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Research article

Mycobiome characterization of "Ghimisone", a Sardinian ancient barley sourdough traditionally used in Sardinia for making "Ogliathu" bread

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Abstract
The sourdough is a biological complex matrix consisting of yeasts and lactic acid bacteria, derived from the spontaneous fermentation of the native microorganisms normally present in flour. In Italy, the microbiota of several hundred sourdoughs have been characterized at molecular and functional level and some of them have received the Protected Designation of Origin annotation (PDO) or Protected Geographical Indication (PGI). Also in Sardinia, until the '50s, the barley carasau bread was produced using a sourdough named "Ghimisone". The importance of keeping regional traditions alive and scientific curiosity have led to this work in which the mycobiota of Ghimisone was investigated for the first time. Three types of Ghimisones have been set up using flours of barley, naked barley, and a mixture of naked barley and "black lentil" of Calasetta. The mycobiota from these sourdoughs were investigated using independent molecular culture identification. Twenty different fungal species were found. The results were unexpected and showed that the mycobiome of Ghimisone is different from all other sourdoughs known in the literature. The diversity in the mycobiome of Ghimisone compared to known sourdoughs might suggests different nutritional and aromatic characteristics of its derived barley bread.

Keywords
Ghimisone; sourdough; mycobiota; barley; bread; Internal Transcribed Spacer

Introduction

The "mother dough", "natural yeast", "natural leavening" or "sourdough" is a complex biological matrix consisting of yeasts and lactic bacteria, derived from the spontaneous fermentation of indigenous microorganisms of the flour in which they live. The best-known sourdoughs are those derived from wheat flour. Overall, the yeasts most commonly identified in various types of sourdough belong to *Saccharomyces cerevisiae*, *Kazachstania exigua*, *Kazachstania humilis*, *Yarrowia keelungensis* and *Debaryomyces delbrueckii*, while lactic bacteria (LAB) belong to Lactobacillaceae, such as *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Fructilactobacillus sanfranciscensis*, *Limosilactobacillus fermentum*, *Latilactobacillus curvatus* and *Latilactobacillus sakei* (Venturini et al., 2012; Palla et al., 2017, Zheng et al., 2020). Some of the lactic acid bacteria species live in a close metabolic association with particular yeast species. For example, *F. sanfranciscensis,* usually the predominant bacterial species in sourdoughs, very efficiently ferments maltose contained in the flour and is often found in symbiosis with yeast species unable to use maltose, such as *K. humilis* and *K. exigua*.

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Various studies have demonstrated the beneficial properties derived from the use of sourdough for bakery products with respect to the use of the industrialized brewer's yeast (*S. cerevisiae*) (Lau et al., 2021). Such properties include the following: inhibition of the lipase activity of the wheat germ which could lead to the rancidity process (Rizzello et al., 2010); fermentation performed by natural yeast which leads to swelling of starch granules and to dissolving of starch linear chain (Nordlund et al., 2016). Moreover, during the fermentation, a lowering of the pH is found to improve the endogenous activity of the wheat protease (Heiniö et al., 2016). An accumulation of thiols has been found due to *F. sanfranciscensis*, which reduces oxidized glutathione to glutathione by glutathione reductase. This latter, in turn, undergoes thiol exchange reactions with gluten proteins leading, therefore, to the decrease in cross-linking of disulfide bonds and to the production of low molecular weight compounds (Gänzle and Zheng, 2019*). Lactiplantibacillus plantarum* allows the release of active phenols and peptides determining a good antioxidant activity (Fois et al., 2019). LAB act on the secondary structure of gluten proteins thus influencing the rheological properties of the dough and the quality of the product (Nutter et al., 2019). Recent studies on pure cultures have shown that LAB fermentation makes it possible to hydrolyze gliadin of gluten and that after fermentation of natural yeast for 72 hours, all the soluble alcohol protein complex extracted from the wheat is broken down (Fraberger et al., 2020). The sourdough LAB allow a decrease in phytic acid, the production of a more digestible product, and an increase in mineral bioavailability (Yildirim and Arici, 2019). In addition, acid fermentation allows the decrease of raffinose (a predisposing factor to internal disorders), condensed tannins, and trypsin inhibitors. Specific LAB (*Lactiplantibacillus plantarum, Lacticaseibacillus rhamnosus, Lactobacillus deuterium*) also have degradative action on some mycotoxins such as aflatoxin B1 (Zadeike et al., 2021). The sourdoughs increase the shelf-life of the product. During the sourdough fermentation, in fact, acetic acid is produced, a very important compound for its antimicrobial and antifungal action, and many LAB such as *Lactobacillus royi* and *Limosilactobacillus reuteri* inhibit the mold (Quattrini et al., 2019; Sadeghi et al., 2019; Sakandar et al., 2019). Antimycotic action is also due to LAB metabolites such as phenyl lactate, benzoic acid, fatty acids, volatile compounds, hydrogen peroxide, reuterin, and proteins (Crowley et al., 2013; Salas et al., 2017). Some aromatic compounds produced by sourdoughs such as carboxylic acids, esters, alcohols, ketones, aldehydes, and heterocycles improve the bread's sensory properties (Axel et al., 2015).

The sourdough in bakery products is mostly used in Italy and France due to their long experience and cultural heritage, but it is also used in other countries with very different traditions, such as China where traditional leavening agents include Laomian, Jiaozi, and Jiuqu / Quzi (Yan et al., 2019). Other examples are Iranian barbarians, Indian Bathura and Kulcha, African Egyptian Balady, Apprehenra, Ethiopian injera, and tortillas (Arora et al., 2021).

Less well-known but existing is the sourdough from barley flour (Zannini et al., 2009). It contains a greater number of lactic bacteria and yeasts than wheat flour sourdough, probably due to the greater concentration of vitamins and minerals available in barley flour. The presence of *L. brevis* and *L. plantarum*, able to produce organic acids, led to a higher total titratable acidity (TTA). In the sourdough from barley flour significant quantities of beta-glucans are also present (Rieder et al., 2012), which certainly contribute to the features of the obtained bread. Despite the decrease in volume and density of barley bread crumb, the consumer has in general accepted the product. The nutritional value and consumer acceptance suggest that the production of barley bread with sourdough should be encouraged.

Even in Sardinia up to the 1950s, barley bread and specifically barley carasau bread ("Orgiathu") was produced using the sourdough known as "Ghimisone". Ogliathu, the ancient barley bread was part of the diet of the long-lived Sardinians (Bertuccioli et al., 2021). In fact, the Sardinian agricultural economy of Ancient Neolithic was characterized by dressed barley, spelled, small spelled, and soft

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and durum wheat. Kernels of naked barley cereals have been found in the Middle Neolithic (4500-4100 BC) archaeological site of Su Mulinu Mannu (Terralba). Recent Neolithic evidences from the archaeological contexts of "Sa 'Ucca de su Tintirriolu" (Sassari), "Grotta del Guano" (Oliena), and Molia (Nuoro) show different plant species remains that witness the that time cultivation of barley both in the dressed and naked form. One hundred and one seeds have been identified belonging to kernels of cereals and legumes at the "Grotta del Guano" in the territory of Oliena. The cereals include naked and dressed barley, hexaploid and tetraploid wheat (*Triticum aestivum / durum*), while Peas (*Pisum sativum*) are the only identified legume (Attene et al., 1996). Further archaeobotanical evidences from the archaeological context of "Su Coddu-Canelles (Selargius)" dating back to the early and late Neolithic period, testify to an agricultural economy based on the cultivation of naked and dressed barley as well as soft and durum wheat. The high percentages of naked barley found would indicate an important role of this cereal in the diet of the community. In some cases, the barley choice adopted by prehistoric communities could also be linked to the type of soil where some cereals, such as barley, were able to better tolerate the high concentration of soil salt. In addition to grains, legumes such as peas and lentils were cultivated (Ucchesu et al., 2018).

The barley continued to be an important crop in the following centuries. In fact, during the Pisan and Genoese domination, the English historian (Day, 1984) reported that the Sardinian people had been forced to eat only barley since the wheat was shipped to the international markets. In particular, five-sixths of barley production and only one-sixth of wheat production were reserved for local consumption (Attene et al., 1996). Globalization has led to the loss of ancient traditions, with a consequent impoverishment of Italian food culture, considered the cradle of healthy eating. The relevance of keeping regional traditions alive and scientific curiosity have led to the present work in which for the first time the mycobiota of "Ghimisone" was investigated by using a next generation sequencing (NGS) approach. Three types of Ghimisones have been set up: one, more usual with dressed barley (B), another, less usual but still used, with naked barley (NB), and one with naked barley and "black lentil" (NBL), a legume vulgarly confused with lentil but identified as *Vicia articulata*.

Material and Methods

Sample preparation

Ghimisone was obtained from a simple blend of flour and water. Its preparation has required several different stages. Three flours were produced in order to prepare three different sourdoughs. The first flour was made using a Sardinian variety of barley (*Hordeum vulgare*) named "Ogliu Sardu", from which 800 g of flour were obtained from one kilogram of barley. A second flour was produced using a Sardinian variety of naked barley (*Hordeum vulgare* var. *nudum*) named "Tridihogliu - Pede voe", from which 900 g of flour were obtained from one kilogram of barley. A third flour was produced using a combination of 70% of naked barley, and 30% of "black lentil" (*V. articulata)*; 900 g of flour were obtained from one kilogram of barley. Three different Ghimisones were then produced, with different proportions of flours and water. B Ghimisone was obtained by mixing 780 g of "Ogliu Sardu" flour and 500 ml water, NB Ghimisone by mixing 900 g of "Tridihogliu - Pede voe" and 900 ml water; NBL Ghimisone by mixing 630 g of naked barley flour plus 270 g of black lentil flour and 600 ml water. The three Ghimisones were then cooked in wood-burning oven off for forty minutes at 250 °C (Fig. 1a). The temperature was releaved by an infrared thermometer (infrared thermometer 98-880 Alpha elettronica, Collecchio, Italy), which measures the temperature in the range of 20-520 °C, with a response time of 500 mS. The detected temperature at the beginning and at the end of the baking procedure was 250 °C and 220 °C respectively. The inner temperature of the Ghimisone at the end of baking was 40 °C. After cooking, the Ghimisones were covered with wool towels for six days

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at room temperature (Figs 1b,c). Thus, a small portion of the inner central part of each Ghimisone was quickly trasnferred in steril falcons, kept at -20 °C until subsequent molecular analyses were carried on by BMR genomics Srl (Padova, Italy). The inner part of Ghimisone is the one that is mixed with water to obtain "Su Druhe" (Fig. 1d), the sweet-taste leavening cream, traditionally used in Sardinia as sourdough for making the barley bread.

a) b) c) d)

Fig. 1 – Ghimisone preparation procedure: a) Ghimisone after the cooking phase; b) procedure of Ghimisone covering by means of a wool towel; c) six-day rest time of Ghimisone d) preparation of the "Su Druhe", the inner part of Ghimisone used as sourdough for making "Ogliathu" barley bread.

Sample processing

The three Ghimisones were processed at BMR genomics Srl which characterized samples through the following steps: DNA Extraction, DNA Quantification; Conventional PCR and Gel Electrophoresis; qPCR assays with Specific and Generic Primers; Analysis of qPCR results; Next Generation Sequencing (NGS) methods; Bioinformatics.

DNA extraction

200 mg of sourdough were added to 800 µl of CD1 solution (Dneasy® 96 PowerSoil® Pro QIAcube® HT Kit, Qiagen) and to about 200-300 µl of zirconium-silica beads (0.1 mm - Biospec), vortexed and incubated for 10 min at 65 °C; vortexed using the Tissue Lyzer (Qiagen) for 10 minutes at 25 Hz. Then the samples were centrifuged at 15000 g for 1 min. 550 µl of the lysate was added to 250 µl of CD2 solution and centrifuged at 3700 rpm for 10 minutes. 550 µl were used as starting material to extract DNA with the Qiacube HT Extractor and Dneasy® 96 PowerSoil® Pro QIAcube® HT Kit. DNA was eluted in 120 µl.

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DNA amplification and sequencing

ITS2 region was amplified with Taq Platinum HiFi (Termofisher) and the primer pair ITS3f-ITS4r (White et al. 1990) with universal tails:

ITS3f:5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGAACGCAGC-3′

ITS4r: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3′

The samples were analyzed on 1.5% agarose gel and a sample concentration of not less than 5 ng μl^{-1} was considered acceptable (data not shown). The amplicons obtained were purified with Thermolabile Exonuclease I (NEB), diluted 1:2, and amplified with a short cycle with the Index Nextera XT which are bound to the universal tails. Amplicons were normalized with SequalPrep (Thermo Fisher) and multiplexed. The pool was purified with Agencourt XP 1X magnetic beads, loaded onto Miseq, and sequenced with V3 chemistry - 300PE strategy.

Bioinformatic analysis

After the run, the raw sequences were divided into R1 and R2 (forward and reverse). The raw sequences R1 and R2 were processed by removing the adapters using Cutadapt v1.14 and then merged using USEARCH v.10. The de-novo UPARSE-OTU algorithm was used to select 97% identity OTUs and remove chimeras. OTUs were aligned against the UNITE database, collected in the .biom file and filtered at 0.005% to eliminate spurious OTUs present at low frequency The sequences were delivered in fastaQ format separate for each sample for the bioinformatic analysis (White et al., 1990).

Shannon's Index (H) was used as diversity index; it was calculated as follows:

$$H = \sum_{i=1}^{n} -p_i \ln p_i$$

where p_i was the relative frequency of *i* species; *n* was total number of different species and ln is natural logarithm. A diversity index is a quantitative measure that reflects how many different types (such as species) there are in a dataset (a community), and that can simultaneously take into account. Then the Shannon's entropy quantifies the eveness of species identity of the dataset.

Results

In this study three Ghimisones were analyzed: Barley (B); Naked Barley (NB); Naked Barley + Black Lentil (NBL). A culture-independent metagenomic analysis of the ITS2 region of the fungal rDNA was conducted for the mycobiota characterization.

All identified species and genera belong to both Ascomycetes and Basidiomycetes (Table 1 and Fig. 2). The predominant species in all three samples is *Filobasidium globisporum* of which the read quantities are approximately the same in the three samples: 41.20% in B, 35.06% in NB, and 23.19% in NBV. *Sporobolomyces roseus* is present for 30% in B and in much lower quantities in the other two samples (about 5 times less). *Vishniacozyma victoriae* is also abundant, with the highest percentage in NBVsample (29.71%) followed by NB (25.14%) and B (11.37%). The same trend is also found for *A. pullulans* (1.72% in B, 6.98% in NB, 17.39% in NBV) and *Cystofilobasidium macerans* (1.79% in B, 15.01% in NB, 17.39% in NBV). The remaining species are reported in very low quantities, so we suppose that they have a negligible role.

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Table 1 – Relative abundance of fungal species detected in the three Ghimisones

Family	Species	B	NB	NBL
Aureobasidiaceae	*Aureobasidium pullulans*	1.72%	6.98%	17.39%
Buckleyzymaceae	*Buckleyzyma salicina*	-	0.21%	-
	Dioszegia hungarica	1.72%	1.77%	5.07%
	Gelidatrema spencermartinsiae	-	0.18%	0.72%
Bulleribasidiaceae	*Vishniacozyma dimennae*	-	0.64%	1.45%
	Vishniacozyma heimaeyensis	0.64%	0.18%	-
	Vishniacozyma tephrensis	-	0.28%	-
	Vishniacozyma victoriae	11.37%	25.14%	29.71%
Cystofilobasidiaceae	*Cystofilobasidium macerans*	5.79%	15.01%	17.39%
Entylomatales	*Tilletiopsis washingtonensis*	-	0.18%	-
Filobasidiaceae	*Filobasidium globisporum*	41.20%	35.06%	23.19%
	Filobasidium oeirense	2.58%	0.92%	-
	Filobasidium wieringae	-	2.87%	-
	Naganishia albida	-	0.32%	-
Holtermanniales	*Holtermanniella takashimae*	4.29%	3.58%	-
Leucosporidiaceae	*Leucosporidium fragarium*	0.43%	0.04%	-
	Rhodotorula graminis	-	0.50%	0.72%
Sporidiobolaceae	*Sporobolomyces roseus*	30.26%	5.84%	4.35%
	Sporobolomyces ruberrimus	-	0.25%	-
Incertiss sedis	*Curvibasidium cygneicollum*	-	0.07%	-

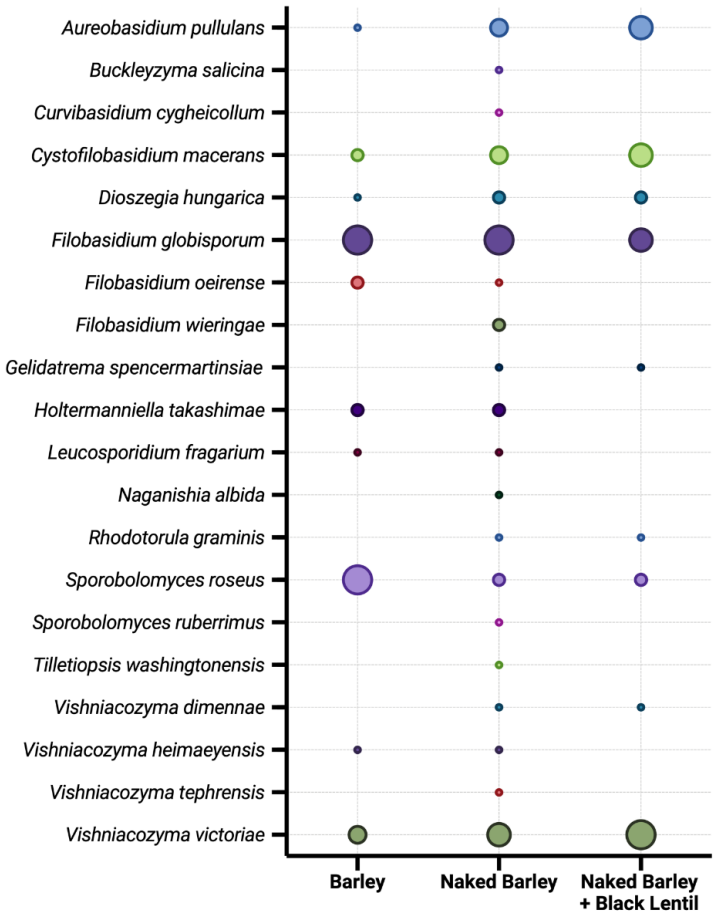

Fig. 2 - Percentage quantities of reads deriving from the single species. Circle's areas were proportional of relative read abundance.

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The Shannon index has a value of 2.11 for Ghimisone B, 2.55 for Ghimisone NB, and 2.25 for Ghimisone NBL, so Ghimisone NB showed higher community heterogeneity

Discussion

Several studies carried out all over the world have long shown that the use of sourdough confers unique sensory and nutritional characteristics to bread, increasing its aroma and taste, improving its volume and consistency, prolonging its shelf-life, and increasing its nutritional and nutraceutical value. In Italy, the microbiota of several hundred sourdoughs has been characterized at a molecular and functional level. Some breads have received the PDO (Protected Designation Origin, e.g. Italian Altamura bread and Tuscan bread) (Ricciardi et al., 2005; Minervini et al., 2012; Palla et al., 2017) or the PGI (Protected Geographical Indication, e.g. Matera bread and Pair of Ferrara bread) (Vernocchi et al., 2004; Minervini et al., 2012). In each sourdough used for the production of the various typical bread and bakery products, populations of peculiar microorganisms develop, in relation to the production process (temperature, pH, mode of refreshments), the type of flour used, and the different environmental conditions. For this reason, each sourdough is closely related to the geographical area in which it is produced. Since very little is known about Ghimisone and given the above-described influence that sourdough can have on the derived bread, we have undertaken its characterization starting from the mycological composition.

In the present analysis, we also included a type of Ghimisone, obtained in the past by adding to the naked barley a legume, confused by farmers for lentil, and named “black lentil” but botanically identified as *V. articulata.* This species of *Vicia* in Italy disappeared in the twentieth century, although in Sardinia an ancient variety is still cultivated in Calasetta. Good nutritional quality of *V. articulata* seed proteins has been demonstrated with a protein content of 20.1%. Among the essential amino acids, the most abundant ones appear Leu, Lys, Phe, Thr, and Val. On the contrary, the content of Trp is limited, between 0.5% and 1.0% as well as the sulfur-containing amino acids, Met and Cys. Total unsaturated fatty acids are in greater quantities than saturated ones and among unsaturated fatty acids the most abundant ones are linoleic, oleic, and linolenic acids. *V. articulata* is rich in phenolic compounds with a greater antioxidant activity than the widely consumed legumes such as soy, chickpeas, or lupine (Pastor-Cavada et al., 2011). An oral testimony handed down in Oliena (Sardinia), tells that in times of famine, during the grinding of the barley, some families added a percentage of lentils to obtain a more abundant flour starting from small quantities of available cereal. At the same time, the added legume mitigated the too bitter taste of barley and made the barley bread more pleasant.

The results obtained in the present investigation showed that the Ghimisone mycobiota is completely different from those of the sourdoughs reported in the literature including sourdough obtained from barley flour (Zannini et al., 2009). This difference is attributable to the completely unique way of preparing this sourdough that perhaps should not even be defined as such. In the traditional and modern overview of the bakery, we did not find any procedure like the one described for Ghimisone preparation. The barley bread obtained using this technique has been the main pillar of the diet of inhabitant of Barbagia, a Sardinian geographical area. Today this ancient preparation has almost disappeared, but due to the climate change and to the global crises of wheat production, it could be a valid alternative, both from a nutritional point of view and to enhance the biodiversity of local cereal, as well as to maintain a tradition with deep roots.

The Ascomycetes and Basidiomycetes that we have identified are largely unknown in the literature, and some of them are only mentioned. Ascomycete yeasts are those mainly used for biotechnological purposes as they are responsible for alcoholic fermentation in the production of bread and beer,

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and for the synthesis of recombinant proteins for pharmaceutical or environmental use. The use of basidiomycete yeasts in biotechnology, have only been evaluated in recent decades with respect to ascomycete yeasts. Basidiomycetes are mainly known for enzyme production in pharmaceutical chemical fields, for the production of some classes of primary and secondary metabolites such as terpenoids and carotenoids, for the aerobic catabolism of complex carbon sources, and for the bioremediation of environmental pollutants (Johnson, 2013).

Among the identified fungi, *F. globisporum* is present in the highest percentage in the three samples. There is not much information in the literature regarding *F. globisporum*, except that it was found in a particular white and marinated artisan cheese from western Serbia, a product with a very complex microbial community (Šuranská et al., 2016).

V. victoriae is also an abundant species present in the three samples. *V. victoriae* yeast has been identified in wheat grain samples from the Canadian prairies (Vujanovic, 2021) and has a very interesting biotechnological potential linked to the biosynthesis of yeast oils, carbon-active and proteolytic enzymes.

Another abundant species characterized in the three samples of Ghimisones is *A. pullulans*. It appears to be of particular nutraceutical interest as it produces fructooligosaccharides (FOS) with antidiabetic activity (Bharti et al., 2013). *A. pullulans* is one of the most abundant species in freshly pressed grape juice; it survives in the fermentation phase of the juice, with the ability to produce polysaccharides and malic acid polymers such as poly-malic acid and enzymes with β- glucosidase, pectinase, and tannase activities (Onetto et al., 2020). *A. pullulans* promotes the synthesis of beta-glucans; specifically, it produces 1-3,1-6 beta-glucan which is secreted outside the cell. It has been found that 1-3,1-6 beta glucan produced by *A. pullulans* plays an important role in enhancing the immune system and thus in decreasing the infections of the respiratory tract. For this reason, it was also used as a nutraceutical supplement to counteract COVID-19 infection. The beta-glucan produced by *A. pullulans* seems to improve immunity by increasing IL-8, sFAS macrophage activity, and NK cells' cytotoxicity, which represent the major defense mechanisms against viral infection. This beta glucan has been listed by the US FDA in the Generally Recognized As Safe category (GRAS) and has been approved by Ministry of Health in Japan, where it is available as a commercial dietary supplement for human consumption since 1996 (Rao et al., 2020).

Filobasidium oeirense, *S. roseus*, *A. pullulans*, *N. albida* and *V. dimennae* have recently been identified in 39 genotypes of durum wheat cultivated in Sicily. The importance of *N. albida* and *V. dimennae* yeasts was highlighted, as their use produces doughs with a greater volume, improving the sensory characteristics of bread when co-inoculated with *Saccharomyces* (Alfonzo et al., 2021). *A. pullulans* and *N. albida* have been identified from wheat and flour of genotypes grown in several US states (Kurtzman et al., 1970) and in Sicily (Gaglio et al., 2020). *S. roseus*, *V. victoriae*, and *A. pullulans* yeasts were characterized in wheat where they represented 20-40% of the total isolated fungi (Rojas et al., 2020). *V. dimennae* has also been detected among genotypes of old local wheat species (Alfonzo et al., 2021) and it is present in NBV-(1.45%) and in NB (0.64%) while is absent in B-sample.

Furthermore, in the research here conducted we identified yeasts not yet studied individually in-depth such as *Gelidatrema spencermartinsiae* and *Filobasidium oeirense*, which are indicated as new species in GenBank in the phylogenetic study on Tremellomycetes conducted by Liu et al. (2015). *Dioszegia hungarica* has also been found in Portugal as a new anamorphic species in the Tremellales lineage of Hymenomycetes Basidiomycota (Inácio et al., 2005). *Dioszegia* yeasts produce orange colonies, compounds similar to starch, that assimilate D-glucuronic acid and/or inositol, and are unable to use nitrates.

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The yeasts *V. victoriae* and *V. tephrensis* were discovered in samples of wheat grains. These yeasts have been tested for plant protection as antagonists of common fungal phytopathogens in post-harvest disease management and for the biological decontamination of agricultural products from mycotoxins. However, the relationship between these Tremellomycetes and their host or wheat is still unknown (Vujanovic, 2021). *Tilletiopsis washingtonensi*s was found for the first time in a research carried out on the assimilation of volatile substances on ripe apples (Vishniac et al., 1996) in which the assimilation by yeasts (*T. washingtonensis*) of acetate, propionate, butyrate and ethyl acetate was studied. It was also found in a study carried out on Ciauscolo, a fermented sausage with PGI status produced in the Italian Marche region, in which volatiloma was characterized during fermentation (Belleggia et al., 2020). In the present study, *T. washingtonensis* was determined in low percentages only in NB sample (0.18%).

Leucosporidium fragarium is a psychrotolerant yeast isolated from the soils of King George Island in the sub-Antarctic region which has been evaluated for its production of extracellular gelatinase, an enzyme with a high potential for applications in different areas, including functional food and medicine (Yuivar et al., 2019). We found it in B (0.43%) and in NB (0.04%) samples, while it is absent in NBV sample. *Curvibasidium cygheicollum* actively participates in the fermentation of Grignolino grapes in a particular vineyard in Piedmont (Italy) (Vaudano et al., 2019). In our study, 0.07% was detected exclusively in the NB sample.

From the performed analysis the NB Ghimisone shows the most heterogeneous fungal population. It, in fact, differs from the other two Ghimisones for the presence (although in small percentages) of the *V. tephrensis*, *T. washingtonensis*, *S. ruberrimus*, *N. albida*, *F. wieringae, C. cygheicollum*, and *B. salicina*. The NB and NBL Ghimisones could have better nutraceutical properties for the presence of the *A. pullans* species found in greater concentration, and important for the production of beta-glucans. The synergy between black Calasetta lentil and naked barley could represent an interesting combination from which a good alternative of functional food could derive, either for nutraceutical or for sensorial and organoleptic characteristics. Moreover, in the NBL Ghimisone *V. dimennae* species (absent in the two others Ghimisones), and *N. albida* (present only in the NB Ghimisone) could confer consistency and volume to the dough. Their absence in Ghimisone B suggests that it is not the type of Ghimisone to make mixtures more easily workable. Finally, we remark that the species most represented in the three samples is *F. globisporum*, which, being a species still unknown in literature, will be adequately investigated in our future researches.

The results reported in this research suggest that Ghimisone is a community of yeasts with fermentation capacity at low temperatures. The research will move to the analysis of the bacterial composition of Ghimisone and especially to the derived bread in the attempt to provide scientific information, to support the local cereal biodiversity and to keep alive a Sardinian culinary tradition.

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