **Plant agro-biodiversity can be resource for the sustainable development of mountain areas: the case of "Copafam" landrace (*Phaseolus coccineus* L.)**
  Contribution to Congress: XIII European Mountain Convention, October 15-18, 2024, Catalonia. **Poster presentation**
  Authors: **Davide Pedrali** [1], Luca Giupponi[1], Annamaria Giorgi[1]
  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

Year 2023:

- **Saffron valorization by NIR spectroscopy**
  Contribution to Congress: International Conference on Near Infrared Spectroscopy (NIR 2023 Innsbruck), August 20-24, 2023, Innsbruck, Austria.
  Poster presentation
  Authors: Irene Locatelli[1], **Davide Pedrali**[2], Silvia Grassi[1]
  1 Department of Food, Environmental, and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Via G. Celoria 2, 20133 Milan, Italy.
  2 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

- **Monitoring of two forests damaged by the Vaia storm reveals a gradual restoration of vegetation greenness and possible changes in mature forest communities**

Contribution to Congress: 118th Congresso della Società Botanica Italiana, September 13-16, 2023, Pisa. **Poster presentation**

Authors: Luca Giupponi[1], Valeria Leoni[1], **Davide Pedrali** [1], Stefano Sala[1], Annamaria Giorgi[1]

1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

- **Landraces can be resources for the sustainable development of mountain areas: the case of "Copafam" bean (*Phaseolus coccineus* L.)**

  Contribution to Congress: 118th Congresso della Società Botanica Italiana, September 13-16, 2023, Pisa. **Oral presentation**

  Authors: **Davide Pedrali** [1], Luca Giupponi[1], Alessia Maria Bernardi, Francesca Cocchi[1] Annamaria Giorgi[1]

  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

- **Landraces can be agri-food resources for the sustainable development of mountain areas: the case of "Copafam" bean (*Phaseolus coccineus* L.).**

  Contribution to Congress: 2nd Conference for Young Botanists (CYBO), February 9-10, 2023, Bolzano. **Oral presentation**

  Authors: **Davide Pedrali** [1], Luca Giupponi[1], Marco Zuccolo[1], Valeria Leoni[1], Alessia Maria Bernardi, Francesca Cocchi[1] Annamaria Giorgi[1]

  1 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

- **The Fagio.Lo project**

  Contribution to Congress: 66th Annual Congress of Italian Society of Agricultural Genetics. September 5-8, 2023.

  Poster presentation

  Authors: Stefano Sangiorgio[1], Elena Cassani[1], Martina Ghidoli[1], Luca Giupponi[2], **Davide Pedrali[2]**, Gloria Coatti[2], Annamaria Giorgi[2], Roberto Pilu[1]

  1 Department of Agricultural and Environmental Sciences-Production, Landscape, Agroenergy, Università degli Studi di Milano, Milano, Italia.

  2 Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas-CRC Ge.S.Di.Mont., University of Milan, 25048 Edolo, Italy.

Co-supervision of thesis

- *“Monitoraggio della qualità dei foraggi e della caseificazione a nostrano Valtrompia*” D.O.P.”. Paolo Viviani; Supervisor: prof. Alberto Tamburini.