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NEW LOW-INPUT MICROBIAL PROCESSES FOR THE ENHANCEMENT OF BREWERS' SPENT GRAIN

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Dedication

"All researchers had a lead teacher,

but only the luckiest find a Master"

Alla Mia Maestra

Declaration

By submitting this thesis, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by the University of Sassari will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Chapters 1, 2 and 3 have been published in peer-reviewed journals. Chapters 1, 2, and 3 are presented in the style of a journal manuscript. Work on Chapter 1 was started in 2018.

Sassari, 5 January 2022

Abstract

Sustainable production is the creation of goods and services using processes compatible with the protection of the environment and the natural resources to be passed on to future generations. In this context, and also supported strongly by EU policies, the production of craft beer must combine the concept of quality with that of sustainability, favoring a short supply chain and zero waste. This new production culture must translate into an increased and equitable distribution of income along the supply chain. To achieve these goals, it is necessary to understand which phases of the production process can be optimized and which innovations can be adopted in terms of sustainability, to obtain economic advantages. It is therefore strategic to seek product quality starting from local materials, to strengthen the link between beer and the production area, and to increase the sustainability through the valorization of the by-products.

The disposal of waste from agro-industrial processes represents a problem with economic and environmental implications. However, waste can be seen as a resource rather than a problem. EU regulations (Directive 2018/851 / EU; Directive 2008/98 / EC) encourage the extraction of valuable components from by-products of the food industry, to produce functional foods, adjuvants, and pharmaceutical preparations, and/or the transformation of waste into soil improvers and fertilizers for agricultural use. In the brewing sector, the management of by-products, which account for approximately 90% of the raw materials used, is one of the critical issues. Brewers' spent grain (BSG) is the most abundant by-product, after water, and it is obtained from the mashing, with an average yield of about 20 kg per hectoliter of beer. The recovery and valorization of BSG would bring considerable savings to the production costs. Currently, many breweries have agreements with local farmers to give their BSG free of charge as a low-cost supplement for animal feed.

Recently, a technological transfer project entitled, "Sustainable Development of Craft Beer in Sardinia" has been financed by the Sardinian Regional Government within the framework of the POR FESR 2014/2020 - PRIORITY AXIS I "SCIENTIFIC RESEARCH, TECHNOLOGICAL DEVELOPMENT AND INNOVATION". This project is being carried out by the researchers of the University of Sassari in collaboration with Porto Conte Ricerche, SOTACARBO, and 22 craft breweries and 12 agricultural companies, with the general objective of making regional brewing production more competitive on

the national and international market by favoring the development of innovative and beneficial models of a circular economy. In particular, it is aimed at the identification of microbial processes for the valorization of brewery waste, such as wastewaters and BSG.

In this context, the aim of this Ph.D project was to identify and optimize environmentally friendly microbial processes for the valorization of BSG for its reuse as an agricultural soil conditioner and a fertilizer. Providing innovative solutions in the enhancement of BSG will serve to increase the profitability and environmental sustainability of craft microbreweries.

The objectives of this research were therefore:

- to present the current literature on BSG from a microbiological point of view;
- to compare the physiochemical and microbiological quality and safety of vermicompost obtained from both BSG and cow manure using the earthworm *E. fetida*;
- to evaluate from a microbiological and chemical perspective a low-input pre-treatment process designed to make raw BSG palatable for these earthworms;
- to evaluate of the possible use of raw BSG as a growth substrate for a microbial-based biofertilizer.

Organization of the Ph.D. thesis

The general introduction provides a comprehensive overview of the initiatives undertaken by international organizations, such as the United Nations, to encourage the circular economy and sustainable development and to minimize the exploitation of natural resources and reduce wastes. Specifically, the beer sector has been considered in greater detail, particularly that of craft breweries, whereby encouraging the reuse of waste after the necessary transformation by innovative biotechnologies can increase the sustainability of production and generate new income.

Chapter 1, “The role of microorganisms on biotransformation of brewers’ spent grain”, provides a bibliographical examination particularly focused on the role of microorganisms in BSG exploitation to (1) produce enzymes and metabolites of industrial interest, (2) supplement human and animal diets, and (3) improve soil fertility. Emerging safety issues in the use of BSG as a food and feed additive have also been considered. A better understanding of the potential of microorganisms as biocatalysts for BSG transformation is essential for its recycling and exploitation. Given the bio-technological and health importance of the various microbial groups that have been isolated from BSG, it is also essential to assess the metabolic relationships among the microbial communities, and to determine their influences on the final transformation of BSG, to exclude, or on the contrary, to favor, new microbial-based processes for BSG valorization. This bibliographical examination was published in “Applied Microbiology and Biotechnology” and is indexed on the SCOPUS and WOS databases (Q1; IF: 3.530)

Angela Bianco, Marilena Budroni, Severino Zara, Ilaria Mannazzu, Francesco Fancello, Giacomo Zara. (2020). *The role of microorganisms on biotransformation of brewers’ spent grain*. Applied Microbiology and Biotechnology, 104(20), pp. 8661-8678.

Chapter 2, “Comparative analysis of vermicompost quality produced from brewers’ spent grain and cow manure by the red earthworm *Eisenia fetida*”, reports on the suitability of BSG as a substrate for the breeding of earthworms to produce a nutrient-rich vermicompost. Vermicomposting is a green technology used to transform a lot of organic waste into an excellent fertilizer. The process uses the work of earthworms, which interact with microorganisms and other soil organisms to fragment, oxidize, and stabilize the organic waste to provide a fertilizer that is permitted for use in

organic farming. The results obtained showed that BSG supports healthy growth of *E. fetida* and that vermicompost from BSG is rich in nitrogen and can be safely used as soil improver. Indeed, vermicompost from BSG respects biological and microbiological safety law parameters, while unprocessed BSG showed ochratoxin A levels that exceeded the legal thresholds. Finally, the enzymatic activities revealed a strict link between microbial populations and the quality of the vermicompost. The results obtained were published in “Bioresource Technology”, which is indexed on the SCOPUS and WOS databases (Q1; IF: 9.642).

Saba Sara, Zara Giacomo, **Bianco Angela**, Garau Matteo, Bononi Monica, Deroma Mario, Budroni, Marilena (2019). Comparative analysis of vermicompost quality produced from brewers’ spent grain and cow manure by the red earthworm *Eisenia fetida*. Bioresource Technology, 293, 122019.

In Chapter 3, “Microbial and chemical dynamics of brewers' spent grain during a low-input pre-vermicomposting treatment”, a low-input pre-processing step was evaluated to better exploit BSG microbiota and to make BSG suitable for vermicomposting. This process was designed by avoiding expensive thermal treatments or chemical and microbiological additives, and to fully exploit the microflora naturally associated with raw BSG. This pre-treatment resulted in accelerated decomposition of BSG, with reduced time required to obtain high-quality vermicompost. In particular, the vermicompost obtained showed good indicators of maturity and respected the legal requirements related to pathogenic microorganisms and mycotoxins, as well as phytotoxicity. Finally, the pre-treatment allowed the growth of fungal and bacterial species that are naturally associated with BSG that eventually shaped the microbiota of the mature vermicompost, thus increasing its biological quality. The results obtained were published in “Science of The Total Environment”, which is indexed on the SCOPUS and WOS databases (Q1; IF: 7.963).

Bianco Angela, Fancello Francesco, Garau Matteo, Deroma Mario, Atzori Alberto Stanislao, Castaldi Paola, Zara Giacomo, Budroni Marilena (2022). Microbial and chemical dynamics of brewers' spent grain during a low-input pre-vermicomposting treatment. Science of The Total Environment, 802, 149792.

In Chapter 4, “Brewers’ spent grain as a no-cost substrate for production of plant-growth-promoting microorganisms in beneficial microbial consortia”, the feasibility of the use of BSG as a growth

substrate to produce microorganism-based biopreparations in solid-state fermentations was investigated.

In the general conclusion, the results obtained from this PhD project have been summarized. An alternative reuse of BSG has been proposed not only as a low-cost growth substrate for biofertilizers, but also as a substrate that enriches the composition of the preparation with beneficial microorganisms for the soil and for plants.

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1. General introduction

1.1. The trend of the beer sector

In the EU, the number of breweries exceeded ten thousand units in 2019. Furthermore, for the first time in a decade (2009/2019), beer production volumes exceeded 400 million hectoliters/year, with consumption per capita close to 70 liters/year (European Beer Trends, Statistics Report, 2020). The effect of the economic crisis and the evolution of the competitive framework led to a concentration of production, which reduced the number of companies in the sector, but not the brands on the market. Europe, including Russia, is characterised by the closest production and consumption tradition of beer in the world, and exports volumes of more than 30 million hectoliters.

The countries of western Europe, which are more traditionally associated with beer, show declining trends in consumption. Germany and Belgium are characterised by a production landscape rich in regional and zonal specialities, some of which are protected by the Protected Geographical Indication and Traditional Speciality Guaranteed labels. In Italy and France, which have a predominantly wine-growing vocation, the production and consumption of beer is lower than the European average. Of particular note, there is the phenomenon of the spread of the consumption of special and craft beers, both national and imported, which highlights the progressive formation of an increasingly evolved beer culture, and which may open new prospects for market development. On the other hand, Spain has been characterised in the recent past by exponential development of the brewing industry and consumption. On the Iberian Peninsula, beer consumption has become a daily occurrence and has become more widespread than in any other Mediterranean country.

1.2. The Italian beer sectors

Analysis of the market trends (2009/2019) shows a growth in volumes, production, and per capita consumption in Italy (AssoBirra Annual Report, 2020). Beer production has grown more than eight-fold in the last 10 years, from 13.2 million hectolitres produced (2013) to 16.4 million hectolitres (2018), with a growth of almost 750,000 hectolitres of beer from 2017 to 2018, equal to 4.7%. With 16.41 million hectolitres in 2018, Italy ranks 9th in Europe in terms of production volumes, while it is 5th in terms of number of breweries (Figure 1). In 2019, production (+5%), domestic consumption (+2.6%). and exports (+13%) increased for the third year running. There were positive repercussions

on employment (+3,300 units), which reached 144,000 workers, including direct and indirect employees. Finally, positive trends have also affected the microbrewery sector, with over 800 microbreweries born in Italy mostly in the last 10 years, the production of which grew by 4.3% in 2018, compared to the previous year.

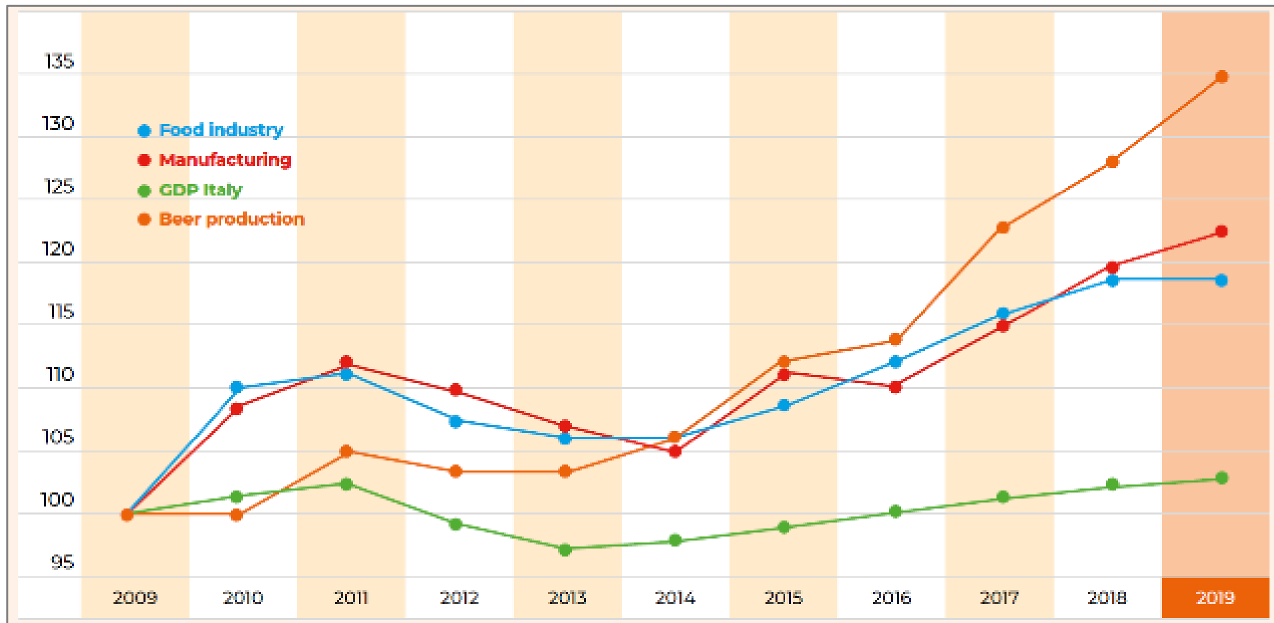

Fig.1: Italian beer production in the last decade (2009 Base =100; data by volume, Source: AssoBirra 2020 processed Istat data (AssoBirra - Italian brewers' association; Istat - Italian Government Statistical Office).

The increase in value has affected the entire sector, also involving the branch of small producers, a sector that in Italy has about 850 facilities, including brew pubs and microbreweries, and which in 2019 recorded a production of 523,000 hectoliters (3.1% of the national figure), up by 3.8% compared to 2018. Beer conveys an image of a beverage that sees its reputation strengthened on the one hand, and its nature as a meal drink on the other. Innovation and the ability to satisfy consumer tastes remain key elements in achieving new goals. On the other hand, the development potential of the Italian beer market is enormous.

1.3. The Italian craft beer sector

The production of beer is regulated by Law 1354/1962 "*Disciplina igienica della produzione e del commercio della birra*" (Hygiene regulations in the production and trade of beer), within which, according to the law of 2016 (28 July 2016, no. 154), for the first time in Italy a legal definition of

"craft beer" was given. There are now 862 microbreweries registered, with a production of 523,000 hectolitres, up 3.1% on 2018 (Assobirra Annual Report, 2019). The organisations surveyed from North to South have 3,000 employees and are divided into craft breweries (692) and brew pubs (170). Their market share is 3.1%.

1.4. The craft beer sector in Sardinia and regional legislation

The consumption of beer in Sardinia has peculiarities that are unique in Italy, both in terms of quantity (60 liters per capita) due to consumption being much higher than other regional averages, and in terms of loyalty, since most consumption was and remains oriented towards a single industrial beer, which shrewdly relies heavily on a regionalist image and marketing strategy (although since 1986, Ichnusa has been a product of the Heineken group).

Sardinian beer scenarios have shown that they have something to say in the evolution of Italian craft beer. The future of the market will depend on the ability of brewers to innovate their offerings for new consumers, with more traditional or special beers.

In a few years, the craft beer sector in Sardinia has gone from 13 activities in 2012 to almost 47 today, of which 9 have suspended production, and 5 are brew pubs (Confartigianato Sardegna Studies Office, based on Union Camere-Infocamere report 2020). At the same time, there is a strong need for measures to protect and enhance the value of existing craft breweries, and therefore for a regional regulatory intervention aimed at developing craft beer and considering the *status quo* to enhance all of the companies that already produce craft beer on the island. The creation of regional laws on craft beer is a phenomenon that began well before the birth of the national legislation in 2016. The development of the craft beer sector does not depend only on the number of breweries and clubs that open in Italy, but also on the support tools that the institutions are able to deploy to the entire supply chain. Fortunately, for some years the various regional bodies have been moving in this direction, approving local laws to promote and incentivize the sector. In the initial period these were characterized by a certain heterogeneity of the different laws, such as for the regions of: Friuli-Venezia Giulia (Regulations on Craft Beer, R.L. n. 23 of 9 June 2017 https://lexview-int.regione.fvg.it/fontinormative/xml/xmllex.aspx?anno=2017&legge=23); Veneto (Promotion and enhancement of the products and activities of craft beer producers, R.L. n. 7 of 16 February 2018

https://bur.regione.veneto.it/BurvServices/pubblica/DettaglioLegge.aspx?id=363923); Marche (Promotion and enhancement of craft and agricultural beer, R.L. n. 6 of 19 February 2020 https://www.consiglio.marche.it/banche_dati_e_documentazione/leggi/dettaglio.php?arc=vig&idl=2135); and Campania (Measures to support quality agriculture and agro-food heritage in the sector of agricultural and craft beer production, L.R. n. 15 of 24 June 2020 http://regione.campania.it/normativa/item.php?7b7fec2087f982d694b26f0cc9f850d6=fc9dde2c31a2358bca429be7a48e2fc8&pgCode=G19I231R1888&id_doc_type=1&id_tema=23&refresh=on). More recently, different region have found a common model with which to model their own regulations, such as for the regions of: Lazio (Measures to enhance and promote the products and activities of craft beer producers, L.R. n. 20 of 23 December 2020 https://innovaluppolo.crea.gov.it/?dt_portfolio=legge-regionale-lazio); Abruzzo (Rules for the promotion and enhancement of agricultural and craft beer L.R. n. 5 of March 15, 2021 http://www2.consiglio.regione.abruzzo.it/leggi_tv/abruzzo_lr/2021/lr21005/Articolato.asp); and Puglia (Rules on the enhancement and promotion of the products and activities of the Apulian craft beer producers, L.R. n. 42 of 30 November 2021 https://www.consiglio.puglia.it/-/produzione-di-birra-artigianale-paolicelli-depositata-proposta-di-legge-per-valorizzare-un-attivit%C3%A0-in-crescita-e-sostenere-le-filiere-locali-). The path taken by these regions is the effect of the strong growth that has affected and continues to affect Italian craft beer. The common objective of the laws is to enhance the local agricultural and craft beers with a brand, a specification that highlights raw materials and production area. This "revolution" in the protection and enhancement of excellent beers, together with the supply chain, wants to take root in Italian culture at all costs. The solution remains the same: work on innovation, quality and sustainability. This requires the opportunity to rethink the development model, and to provide a cutting-edge supply chain in terms of sustainability.

1.5. Circular economy and ecological footprint of beer production

Brewers have two competing imperatives. The first is the need to maintain quality all the way to the beer drinker, and the other is to cut back on their environmental impact. The brewing industry uses malted barley, hops, water, energy, and other inputs to produce an alcoholic beverage through

fermentation (Karlovic et al., 2020). During the production process of the beers, solid waste, wastewater, and gaseous emissions are generated (Olajire, 2020). These environmental issues can cause eutrophication and contamination of soil and groundwater, and can contribute to the greenhouse effects. The carbon footprint of a 6-pack 330 ml beer bottle is 3,188.8 g of CO_2 equivalents (g CO_2e). An average adult tree absorbs 59.65 g of CO_2 per day. A single tree would take 53.46 days - or nearly 2 months - to offset the carbon emissions of a single six-bottle pack of beer (Cimini and Moresi, 2016). The main source of greenhouse gases is bottle production (35%), retail transport (26%), and malt production (17%) (Munoz et al., 2012). Glass production, barley production (Cordella et al., 2008), and malt make up the largest proportion of the environmental impact of beer production. Packaging is a major source of greenhouse gas emissions, as it represents up to 85% of the required energy. Stainless-steel drums have a lower impact than glass as they are reusable. A marketing strategy that uses refillable containers and drums could have enormous benefits for the environment (Shin and Searcy, 2018).

Traditional growing methods for barley require repeated tilling of the land and application of fertilizers and pesticides, which also have a heavy environmental footprint. Low and no-till methods could decrease this impact, and organic malts would further reduce the overall equation, as fertilizers have a heavy carbon footprint of their own. As intensive tilling practices disrupt the normal storage of organic carbon in the soil, the assumed carbon offset from this natural process is less likely to be realized. Brewers desire a plump barley kernel, which makes irrigation a general practice, although barley can be grown as a dry land crop. Barley is steeped, germinated, dried, and sometimes roasted in the production of malt for brewing. Drying and roasting are the most energy-intensive parts of this process, using both electrical and heat energy.

Production of beer at the brewery is the smallest part of the environmental impact calculation. If generally accepted practices are adopted—such as heat exchange for cooling the wort and attention to energy and water consumption and conservation—brewery operations account for less than 20% of the overall environmental impact. Electrical energy production is a significant factor in this calculation, so subscription to high-quality renewable energy programs can provide a measurable decrease in overall carbon consumption and emissions. Breweries that are environmentally committed can have carbon emissions around 5% of the total carbon impact of the beer.

Breweries use a lot of water to make beer, especially due to the rigorous and constant cleaning that is necessary during almost every part of the brewing process. A ratio of 3.25 to 1 is considered excellent throughout the world. Many international breweries have set aggressive targets around water usage.

Understanding the life cycle of beer can help focus attention on areas with the greatest potential for reducing the environmental impact. Identifying these can help guide improvement efforts to those parts of the beer value chain where they are likely to have the greatest impact, while also identifying potential trade-offs or unwanted consequences.

The beer production chain – which starts with the agricultural crops and ends with the final product – has been modernized before the others, driven by the first-class production and quality standards of Italian brewing. Ninety percent of the total malting barley cultivated in Italy today is covered by sustainable agriculture certificates: for this 90% of the production, we know what the carbon footprint, the carbon food print, the ecological footprint, and the water footprint. We know exactly what the consumption and emissions are on almost all crops. After the UN 2030 Agenda for Sustainable Development, between 2015 and 2018 the EU defined the so-called Circular Economy Package, as four directives that profoundly reviewed the EU legal regime of waste (European Parliament and Council, 2018 (UE 2018/849, 2018/850, 2018/851, 2018/852 https://www.europarl.europa.eu/RegData/etudes/BRIE/2018/625108/EPRS_BRI(2018)625108_EN.pdf)), whereby any waste must be handled in a manner that does not have a negative impact on the environment or human health. A new approach to waste management was born that corresponds to a new hierarchy: waste prevention, preparation for reuse (people needs, animal feed), recycling of materials (extraction of valuable compounds), other types of recovery (anaerobic digestion, composting, thermal valorization or spread for land fertilization or improvement), and disposal (Cimini and Moresi 2021). The long-term strategy is to involve companies in making products with new materials that are entirely reusable and therefore do not generate waste, while the short-term and medium-term strategies are to manage the waste produced in a more responsible way, through reuse and recycling.

Through the study of the Life Cycle Assessment (LCA), an analytical and systematic methodology, it is possible to evaluate the environmental footprint of a product or service along its entire life cycle

(Morgan et al., 2021). Measuring the environmental impact generated by the various production processes included in it serves to identify those with the greatest impact and to be able to carry out subsequent management through their reduction and compensation. Traditionally, the classical model of the industrial economy has always been linear, characterized using raw materials extracted or obtained to produce consumer goods. This classic model did not focus on what is one of the biggest problems facing our planet in the 21st century: waste (Figure 2).

The increasingly massive presence of waste has led to a revision of the linear model, which is in fact unsustainable from an environmental point of view (Androniceanu et al., 2021). The excess of waste and its impact on public health have made global intervention on economic change towards a new approach in which raw materials are reused countless times. The fundamental principles of circular models are the creation of value through the reduction of external inputs and the enhancement of by-products, looking beyond the simple service to the final consumer and considering the ecological and social impacts. The circular economy therefore aims to ensure that the materials that make up the products can be reused as much as possible, ideally an infinite number of times.

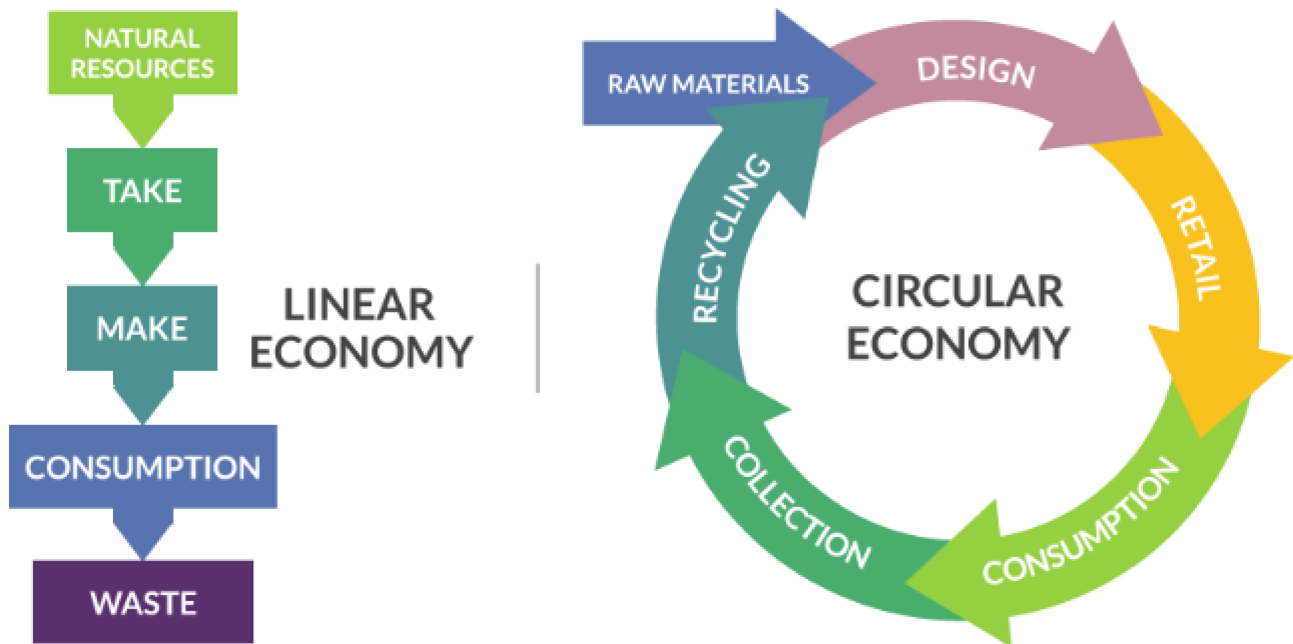

Fig.2: Linear economy *versus* circular economy (Source https://endofwaste.com).

In this regard, craft breweries are characterized by a high propensity for innovation and change, and constitute an interesting sector in which to apply this change of mentality/objectives of the circular economy.

The carbon footprint generated by the beer production process consists of three phases: **Upstream** processes: these are all of the processes upstream of the actual production process, concerning the production of the main raw materials used (packaging production, cultivation and processing of barley, spelt and hops, other raw materials, etc.); **Core** processes: these include all of the brewing processes, from the entry of raw materials into the plant to the exit of the finished and bottled beer, including secondary packaging (energy and water consumption, production waste, refrigerant gas emissions from refrigeration systems, transport to the disposal and disposal centers of the waste generated, transport of all raw materials); and **Downstream** processes: these concern the finished product that leaves the production plant (distribution to the customer, recycling, incineration, and disposal of primary and secondary packaging) (Holland 2021).

The largest proportion of the carbon footprint is the packaging materials, the transport of raw materials from the place of cultivation to that of processing, and finally the disposal of the by-products, such as brewers' spent grain (BSG), exhausted yeast, and wastewater. A circular economy and sustainability of the process would guarantee added value to the finished products, with a greater profit margin for breweries (Holland 2021). With reference to the beer supply chain, the concept of a circular economy can be applied to the reuse of abiotic materials, such as material packaging, mainly made of glass bottles, and the reuse of biotic materials, i.e., the main by-products of the brewing process (Cimini and Moresi 2021). One of the main challenges of the brewing sector is the possible reuse and exploitation of the by-products.

1.5.1 Enhancement of beer by-products

To achieve a zero-waste goal, it is necessary to understand which phases of the beer production process can be optimized and which innovations can be adopted in terms of sustainability, to obtain economic benefits (Holland, 2021). One of the critical issues identified is the management of production waste, which represents approximately 90% of the raw materials used.

It follows that one of the main challenges of the beer sector is the enhancement of the by-products, including BSG, which represent the most abundant by-product, with an average yield of about 16 kg per hectoliter of beer produced (Assobirra Annual Report 2020). BSG average annual worldwide production is around 39 million tons, of which 3.4 million are produced in Europe. Only in Italy in 2020, the BSG produced was 219,965 tons – (20% dry) (Assobirra Annual Report 2020) of which only 30% is reused, mainly in the livestock sector. This wide availability makes BSG a very economical by-product, with an average EU market price of €35/ton (Baffington, 2014). The possibility of recovering and enhancing BSG directly within the company would lead to significant savings on production costs.

1.5.2. Brewers' spent grain

The composition of BSG varies and depends on various factors, such as: variety of barley, malting process, and type and quality and formulation (malted or not) of any other cereals added to the brewing process (Santos et al., 2003). The grains are made of lignocellulosic polymers, of which the major components are the fiber (cellulose and hemicellulose, as 30%-50% dry weight), protein (19%-30% dry weight), and lignin (12%-28% dry weight) (Mussatto & Teixeira, 2010; Xiros and Christakopulous, 2012; Mussatto, 2014). Together with cellulose, both β-D-glucan (a linear polysaccharide made up of glucose molecules) and starch can be present, while the most abundant monosaccharides are xylose, glucose, and arabinose, and to a lesser extent ramnose and galactose (Mandalari et al., 2005; Forssell et al., 2008). Hemicellulose is mainly composed of arabinoxylan, the main non-cellulose polysaccharide of cereals (Mandalari et al., 2005). Lignin is a polymer of cinnamic alcohol derivatives, which consists of phenolic compounds and gives rigidity and resistance to the cell wall. The most abundant proteins in BSG are ordeines, glotulins, globulins, and albumins. Thirty percent of the total protein content is made up of different amino acids, of which the most abundant are histidine (26%), glutamic acid (16%), lysine (14%), leucine (6%), phenylalanine (4%), and isoleucine (3%) (Waters et al., 2012). Lysine has a significant role, as cereal-based foods usually have a low content (Blandino et al., 2003). Among the minerals, the most abundant are silicon, phosphorus, calcium, and magnesium (Mussatto & Roberto, 2006; Waters et al., 2012; Meneses et al., 2013). There are also vitamins in BSG, which include: biotin (0.1 ppm), choline (1800 ppm) folic

acid (0.2 ppm) niacin (44 ppm), pantothenic acid (8.5 ppm), riboflavin (1.5 ppm), thiamine (0.7 ppm), and pyridoxine (0.7 ppm) (Huige, 2006; Mussatto et al.,2006).

1.5.3. Preservation

As it comes from the mashing process, BSG has a high moisture content (80%-85% fresh weight), and this makes it easily contaminated by microorganisms, with subsequent deterioration over a short period of time (about 7-10 days; Stojceska et al., 2008). Several methods of preservation have been proposed to prolong the shelf-life of BSG. Direct drying at 60 °C is the most effective method. However, due to the high energy cost, many breweries cannot cover the costs related to this treatment (Ikurior, 1995). Drying has the advantage of reducing the volume of the product. Many industrial breweries have systems for processing BSG and use the drying technique in two phases: squeezing the BSG to reduce the water content to <60%, followed by drying to guarantee a humidity value <10% (Santos et al., 2003). This process has high management costs because it is energy intensive. Bartolome et al. (2002) compared the effects of BSG conservation using three methods: freeze-drying, drying, and freezing. The results obtained showed that storage by drying or freeze-drying reduces the volume of the product and does not alter its composition, while freezing is inappropriate as alterations in the content of some sugars can occur. Freeze-drying is an expensive method and cannot be used on a large scale. As an alternative method, thin layer steam drying has been proposed (Tang et al., 2005). Steam circulation occurs in a closed cycle system; this reduces the waste of energy that occurs with hot air drying. Furthermore, the exhaust steam produced by the water evaporation contained in the BSG can be used in other operations. Thus, the steam current method has several advantages, including reduced environmental impact, improved drying efficiency, elimination of the risk of fire or explosion, and recovery of valuable volatile organic compounds. Another method is the use of a filter press membrane. In this process, BSG is mixed with water and filtered at a pressure of 3 to 5 bar, washed with hot water (65 °C), filtered with a nylon membrane, and dried under vacuum to humidity levels between 20% and 30% (El-Shafey et al., 2004). Recent developments in the agronomic field highlight the possible use of BSG as a fertilizer (Assandri et al., 2021a,2021b; Saba et al., 2019).

1.6. Vermicomposting biotechnology

Recently, the stabilization of organic waste materials using various earthworm species has become a viable and environmentally friendly technique to minimize biowastes. Vermicomposting is an uncomplicated and low-cost technology, carried out with the available space and or in containers or vermireactors, with epigeic earthworms frequently used for the vermiconversion. Vermicomposting is an eco-sustainable process that does not require high technological levels. The process is based on complex interactions between microorganisms, earthworms, and other soil organisms (meso and macrofauna) that fragment, bio-oxidize, and stabilize the organic substance (Dominguez, 2004; Lazcano & Dominguez, 2011). In vermicomposting, earthworms have a crucial role, as they influence the activity of the microorganisms through the fragmentation and ingestion of organic matter (Dominguez et al., 2010). Earthworms (Annelida, Oligochaeta, family Lumbricidae) are the main actors within the vermicomposting process. It is essential to focus on these animals to better highlight the essential parameters and possible process problems that can arise when the environmental, climatic, and organic substrate characteristics are not optimal for the success of the process.

Earthworms are animal organisms with bilateral symmetry that are characterized by a body segmented into several septa, each characterized by an external gland (clitellum) that produces cocoons that collect the earthworm eggs (cocoon), from a sensory lobe located in front of the mouth (prostomium), to an anus in the final posterior part of the earthworm, and with a series of small bristles (chaetae), positioned in pairs on each septum of the body. They are insufficient hermaphrodites and reproduction normally occurs through cross-reproduction between two specimens in which seminal fluid passes from both sides, and therefore there is fertilization of both organisms with the production, for each, of cocoons (ootecas). The ootheca contain from one to 20 fertilized eggs. After an incubation period that varies according to the species and the climatic conditions, the young earthworms emerge from the ootheca, with a much lighter color than the adults, as well as being much smaller and not yet sexually mature. Sexual maturity in most species and under suitable environmental conditions occurs a few weeks after leaving the ootheca, even though it has been shown that a longer period may pass. Sexually mature individuals can be easily distinguished from young ones by the presence of the clitellum, a band that can take on a pale or

dark color, which is located behind the septa that host the genital pores. After fertilization, the clitellum secretes cocoon fibrosis and the clitellar glands produce a nourishing albuminal fluid that covers the cocoon. Earthworms can continue to grow even after completing their sexual development phase, but not to grow in length by generating new septa (Edwards & Bohlen, 1996).
Through trophic activity, the construction of tunnels and the production of cast, earthworms modify the chemical, physical, and biological properties of organic matter and soil (Aira et al., 2008). The physical properties most affected are particle aggregation, stability, and porosity; at the chemical and biological levels, the properties that can be modified are the nutrient cycle (mainly P and N), the decomposition rates of the organic substance, and the chemical forms and bioavailability of nutrients in the soil (Ahmad et al., 2021). They are also able to modify the soil pH, the dynamics of the organic substance in quantity and quality, the microbial activity, and other soil invertebrates (including the production of enzymes and plant growth regulators), and thus the abundance, biomass, species composition, and diversity of soil microflora and fauna (Lu et al., 2021; Lavelle et al., 1998).
Only species that fall into epigeic behavior can be considered for vermiculture and vermicomposting. The characteristics common to all of the species used for the vermicomposting process are:

1. Natural ability to colonize organic waste.
2. High rates of consumption, digestion, and assimilation of the organic substance: this characteristic is essential to shorten the time of the vermicompost production process and to be able to quantitatively increase production.
3. High ability to adapt to adverse environmental factors. One of the most important factors that influence the vital conditions of the earthworm is the temperature, the humidity of the heap of organic matter, and the salinity of the organic waste in which they are found.
4. High reproductive rates, with the production of a high number of cocoons, with reduced hatching times, and high rates of sexual growth and maturation. This results in a speeding up of the vermicomposting process.

Vermicomposting is regarded as an environmentally friendly biotechnology for the biooxidation of agricultural, industrial, rural, and urban generated organic solid wastes, which are serving as reservoirs of environmental pollution (Yuvaraj et al., 2021). There are many epigean species found in temperate climates and present in the European and Mediterranean area, but the most suitable for vermicomposting are: *Eisenia fetida and E. andrei, Dendrobaena rubida and D. veneta*, and *Lumbricus rubellus*. The two most used in vermiculture are *E. fetida* (Savigny, 1836) and *E. andrei* (Bouchè, 1972).

1.6.1. The earthworm *Eisenia fetida*

Among the many species of earthworm, *E. fetida* is an efficient bio-degrader and vermicompost producer (Bansal and Kapoor,2000). It is a species that tolerates wide variations of environmental factors (temperatures from 15-30 °C; pH 5.5-8.0; humidity 50%-70%), and is small, and characterized by a short life cycle and a high reproductive rate. The vermicomposting process is mainly divided into two mesophilic phases: the active phase, where the earthworms process the organic substrate, changing the physical state and microbial composition (Dominguez et al., 2010); and the maturation phase, where the earthworms leave the layers of the organic substance already digested to migrate towards the more superficial layers, made up of organic substance not yet digested and microorganisms associated with the substrate, where the earthworms have key roles (Dominguez & Brandón 2012; Aira et al. 2007). The maturation period varies in duration and essentially depends on the efficiency of the active phase. This transforms the new microbiologically stable product into an evaluable (marketable) organic soil improver with a strong and diversified microbial activity and a high concentration of humic substances, which increases the presence of plant growth promoters (Atiyeh et al, 2002).

More specifically, the process is attributable to the phases known as gut-associated processes (GAPs) and cast-associated processes (CAPs):

- The GAPs are the phase that includes all of the changes that the organic substance undergoes during its transit through the digestive system; i.e., physical harrowing transformation of nutrients, modifications and increases in diversity and microbial activity, changes in microfauna populations,

homogenization, processes of digestion, assimilation, and excretion (Gómez-Brandón & Domínguez, 2014).

Specific microbial groups respond differently to the environment of the digestive system, and there are selective effects on the presence and/or abundance of microorganisms throughout the passage of the organic substance through the digestive system of these various earthworm species (*E. andrei*, *E. fetida, Eudrillus eugeniae* etc ...). Several bacteria are activated during the passage through the intestine, while others remain inactive, and others are digested by earthworms (thus decreasing the bacterial population density). These modifications can alter the decomposition phase of the organic substance, as the microbial biomass is strongly influenced by the casts released by earthworms (Gómez-Brandón et al., 2011). Given that the microbial population of the casts has, in general, a greater population density and a difference in its specific composition, it is more than appropriate to think that the inoculation of these communities into fresh organic substance can promote changes like those that occur when earthworms are actively present in the soil (Parthasarathi et al., 2007).

- The CAPs are the phase in which the effects of earthworms are mostly indirect, and include aeration of the substrate through the tunnel complexes excavated in the organic matter and the microbial activity promoted by the cast community. In this phase, the casts of earthworms are subject to aging and the actions of microorganisms and micro invertebrates present in the substrate, thus mixing these with the materials that have not been ingested by the earthworms. In this way, the vermicompost reaches its optimum in terms of biological properties, thus promoting the growth of plants and suppressing their potential pathogens (Dominguez et al., 2010).

1.6.2. Effects of vermicompost on plant growth

Vermicompost is a fine-sized amending material, like peat, that is microbiologically stable and active, has low C/N ratio, high porosity, and high water-retention capacity, and is rich in highly mineralized organic substance. Vermicompost significantly stimulates the growth of several plant species (Singh et al., 2020), including various vegetable crops, such as tomato (Gutiérrez-Miceli et al., 2007), pepper (Arancon et al., 2005), garlic (Argüello et al, 2006), eggplant (Gajalakshmi and Abbasi, 2004), strawberry (Arancon et al., 2004), sweet corn (Lazcano et al, 2011), and bean

(Karmegam et al., 1999). Furthermore, there have been positive results on some aromatic and medicinal plants (Prabha et al., 2007), on cereals, such as sorghum and rice (Fritz et al., 2012), on tree crops, such as banana and papaya (Cabanas-Echevarria, et al., 2005), and on ornamental plants, such as geranium (Chand et al. 2007), marigold (Atiyeh et al., 2002), petunia (Arancon et al., 2008), chrysanthemum (Hidalgo and Harkess 2002a), and poinsettia (Hidalgo and Harkess, 2002b). Positive effects have also been shown for forest species, such as *Acacia* spp., *Eucalyptus* spp., and *Pinus* spp. (Lazcano et al., 2010a, 2010b).

Vermicompost has beneficial effects when it is used as a total or partial substitute for mineral fertilizers together with the compost used in nurseries and as a soil improver. Furthermore, some studies have shown that the aqueous extracts of vermicompost (i.e., compost tea) used as a foliar spray can promote the growth of plants, such as tomato (Tejada et al. 2008), sorghum (Gutiérrez-Miceli et al., 2008), and strawberry (Singh et al. 2010). The positive effects of vermicompost include: stimulation and germination of the seeds of various plant species, such as beans, tomatoes, petunias and *Pinus* spp., and of vegetative growth, both as regards the aerial and root parts of plants (Arancon & Edwards, 2010). The effects include alterations in the morphology of seeds, and increases in leaf area and root capillization (Lazcano et al., 2009). Vermicompost also stimulates flowering, and increases flower biomass and fruit yield. In addition to increased productivity and growth of plants, vermicompost can also increase the nutritional quality of some crops, such as Chinese cabbage, spinach, lettuce, and sweet corn (Lazcano & Dominguez, 2011).

Some of the indirect effects of vermicompost are related to the indirect changes in the microbiological properties of the soil (Pathma & Sakthivel, 2012). Vermicompost has a different microbial community than the organic matrix from which it is created, with a lower level of activity and biomass, but with increased metabolic diversity. A substrate so active at a microbiological level can have important effects on the microbial properties of the soil to influence plant growth. However, information regarding the impact of vermicompost on soil microbial properties is severely limited.

However, although various studies have reported the beneficial effects of vermicompost, other studies have report that vermicompost can decrease plant growth or even result in plant death. The variability in the effects of vermicompost can depend on the cultivation system within which it is

incorporated, which strongly depends on the basic organic matrix, on the species of earthworms used in the composting, in the production processes, and on the age of the vermicompost (Rodda et al., 2006, Roberts et al., 2007; Warman and AngLopez, 2010). Considering this evidence, vermicompost constitutes a promising alternative to inorganic fertilizers. However, more research is needed to better understand the mechanisms and exact circumstances that exert the positive effects of this soil conditioner.

Various types of crop residues and agro-industrial wastes have been successfully converted into vermicompost. Chauhan and Singh (2013) studied the effects of different types of crop residues (straw and bran) on the growth and reproduction of *E. fetida*. Successful conversion of cattle manure, orange peel, and filter cake into vermicomposts was reported by Pigatin et al. (2016). Bhat et al. (2016) investigated the vermicomposting potential of pressmud, a residue of the sugarcane industry, and cattle dung. Recently, Amouei et al. (2017) reported vermicomposting of household and paper industry waste. Sharma and Garg (2018) used rice straw and paper waste for vermicomposting after mixing with cow dung, using the earthworm *E. fetida*. Mago et al., (2021) reports vermicomposting of banana crop waste biomass by *E. fetida*,

In the agronomic sector, Saba et al. (2019) recently proposed BSG as a raw material to produce vermicompost, highly efficient soil improvers, and fertilizers.

1.7. Microorganisms have crucial roles in the maintenance of life on Earth and agroecosystem sustainable development

Increased energy demands, sustained consumption of fossil fuels, and climate change have favored the use of renewable energy and sustainable production models. For several years now, initiatives have had to be taken to remedy situations that are becoming critical, such as global warming and the accumulation of waste. Achieving the 2030 targets implies a series of radical transformations in the energy, transport, and agricultural sectors. In this context, microorganisms can contribute significantly to mitigate climate change, improve green production technologies, improve crop productivity, and provide a means of income (Figure 3 and Table 1).

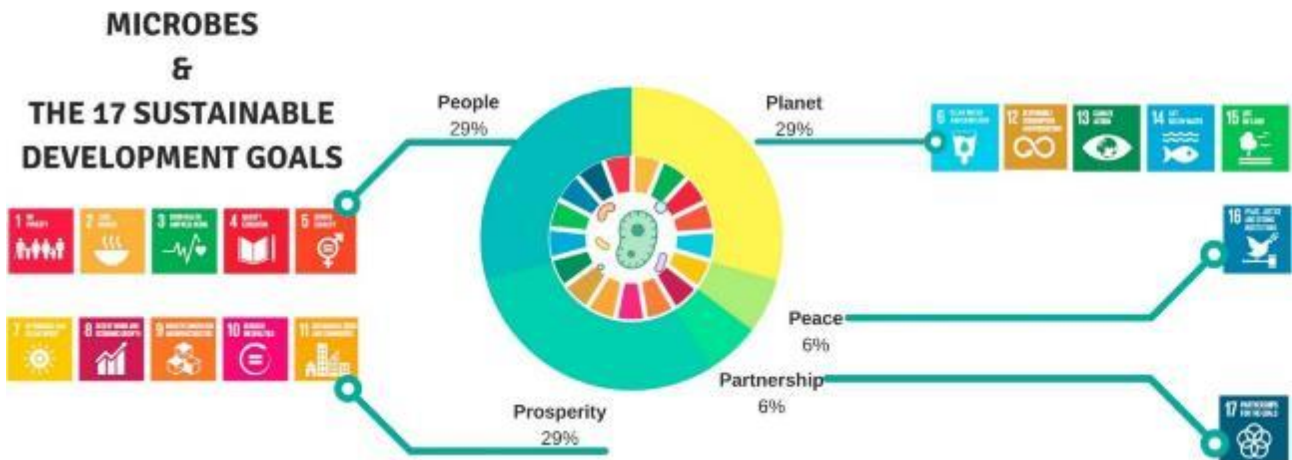

Fig.3: Microbes and the sustainable development goals (Akinsemolu, 2018).

Table 1 Summary of the role of microorganisms in achieving sustainable development goals (SDG) 1-17 (Akinsemolu 2018).

SDG	Goal	Application of Microorganisms	References
1	No Poverty	**1. Income Generation** **Production of Gobar Gas** by methane-producing organisms such as *Methylosinus, Methylocystis, Methanomonas, Methylomonas, Methanobacter, and Methylococcus*), traditional food in Nigeria like Ogi, Garri, Pito, and Iru, and **Vinegar** (Lactic Acid Bacteria and Yeast).	(Adesulu and Awojobi, 2014; Bhutto et al., 2011; Encyclopedia.com, 2017; Rainieri and Zambonelli, 2009)
2	Zero Hunger	**2. Fermentation** **Pickles** (*Leuconostoc mesenteroides, Lactobacillus plantarum, Pediococcus cerevisiae*), **Milk** (*Lactobacillus*), **Tempe** (*Acetobacter and Rhizopus oligosporus*), **Cheese** (*Penicillium roqueforti, Streptococcus salivarius subsp thermophilus*), **Soy Sauce** (*Aspergillus oryzae, Aspergillus soyae, Aspergillus glaucus*), **Yogurt** (*Lactobacillus delbruekii subsp bulgaricus, Streptococcus salivarius subsp thermophilus*) and **Bread** (*Saccharomyces cerevisiae*) **3. Soil Fertility** Arbuscular mycorrhizal symbiotic fungi and phosphate-solubilizing microorganisms such as *Pantoea agglomerans, Microbacterium laevaniformans and Pseudomonas putida*	(Beuchat, 2008) (Averill et al., 2014; Khan et al., 2009)
3	*Good Health and Well-being*	**4. Prebiotics and Probiotics** (*Lactobacillus acidophilus, Aspergillus niger, Saccharomyces carisbergensis*) **5. Development of New Drugs** Penicillin (*Penicillium notatum*) Chloramphenicol (*Streptomyces venezuelae*) Griseofulvin (*Penicillin griseofulvin*) **6. Immunoregulation in Humans** (*Bifidobacteria and Lactobacilli*) **7. Gastrointestinal Flora Prevention of diseases like gastroenteritis** (*Prevotella, Bacteroides, Faecalibacterium prausnitzii and Ruminococcus*) **8. Controlling Pollution related diseases** *Burkholderia, arthrobacter, Chromobacterium, Micrococcus, Candida, Pseudomonas, and Bacillus*	(De Vrese and Schrezenmeir, 2008; Manning and Gibson, 2004; Mizock, 2015; Wang, 2009; Williams, 2010) (Balbi, 2004; Bergmann and Sicher, 1952; Finkelstein et al., 1996; Fleming, 1944; Lerner, 2004; Rosefort et al., 2004) (Romano-Keeler and Weitkamp, 2015) (Kaplan et al., 2011) (Das et al., 2011; Zabochnicka-Światek and Krzywonos, 2014).
4	*Quality Education*	**9. Teaching, Research and Innovation** • Built Environment, Molecular Biology, Education on Infant Immunity (breastfeeding and hygiene) • **Enzymes:** *Humicola lanuginose* **(thermostable lipase)**, *Aspergillus niger* **(lipase, protease, cellulase, and xylanase)**, *Aspergillus flavus* **(cell-bound lipase)**, *Mucormiehei* **(lipase)**, *Bacillus megaterium* **(organic solvent tolerant lipase)**, *Cellulomicrobium* **(alkaline protease and keratinase)**, *Geobacillus thermodenitrificans* **(thermostable lipase)** **10. Renewable energy sources for reading in rural communities** Microbial Fuel Cell, Biogas	(Cortese, 2003; O'Sullivan et al., 2015; Simonneaux, 2000). (Gautam et al., 2009; Logan et al., 2006; Mshandete and Parawira, 2009; Nagamani and Ramasamy, 1999; Santoro et al., 2017)
5	*Gender Equality*	**11. Sexual and Reproductive health** • About 290 million women have a human papillomavirus (HPV) infection • STIs have grave reproductive health consequences such as infertility or mother-to-child transmission • In 2012, more than 900 000 pregnant women were infected with syphilis resulting in roughly 350 000 adverse birth outcomes including stillbirth **12. Income generation** Through production and preservation of foods and pharmaceutical products, as well as the production of Gobar Gas and biofuel. **13. Empowerment (STEM)** A high percentage of women graduating from these fields end up employed in other areas **14. Well-being of the family** Promoting education among women will improve the well-being of their children, families, and society	(de Sanjosé et al., 2007; Newman et al., 2013; World Health Organization, 2016b) (Palanithurai, 2007; Rao, 2005) (Xie and Killewald, 2012) (Roudi-Fahimi and Moghadam, 2003)
6	*Clean Water and Sanitation*	**15. Controlling Poor-Sanitation Related Diseases** Microbes are essential in the production of Rotavirus vaccine, zinc and vitamin A and in improving water sources. **16. Regulation of microbial load** *Escherichia coli, Salmonella, Vibrio*, and *Shigella* can significantly contribute in controlling water-borne diseases	(Chola et al., 2015) (Cabral, 2010)
7	*Affordable and Clean Energy*	**17. Electricity generation (Bioenergy and Biofuel)** • *Botryococcus* can store up to 50% of the biomass in the form of long-chain hydrocarbons.	(Hannon et al., 2010) (Lal, 2013) (Appels et al., 2011; Elshahed, 2010)

Table 1 (*continued*)

SDG	Goal	Application of Microorganisms	References
		• Utilizing of organic matter by *Shewanella oneidensis* and *Geobacter sulfurreducens* to produce utilizable electricity. • Utilizing waste products such as sewage sludge, municipal solid waste and agricultural waste by *Trichoderma, Aspergillus, Penicillium*, and *Clostridium* to produce bioenergy.	
8	*Decent Work and Economic Growth*	**18. Research and development** • Dinoflagellate *Karlodinium veneficum* is under investigation for its potential uses in human health. • Organisms like *Penicillium* produces the first discovered antibiotic: Penicillin and bacteria like *Streptomyces sp.* produce the aminoglycoside antibiotics. • In Denmark, biogas production policy contributes to the nation's bioenergy concept and reduction of carbon emissions. • Biogas stove providing jobs in Sub-Sahara Africa	(Waters et al., 2010) (de Lima Procópio et al., 2012; Fleming, 1944; Hersbach, 1983; Lerner, 2004) (Mæng et al., 1999) (Tumwesige et al., 2014)
9	*Industry, Innovation and Infrastructure*	**19. Biofertilizers** *Pseudomonas and Rhizobia* **20. Microbial products** **Vitamin C** (*Corynebacterium sp.*), **Coenzyme** (*Brevibacterium ammoniagenes*), **Riboflavin fermentation** (*Eremothecium ashbyii, Ashbya gossypii, Bacillus sp.*), **Vitamin B12** (*Propionibacterium shermanii, Pseudomonas denitrificans*), **S-Adenosylhomocysteine** (*Alcaligenes faecalis*), **Biotin** (*Serratia marcescens, Bacillus sphaericus*)	(Mohammadi and Sohrabi, 2012; Sahoo et al., 2013; Vessey, 2003) (Shimizu, 2008)
10	*Reduced Inequality*	**21. Agricultural and land management practices** Women can be taught the importance of soil microflora like *Burkholderia, Pseudomonas, Rhizobium, Trichoderma, Bradyrhizobium*, and *Azospirillium* in improving crop productivities	(Pereg and McMillan, 2015)
11	*Sustainable Cities and Communities*	**22. Ecology of urban cities and Solid waste management** Composting of the solid waste uses microorganisms like *Pseudomonas, Bacillus, Microbispora, Actinobifida*, and *Thermoactinomycetes* for converting its organic constituents into useful end products.	(Finstein and Morris, 1975; King, 2014)
12	*Responsible Consumption and Production*	**23. Responsible production through bacterial cellulose** Bacterial Cellulose (BC) from *Komagataeibacter* can be used in the production of fuel and Li-ion batteries **24. Microbial and Environmental education** Personal hygiene, cleanliness, restricting self-medications, separating organic and inorganic household waste, and minimizing the use of fossil fuels. **25. Bioremediation** Bioremediation is an fascinating and successful cleaning technique for polluted environment.	(Jang et al., 2017) (Barberán et al., 2016; Chawla and Cushing, 2007; Dewan et al., 2010; Tilbury, 1995) (Atlas and Philip, 2005; Ayotamuno et al., 2006; Karigar and Rao, 2011; Rajendran et al., 2003; Rosenberg and Ron, 1996)
13	Climate Action	*26. Biogeochemical cycles* *They are key drivers in biogeochemical cycles like nitrogen, carbon and phosphorus cycles.* **27. Nitrogen fertilizers** Microbial community structure is important when assessing the impact of environmental perturbation (e.g. nitrogen fertilizers) on biodiversity loss and ecosystem functioning. **28. Biofuel production** Algae like *Saccharomyces* or modified *Escherichia coli* can be used for third-generation biofuel production. It is practical, economically-feasible, and achievable solution for the development of sustainable environment (Singh et al., 2010).	(Auguères and Loreau, 2015; Falkowski et al., 2008; Grandy et al., 2016) (Mohanty et al., 2006) (Singh et al., 2010)
14	*Life below Water (Marine Ecosystems)*	**29. Aquaculture** Microbes can effectively boost the production of fisheries by controlling the pathogenic outburst and water quality, as well as regulating the environmental impact	(Martínez-Córdova et al., 2016)
15	*Life on Land (Ecosystems)*	**30. Ubiquitous functions** Microbes stabilize the soil structure, enable nutrient uptake by plants, control pests and diseases, decompose organic material and degrade harmful chemicals, as well as being an indicator of the soil health.	(Wachira et al., 2014)
16	*Peace, Justice and Strong Institutions*	**31. Combating bioterrorism** Microbial forensics can be used to trace perpetrators and sources of biohazard, thereby being as a powerful tool for combating bioterrorism	(Budowle et al., 2005; Sijen, 2015)
17	*Global Partnerships for Sustainable Development*	**32. Capacity building on trade-related aspects** The benefits of microbes and microbial technology can only reach the masses through globalization and overcoming land barriers. This will	(Chambers et al., 2004)

Soils are ecosystems with the most diverse composition of microbiota on Earth, because of the presence of so many different niches. Nowadays, agricultural production faces many challenges, such as climate change, demographic development, and the consequent increase in food demand, and there is a growing demand for sustainable production and the need for continuous innovation. Soil is the production base of our food, and it represents the biobank of microbial diversity from which a plant selectively takes its microbiome to meet its needs. It is a precious natural resource that hosts microbial hotspots, with a fundamental role in the maintenance of the global balance of nutrients and function of ecosystems. In the soil, there are numerous and different microorganisms that perform essential functions in the cycles of the elements (C, N, S, P, others) and that contribute substantially to the well-being of the ecosystem, in terms of both soil health and plant development. Rhizobacteria, symbionts, and endophytes participate in the life of plants, to promote their growth through multiple mechanisms, such as nitrogen fixation, phosphorus solubilization, and production of indolacetic acid, siderophores, and antibiotics. The different groups of microorganisms therefore represent the key component of the soil–plant system, where an intense network of interactions is involved in the rhizosphere, in the internal tissues of the plant, and in the phyllosphere (Figure 4) (Barberán et al., 2012; Hassani et al., 2018).

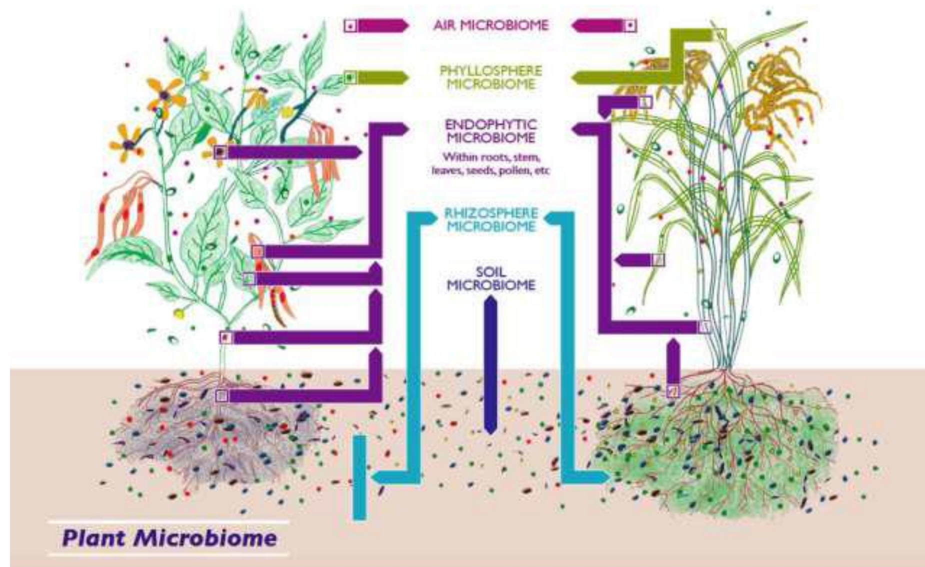

Fig. 4. The ‘plant microbiome’ can be described as the sum of the genomic contributions made by the diverse microbial communities that inhabit the surface and internal tissues of the plant parts. The rhizosphere, endosphere, and phyllosphere constitute the major compartments in which the microbial communities reside in the plant. The soil

microbiome is the main source from which the plant selects and builds its microbiome profile. The plant genotype (e.g., as shown: dicot bean plant, monocot rice plant), its root exudates (indicated by blue shading for bean and green for rice), the soil types and properties, and the environmental factors influence the make-up of the plant microbiome (indicated by different colored microbes inhabiting the plant compartments in bean and rice plants). Mycorrhizal association in both plants is indicated by thin lines extending from the roots into the surrounding soil (image taken from Gopal and Gupta, 2016).

The soil and plant microbiomes have important roles in plant growth and development, and in soil health: they provide the plant with a secondary genome that offers key ecological functions and favors the host; they can influence plant health and productivity by improving tolerance to stress, and thus providing an adaptive advantage; they mediate different functional traits of plants; they influence the phenotypic plasticity of plants; and they are essential to ensure the quality and safety of primary plant production, including fruit and related processed foods (Compant et al., 2019; Timmusk et al., 2017; Panke-Buisse et al., 2015). The use of the functional potential of soil and plant microbiomes can lead to a reduction in chemical inputs, to increase the quality and safety of crops and food products with the help of 'beneficial' microorganisms that can improve the yield and quality of crops. This can also counteract the impoverishment of soils, reclaim contaminated land and reduce the use of water, fertilizers and pesticides, as the strategy to be followed for sustainable development.

The use of synthetic microbial consortia of beneficial microorganisms for plants that have different functions has become an ecologically favorable alternative to integrate inorganic inputs and favor the development of plants. These functions can include nitrogen fixing bacteria, microorganisms that solubilize phosphates, plant growth promoting rhizobacteria, and arbuscular mycorrhizae, that can act synergistically.

There are therefore several paths that can lead to a new generation of inoculants and the application of microbiomes in agriculture that could give rise to a new green revolution, much more sustainable than the previous one (Qiu et al., 2019). We can imagine a future that is based on the precise determination of the soil and plant microbiomes, whereby it is possible to outline a rational intervention with microbial inoculants in the field, optimize yields, and at the same time, reduce expensive and unsustainable inputs of chemicals for agriculture (Schlaeppi & Bulgarelli, 2015).

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Chapter 1

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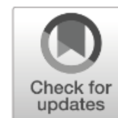
MINI-REVIEW



The role of microorganisms on biotransformation of brewers' spent grain

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Abstract

Brewers' spent grain (BSG) is the most abundant by-product of brewing. Due to its microbiological instability and high perishability, fresh BSG is currently disposed of as low-cost cattle feed. However, BSG is an appealing source of nutrients to obtain products with high added value through microbial-based transformation. As such, BSG could become a potential source of income for the brewery itself. While recent studies have covered the relevance of BSG chemical composition in detail, this review aims to underline the importance of microorganisms from the stabilization/contamination of fresh BSG to its biotechnological exploitation. Indeed, the evaluation of BSG-associated microorganisms, which include yeast, fungi, and bacteria, can allow their safe use and the best methods for their exploitation. This bibliographical examination is particularly focused on the role of microorganisms in BSG exploitation to (1) produce enzymes and metabolites of industrial interest, (2) supplement human and animal diets, and (3) improve soil fertility. Emerging safety issues in the use of BSG as a food and feed additive is also considered, particularly considering the presence of mycotoxins.

Key points

• *Microorganisms are used to enhance brewers' spent grain nutritional value.*

• *Knowledge of brewers' spent grain microbiota allows the reduction of health risks.*

Keywords Microbial communities · Microbial quality · Mycotoxins · Single-cell protein · Vermicompost · Brewers' spent grain

Introduction

The recycling and exploitation of brewing residues, such as brewery wastewater, surplus yeast, and brewers' spent grain (BSG), are critical goals for reduction of energy consumption and residue disposal costs, and also for reduction of the associated carbon foot-print (Zupančič et al. 2017). BSG is the most abundant by-product from the brewing process (i.e., 85% of total by-products) (Fig. 1). Some 100 to 130 kg of fresh BSG (humidity, 70–80%) are obtained from 100 kg of malt, which also equates to 21 to 22 kg BSG per hectoliter of beer brewed (Kunze 2004). The global production of BSG has reached 39 million tons per year on average (Birsan et al. 2019). Of this, 3.4 million tons are produced in Europe, which ranges from 2 million tons/year in Germany (Steiner et al. 2015) to 288,000 tons/year in Italy (Assobirra Annual Report 2018). Around 70% of the BSG produced is used as animal feed, with 10% used to produce biogas and the remaining 20% disposed of as landfill. Its relatively low cost makes BSG an interesting raw material that has the potential to be used for the production of goods with high added value.

Brewers' spent grain comprises the glumes, pericarp, and teguments of the outer layers of the barley kernels and of the other cereals, which contain nutrients that are not extracted during the malting and mashing processes (Fig. 2) (dos Santos Mathias et al. 2014). The chemical composition of BSG (Table 1) varies depending on barley cultivar, malting process, and quality and formulation of the brewing cereals (Gupta et al. 2010; Santos et al. 2003). Notwithstanding, BSG composition always includes high levels of dietary fiber, protein, and essential amino acids, as well as appreciable levels of minerals, polyphenols, vitamins, and lipids (Fărcaş et al. 2014). The high polysaccharide, protein, and moisture content of BSG makes it susceptible to microbial deterioration over a short period of time (i.e., from 2 to 7 days) (Wang et al. 2014; Gupta et al. 2013).

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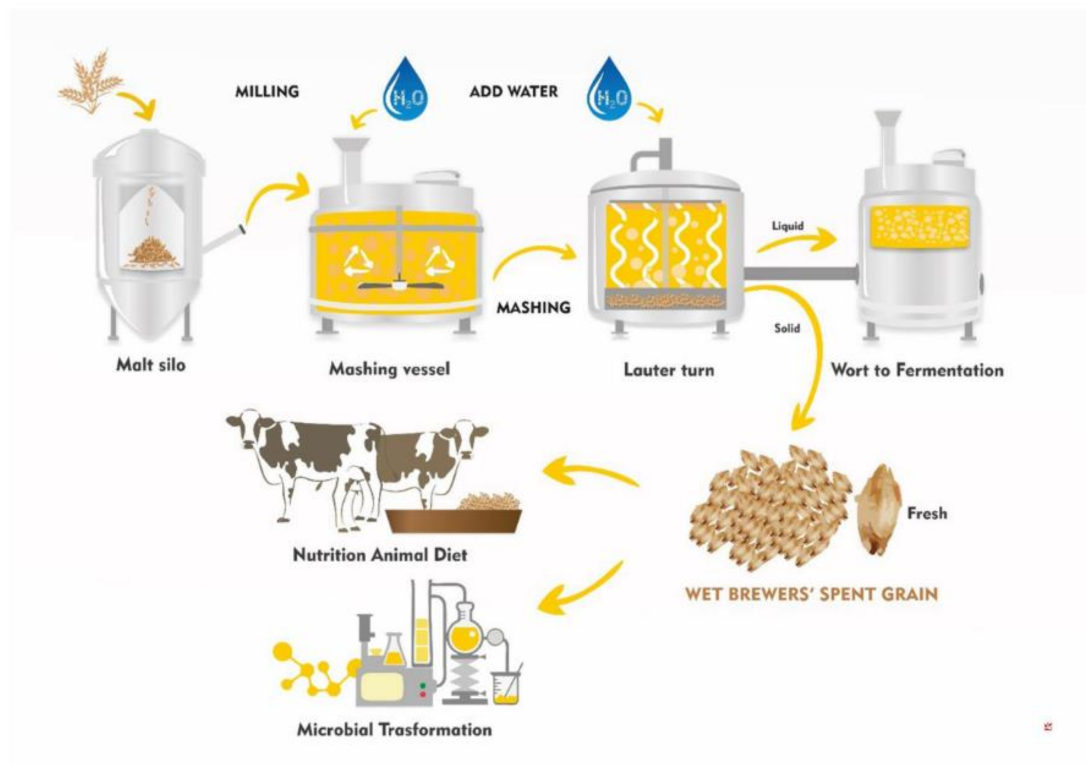

Fig. 1 Scheme of the brewing process and consecutive production of brewers' spent grain

Currently, the main solution for the exploitation of BSG continues to be its supply to local farmers for livestock feed. However, BSG production often exceeds the demand for local feed, which results in disposal issues in terms of the sustainability and environmental impact of breweries (Mussatto 2014). Research into new conservation systems and new uses and enhancement technologies, together with better understanding of the use of microorganisms as biocatalysts, are important in the search for new ways to exploit BSG in foods and animal feed, and in the pharmaceutical and cosmetic sectors. Consequently, patents have been filed recently in connection with BSG (Table 2), while others were originally filed from 1965.

In recent years, different aspects related to the chemical composition of BSG have been reviewed, from its use as a promising integrated feed source of prebiotic compounds for livestock (Lao et al. 2020) and a concentrate feed for lactating dairy cows (Chanie and Fievez 2017), to the best extraction methods to preserve its bio-active compounds (Bonifácio-Lopes et al. 2019; Guido and Moreira 2017). In particular, the antioxidant, anti-atherogenic, anti-inflammatory, and anti-carcinogenic activities of chemical compounds in BSG have been considered (Ikram et al. 2017; Lynch et al. 2016; McCarthy et al. 2013). Finally, methods for exploitation of BSG in terms of food and energy production, and in the chemical and agronomic sectors have been reviewed (Cancelliere et al. 2019; Mussatto 2014; Xiros and Christakopoulos 2012; Aliyu and Bala 2011; Mussatto et al. 2006). In this context, the aim of this mini-review is to present the current literature on BSG from a microbiological point of view. Indeed, the knowledge of different BSG-related microbiological aspects is still limited, particularly when compared with the above-cited literature regarding the exploitation of its chemical components. However, BSG can support the growth of spoilage microorganisms as well as beneficial microorganisms, and these diverse microorganisms and their interactions can determine the safety aspects and full exploitation of BSG.

Brewers' spent grain storage and stabilization

Immediately after lautering, BSG has limited microbial contamination (10^2–10^3 CFU g^{-1}; Robertson et al. 2010a) and it can be considered as microbiologically stable and within

Table 1 Chemical composition of brewers'spent grain

Group	Units	Compound	Content
Major components	g kg^{-1} dry weight	Cellulose (glucan)	3–330
		Hemicellulose	192–419
		Xylan	136–206
		Arabinan	56–419
		Starch	10–120
		Lignin	115–278
		Lipids	30–106
		Proteins	142–310
		Ashes	11–46
		Extractives	58–107
		Phenolics	7–20
		Ferulic acid	1860–1948
		p-Coumaric acid	565–794
Minerals	mg kg^{-1} dry weight	Silicon	1400–10,740
		Phosphorus	4600–6000
		Calcium	2200–3515
		Magnesium	1900–2400
		Sulfur	1980–2900
		Potassium	258.1–700.0
		Sodium	100.0–309.3
		Iron	100.0–193.4
		Zinc	82.1–178.0
		Aluminum	36.0–81.2
		Manganese	40.9–51.4
		Cobalt	17.8
		Copper	11.4–18.0
		Strontium	10.4–12.7
		Iodine	11
		Barium	8.6–13.6
		Chromium	<0.5–5.9
		Molybdenum	1.4
		Boron	3.2
Non-essential amino acids	% total protein	Histidine	26.27
		Glutamic acid	16.59
		Aspartic acid	4.81
		Valine	4.61
		Arginine	4.51
		Alanine	4.12
		Serine	3.77
		Tyrosine	2.57
		Glycine	1.74
		Asparagine	1.47
		Glutamine	0.07
Essential amino acids	% total protein	Lysine	14.31
		Leucine	6.12
		Phenylalanine	4.64
		Isoleucine	3.31
		Threonine	0.71
		Tryptophan	0.14

Table 1 (continued)

Group	Units	Compound	Content
Vitamins	ppm	Biotin	0.1
		Choline	1800
		Folic acid	0.2
		Niacin	44
		Phantotenic acid	8.5
		Riboflavin	1.5
		Thiamine	0.7
		Pyridoxine	0.7

acceptable limits for food use. However, BSG takes several hours to cool down to room temperature (20 °C), and during this period microbial activity continues. The extensive changes in its structure that occur during malting and mashing make it accessible to hydrolytic enzymes, thus making BSG an easier substrate for microbial attack. Consequently, after only 5 days of storage at 20 °C, the microbial concentrations can increase to 10^6 CFU g^{-1}, with microaerophilic, strictly anaerobic, and aerobic, mesophilic, and thermophilic bacteria representing the predominant naturally associated microflora (Robertson et al. 2010a, 2010b). In addition, filamentous fungi are frequently isolated after storage of BSG at room temperature, such as *Aspergillus* spp., *Fusarium* spp., *Mucor* spp., *Penicillium* spp., and *Rhizopus* spp. (Robertson et al. 2010a). Therefore, BSG must be stabilized and stored under the appropriate conditions if it is to be used at a later stage.

Many breweries are discouraged from drying BSG at 60 °C due to the high associated energy costs (Aliyu and Bala 2011; Tang et al. 2009). Robertson et al. (2010b) compared different methods of BSG storage with respect to microbial proliferation, from the fresh material at 20 °C to that refrigerated at 4 °C, autoclaved at 120 °C for 1 h, and frozen. They reported that under storage at 4 °C over 16 days, the numbers of aerobic bacteria in BSG remained < 10^6 CFU g^{-1}, while in the frozen and autoclaved samples, there was no evidence of microbial activity. Indeed, when it was stored at 4 and 20 °C, BSG showed sugars loss that was ascribable to the activities of microbial enzymes, such as xylanases, esterases, and cellulases (Robertson et al. 2010b). Autoclaving was seen as effective for long-term BSG stability (Lynch et al. 2016).

Brewers' spent grain in industrial biotechnology

Brewers' spent grain is a valuable substrate for microbial growth, and it fulfills some of the requirements demanded for biotechnological exploitation, including regular availability and low market price. Bacteria, fungi, and yeast have been successfully used for the biotechnological exploitation of BSG to produce enzymes and metabolites, microbial biomass, pharmaceuticals, and substrates for biocontrol agents (Fig. 3). BSG has also been used as a substitute to expensive carbon sources for industrial production of lactic acid by *Lactobacillus delbrueckii* UFV H2B20 (Mussatto 2014; Mussatto et al. 2007, 2008), *Lactobacillus pentosus* CECT-

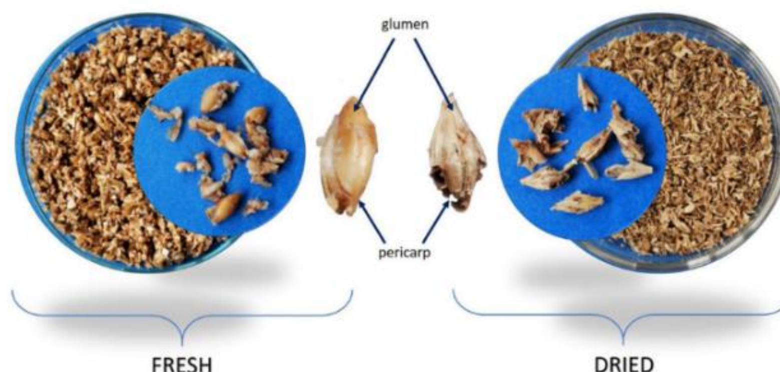

Fig. 2 Brewers' spent grain fresh from production and after drying

Table 2 Patents held for brewers' spent grain

Title of patent	Number	Source	Brief description	References, Inventor, Year
Process for producing a filler from brewer's spent grain (BSG), filler, use of a filler and foodstuff	US Patent Application: US-2020-0138065-A1	http://www.freepatentsonline.com/y2020/0138065.html	A process for producing a filler from BSG. The process comprises (1) comminuting the BSG; (2) heating the BSG; (3) optionally, fermenting the BSG; (4) optionally, reducing the moisture content of the BSG; and (5) optionally, mixing the BSG with at least one flavor-modifying food additive, such as a sugar substitute(s) and/or an aroma. Also disclosed are a filler obtained or obtainable by this process, the use of such a filler, and a foodstuff comprising at least one such filler	Petry and Olwal (2020)
Intermittent infrared drying for BSG	US Patent Application: US-105-78,358-B2	http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PALL&s1=10578358.PN	A system for processing BSG that includes a specific intermittent infrared heating and stirring protocol designed to produce a unique dried BSG product that can be used whole or ground up and used as a quality flour suitable for human consumption	McHugh et al. (2020)
Method for reducing moisture in BSG	US Patent Application: US-2019-0254315-A1	http://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PG01&s1=20190254315.PGNR	A method for reducing the moisture content of BSG comprising the exposure of different BSG to infrared radiation	Willis (2019)
Process for a prepared beverage or beverage component from BSG	US Patent Application: US-2019-0200640-A1	http://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PG01&s1=20190200640.PGNR	A process for preparing a beverage or a component of a beverage. Enzymatic treatment of BSG with addition of one or more enzymes, with α-amylase, gluco-amylase, cellulase, xylanase, protease, and/or β-glucanase activities, and subsequent fermentation with a strain of lactic acid bacteria	Gil-Martinez and Arendt (2019a)
Spent grain fuel product and process	US Patent Application: US-10-364-400-B2	http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PALL&s1=10364400.PN	A process of making a fuel product from BSG. After production, the dried BSG is fed into a combustion chamber of a steam boiler that is used for beer brewing, and the BSG is separated during combustion by agitation, such as spraying of the BSG in the combustion chamber	Larson et al. (2019)
A process for microbiological stabilization of BSG, microbiologically stabilized BSG, and the use thereof	Patent Cooperation Treaty: WIPO (PCT); WO-2019-034567-A1	http://patentscope.wipo.int/search/en/WO2019034567	A process to microbiologically stabilize the spent fresh beer beans (BSG). The process includes acidification of the BSG to a pH 4 or lower, on the basis that the BSG is acidified before reaching mycotoxin levels above the limits of reads and/or counts of colonies, not exceeding 10^3 CFU g^{-1} aerobic bacteria, fungi, yeast, mesophilic aerobic	Gil-Martinez and Arendt (2019b)

Table 2 (continued)

Title of patent	Number	Source	Brief description	References, Inventor, Year
			bacteria, or total anaerobic bacteria, after a week of storage at 25 °C	
Composition of BSG and polylactic acid	US Patent Application: US-10-285,422-B2	http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PALL&s1=10285422.PN.	A composition consisting of BSG and polylactic acid (PLA) is produced by the following steps: processing BSG; mixing BSG with PLA at a specific proportion; BSG and PLA are well mixed by a binder and then subjected to a granulation process to form plastic granules. Adding BSG to PLA can reduce the use of PLA. Furthermore, the composition of BSG and PLA and the method for making this are further used to make utensils, bottles, cans, containers, parts, and other biological plastic products, to provide added value to BSG	Chen et al. (2019a, b)
Composition consisting of BSG and PLA and a method for making the same	US Patent Application: US-10-201,177-B2	http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PALL&s1=10201177.PN	This method for manufacturing a composite consisting of BSG and PLA includes the steps of: providing the raw material containing BSG; providing the raw material containing PLA; mixing the BSG with the PLA at a specific proportion; and providing a binder to enable the BSG and the PLA to be well mixed, and to maintain the desired tensile strength. A pretreatment unit includes dehydration, desiccation, drying, grinding, and sieving. The granulator includes a double screw extruder connected to a cutting machine. The double screw extruder mixes the BSG and the PLA and extrudes them into plastic bars that are then cut into plastic granules by the cutting machine	Chen et al. (2019a, b)
Systems and methods for making BSG dough products	US Patent Application: US-2019-0223457-A1	http://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PG01&s1=20190223457.PGNR	The invention provides methods to produce dough and bread products made at least in part of BSG. The BSG is removed from the brewing or distilling process, dried, frozen, and then further processed into a BSG dough product. More specifically, the invention relates to the systems and methods for making frozen pizza dough balls composed at least in part of grain products from the process of brewing beer or distilling sprits, such as whiskey	Brown and Allgeier (2019)
Process for producing protein concentrate or isolate a cellulosic	US Patent Application: US-2018-0199593-A1 US-2018-0199594-A1 European	https://data.epo.org/publication-server/search http://www.freepatentsonline.com/y2018/0199593.html	A process to obtain a high-value protein product and a cellulosic residue from BSG. The high-value protein product is useful as a	Mackay and Greden (2018)

Table 2 (continued)

Title of patent	Number	Source	Brief description	References, Inventor, Year
thermochemical feedstock from BSG	Patent Application: WO-2018-136,235-A1, WO-2018-136,234-A1		protein supplement or as feed for livestock and poultry, and the cellulose residue has value as a raw material for a thermochemical process unit, to produce a biofuel	
BSG-based protein powder	US Patent Application: US-2018-0014555-A1	http://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PG01&s1=20180014555.PGNR	Production of a BSG-based protein powder and the related methods for using the powder in protein-enriched foods. The BSG-based protein powders are highly soluble and therefore easily wettable, easily dispersible, and mixable at concentrations up to 50% by weight. They can be used alone or as a protein enhancer in food for human consumption, pet food, and commercial feed	Mackay et al. (2018)
Bio-plastic composite containing BSG and a method for making the same	US Patent Application: US-2017-0306153-A1 US-10-030-148-B2	http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PALL&s1=10030148.PN	Method for obtaining a bioplastic composite from BSG	Chen et al. (2018)
Integrated process for extracting proteins and arabinoxylans from BSG	Patent Cooperation Treaty: WIPO (PCT); WO-2012-069889-A1	https://worldwide.espacenet.com/patent/search/family/044479068/publication/WO2012069889A1?q=pn%3DWO2012069889A1	The present invention proposes an integrated process for extracting proteins and arabinoxylans from BSG, without the need to subject the BSG to any pre-treatment, through the use of alkaline reagents followed by selective precipitation by acidification of the medium and addition of ethanol. The present invention is applicable in the areas of re-use or exploitation of BSG, with the aim to obtain products that can be used as ingredients in the food industry, and in the production of dietetic and pharmaceutical products. The final residue obtained after extraction of the proteins and arabinoxylans can be used as a source of cellulose, as an insoluble dietetic fiber, or possibly as a fuel or raw material for the paper-making industry	Saraiva et al. (2012)
Integrated process of filtration to dry BSG	Patent Cooperation Treaty (PCT): WO-2010-117,288-Al WO-2010-117,288-A8	https://worldwide.espacenet.com/patent/search/family/042026531/publication/WO2010117288A1?q=pn%3DWO2010117288A1	A process and the corresponding equipment, to dehydrate BSG from 85 to 15% humidity, to obtain a stabilized product with the same content of protein, fiber, and lipids. The dehydration process involves several phases, two of which are mechanical (membrane filtration, compression) and the last one of which consists of vacuum drying using hot water or low-pressure water vapor as a heat	De Carvalho et al. (2010)

Table 2 (continued)

Title of patent	Number	Source	Brief description	References, Inventor, Year
			source. Energy for the process is available at no cost in the brewing industry, through the use of hot process water or low-pressure steam from cogeneration units. Dehydrated BSG is a stabilized product and can be used as food for humans and animals, ruminants, and non--ruminants, and as a raw material for biotechnological and pharmaceutical applications	
Process for drying BSG	US Patent Application: US-2012-0005916-A1 Patent Cooperation Treaty (PCT): WO-2010-053493-Al	http://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/srchnum.html&r=1&f=G&l=50&d=PG01&s1=20120005916.PGNR	A process for drying BSG, to obtain a product with a moisture content ≤ 15% by weight, biologically stable over time, with high nutritional value, commercially profitable, and environmentally safe, comprising two phases: the first is a mechanical pressing operation to reduce the initial humidity of the BSG by at least 75–80% by weight, up to a humidity < 70% by weight; the liquid obtained is transported to an effluent treatment plant; the solid obtained undergoes a second phase that consists of thermal drying, with two sub-phases: during the first, the product is transported through a stream of hot air, while during the second, it is transported by an air flow at room temperature	Lopez et al. (2012)

4023T, and *Lactobacillus rhamnosus* CECT-288 (Cruz et al. 2007).

Plaza et al. (2017) studied the feasibility of using BSG as a raw material to obtain butanol through an acetone–butanol–ethanol fermentation process using *Clostridium beijerinckii* DSM 6422. Although their results confirmed the suitability of BSG for the production of butanol, further studies are needed to scale-up this process. *Neurospora crassa* DSM 1129 and *Fusarium oxisporum* F3 isolated from cumin were used to convert the cellulose and hemicellulose in BSG directly into bio-ethanol, through successive phases of hydrolysis of the polysaccharides and fermentation of the monosaccharides (Xiros and Christakopoulos 2009; Xiros et al. 2008). The hydrolysis of BSG recalcitrant biopolymers (such as lignin and cellulose) during a pre-fermentation phase carried out by *Aspergillus oryzae* has been shown to improve the availability of nutrients, thus providing the complete requirements for growth of the yeast *Rodosporidium toruloides* (strain CBS 5490), a natural producer of carotenoids (Cooray et al. 2017).

Alternatively, the hydrolysis of BSG can be carried out through alkali, acid, hydrothermal, or enzymatic pre-treatments (Rojas-Chamorro et al. 2020; Paz et al. 2019). Ethanologenic microorganisms have been used to produce ethanol from these hydrolyzed BSG, including *Saccharomyces cerevisiae* and *Zigosaccharomyces rouxii* (Liguori et al. 2015). In particular, the high gravity brewing yeast *S. cerevisiae* BLGII 1762 and *S. cerevisiae* PE-2 isolated from the Brazilian bioethanol industry produce ethanol with yields of between 23 and 81% (Pinheiro et al. 2019). In addition, hemicellulosic hydrolysates of BSG have been used to produce microbial biomass and xylitol using *Debaryomyces hansenii* CCMI 941 (Carvalheiro et al. 2007) and *Candida guilliermondii* FTI 20037 (Mussatto and Roberto 2005). Banjo et al. (2018) used BSG to produce ascorbic acid by submerged fermentation culture of *Aspergillus flavus* and *Aspergillus tamari*, whereby they obtained 6.25 and 7.25 g L^{-1} ascorbic acid, respectively.

Brewers' spent grain has also been used for isolation and maintenance of actinobacteria, such as *Streptomyces coelicolor* A3 (Szponar et al. 2003) and *S. malaysiensis* AMT-3, and their production of cellulases (Nascimento et al. 2009). *Bacillus* sp. KR-8104 has been grown on BSG to produce α-amylase (Hashemi et al. 2011).

Regarding its exploitation in the pharmaceutical sector, BSG has been used as a culture medium for *Cordyceps militaris* (Gregori 2014) to produce cordycepin, a nucleoside analog with anti-tumor, anti-proliferative, anti-metastatic, insecticidal, and antibacterial activities (Gregori 2014). In addition, polysaccharides from *C. militaris* have shown significant antitumor activities against cervical and liver cancer cells in vitro (Yan et al. 2014), and extracts of its fruiting bodies show antioxidant, antibacterial, antifungal, and anti-tumor activities against human cell lines (Yan et al. 2014; Reis et al. 2013; Rao et al. 2010), and also anti-inflammatory (Rao et al. 2010), anti-fibrotic (Nan et al. 2001), anti-obesity (An et al. 2018), and anti-angiogenetic (Yoo et al. 2004) and insulin-secreting (Choi et al. 2004) activities. Thus, the use of BSG for cordycepin production by *C. militaris* has been shown to be a very effective technique for the production of high-value food and feed additives (Gregori 2014).

In recent years, the food industry has focused on the production of natural pigments from plants and microbial sources to overcome the use of synthetic pigments that can be hazardous to human health and to the environment. Silbir and Goksungur (2019) studied the production of natural red pigments by *Monascus purpureus* strain CMU001 in a submerged fermentation system using BSG as the raw substrate. Pigments from *Monascus* spp. have been used as natural coloring agents and natural food additives in eastern Asia, and have applications as pharmaceuticals, as they have been reported to have anti-mutagenic, anti-cancer, anti-obesity, anti-inflammatory, anti-diabetes, and cholesterol-lowering activities (Silbir and Goksungur 2019).

Brewers' spent grain in human and animal diets

The ever-increasing world population and the consequent requests for more food to meet nutritional requirements are two of the factors that define the need for sustainable food production. In particular, the possibility to use waste from agro-industrial processes as raw materials to produce food and feed has drawn the attention of numerous researchers. In this context, BSG provides numerous compounds that can serve as the basis to produce functional food and feed (Bonifácio-Lopes et al. 2019; Skendi et al. 2018). Thus, the use of BSG as a substrate for microbial growth to produce single cell proteins, to enhance its techno-functional properties, and to increase its commercial value has been proposed (Connolly et al. 2019; He et al. 2019; Ibbett et al. 2019; Luft et al. 2019; Marson et al. 2019; Martín-García et al. 2019; Shen et al. 2019).

Single cell proteins are an important example of new healthy proteins with low environmental impact. Their sustainable production represents one of the possible solutions to the problem of protein supply for future generations (Finnigan et al. 2017; Suman et al. 2015). BSG can be used as a low-cost raw material to produce single cell proteins from non-mycotoxigenic filamentous fungi in a sustainable and economic way. Fungi such as *Rhizopus* spp., *Trichoderma* spp., and *Mucor* spp. have been shown to hydrolyze BSG to obtain nitrogen and carbon sources for their growth without the need for costly pre-treatments or enzymatic hydrolysis (Bekatorou et al. 2015).

Brewers' spent grain has been proposed as a food additive due to its beneficial nutritional properties. These are mainly

due to the water-extractable part of BSG, which includes arabinoxylans (Mendis and Simsek 2014) and β-glucans (Steiner et al. 2015) that can regulate the gut microbiota. Possibly due to the release of xylo-oligosaccharides, arabinoxylans promote the growth of *Bifidobacterium* spp. and *Lactobacillus* spp., which have positive effects on the human gastrointestinal tract (Adamberg et al. 2014). Similarly, β-glucans can enhance growth and metabolic activity of beneficial microorganisms, such as *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium animali subsp. lactis* (Jayachandran et al. 2018). To further stimulate the grow of probiotic microorganisms, BSG has been used as a substrate to cultivate the fungus *Pleurotus ostreatus*, which represents an interesting source of β-glucans with prebiotic activities (Wang et al. 2001). In addition, it has been suggested that the intestinal microbiota, particularly *Lactobacilli* (e.g., *L. salivarius*, *L. paracasei*, *L. rhamnosus*) and *Bifidobacteria* (e.g., *B. adolescentis, B. breve*, *B. longum*), can partially degrade the lignin in BSG and metabolize the compounds released (Niemi et al. 2013). More recently, partial lignin degradation mediated by human fecal microbiota was shown in a colon intestine model (Ohra-aho et al. 2016; Aura et al. 2013). Here, it was suggested that lactic acid bacteria can easily adapt in BSG-based broth, thus paving the way for the use of probiotic strains as starter cultures for further improvement of the bioactive properties of fermented BSG (Gupta et al. 2013).

In addition, phenolic acids of BSG have shown antimicrobial activities against pathogenic bacteria. Ferulic acid is active on *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter koseri*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, and *Shigella sonnei* (de Oliveira and Batista 2017). *p*-Coumaric acid inhibits several pathogenic bacteria, such as *Staphylococcus aureus 6538*, *Streptococcus pneumoniae* ATCC49619, *Bacillus subtilis* 9372, *E. coli* ATCC25922, *Shigella dysenteriae* 51302, and *Salmonella typhimurium* 50013 (Lou et al. 2012), and it contributes to the definition of the distribution and abundance of microbial populations in the digestive tract (Reverón et al. 2012).

The nutritional value of BSG can be increased through low-cost microbe-mediated transformation processes. Natural degraders of lignocellulosic materials have been reported to significantly increase the nutritional content of BSG by fermentation (e.g., *Aspergillus* spp., *Trichoderma* spp., *Fusarium* spp., *Neurospora* spp.) (Bekatorou et al. 2007). Solid-state fermentation of BSG with *Rhizopus oligosporus* CCT 4134 and *Rhizopus microsporus* var. *oligosporus* (DMSZ1964) successfully increased the levels of amino acids, citric acid, vitamins, and antioxidants, and reduced the levels of carbohydrates, fats, and dietary fiber (Cooray and Chen 2018; Canedo et al. 2016). Further optimization of the operating parameters of solid-state fermentation can significantly increase the protein content, soluble protein, antioxidant and antibacterial activities, and total phenolic content, all of which are characteristics that are highly appreciated for feed and food applications (Ibarruri et al. 2019). *Bacillus subtilis* WX-17 (NCIMB 15204) isolated from a traditional food in Japan has been used for enrichment of the BSG content of triglycerides, palmitic, oleic, linoleic and stearic acids, polyphenols, and flavonoids (Tan et al. 2019).

Data on the digestive parameters and fecal microbial composition of dogs have also suggested that BSG can be used as a source of fiber in the canine diet (Eisenhauer et al. 2019). Also, recently, BSG was used as a substrate to rear different insects, such as the black soldier fly larva (*Hermetia illucens*) and mealworm larva (*Tenebrio molitor*) (Mancini et al. 2019; Melis et al. 2019; Shumo et al. 2019). These studies have confirmed that it is possible to take advantage of BSG to produce a nutrient-rich feed derived from the black soldier fly larva (Mancini et al. 2019; Melis et al. 2019; Shumo et al. 2019). Microbiological analyses of the larvae reared on the different substrates indicated that BSG administration resulted in decreases in *Staphylococci*, yeast, and mold, and the absence of bacterial endospores (Mancini et al. 2019).

Brewers' spent grain in agriculture

Brewers' spent grain has valuable applications in agriculture. It has been proposed as a promising substrate for production of biocontrol agents, such as the entomopathogenic fungus *Beauveria bassiana*, which is active against *Galleria mellonella* (Qiu et al. 2019). In particular, the high content of starch and fiber in BSG facilitates the germination and mycelial growth of *B. bassiana* conidia. Moreover, *B. bassiana* produced metabolites that promote plant growth (Qiu et al. 2019).

Brewers' spent grain has direct applications for the soil. Here, BSG increases organic substances, stability of aggregates, water retention, and available water, and also lowers the C/N ratio, which favors mineralization (Aboukila et al. 2018; Mbagwu and Ekwealor 1990). Addition of BSG for an earthworm diet produced enriched soil in Paenibacillaceae, Enterobacteriaceae, Chitinophagaceae, and Comamonadaceae (Budroni et al. 2020). In particular, the low pH and high organic carbon content of BSG were associated with higher abundance of bacterial taxa involved in cellulose degradation and showed high assimilation of ammonia and nitrates. When applied to bioremediation of soil polluted by engine oil, BSG enhanced plant growth and encouraged microbial degradation of hydrocarbons, using bacteria such as *Acinetobacter* spp., *Micrococcus* spp., *Pseudomonas* spp., *Nocardia* spp., and *Bacillus* spp. (Abioye et al. 2010). In particular, with the addition of BSG, the counts of hydrocarbon-

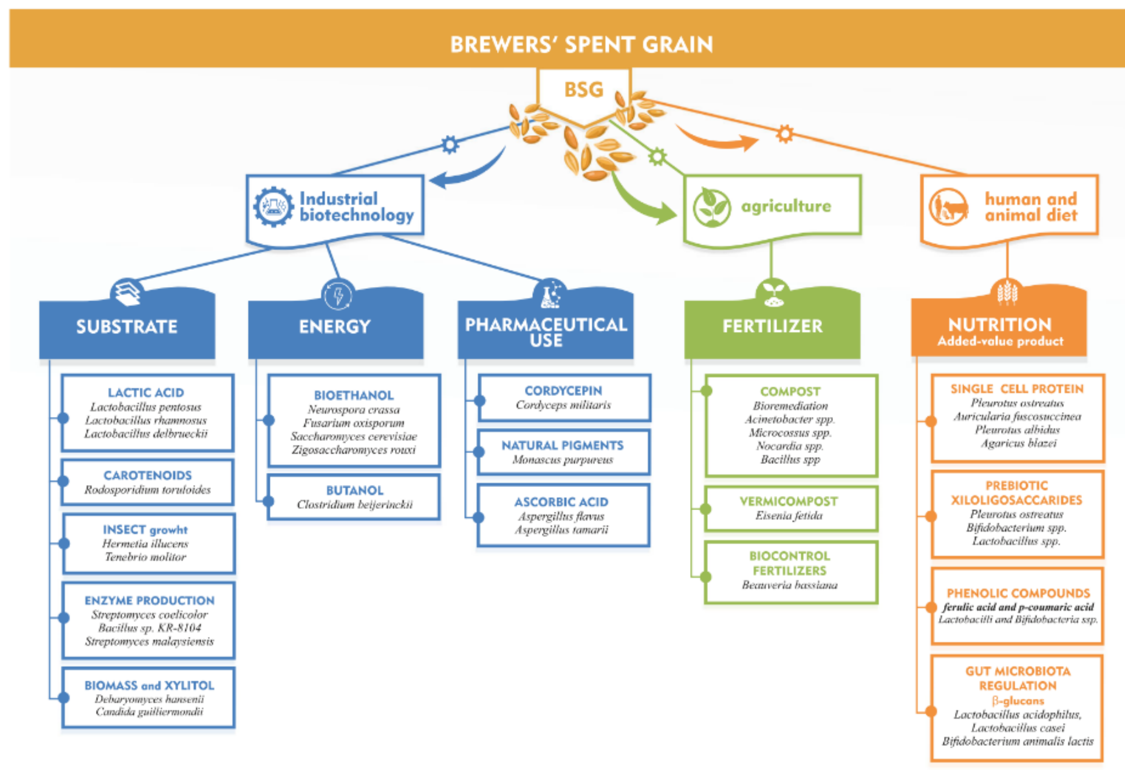

Fig. 3 Microbial exploitation of brewers' spent grain in industrial biotechnology, agricultural processes, and human and animal diets

using bacteria in the soil were about 5% higher than those without BSG addition.

Brewers' spent grain was recently used for vermicomposting by the earthworm *Eisenia fetida*, to produce a biological fertilizer (Saba et al. 2019). The vermicompost obtained from BSG respected the parameters of the safety laws: *E. coli* and *Salmonella* spp. were absent in 25 g of the vermicompost, and mycotoxins were degraded during its formation, with ochratoxin A levels below the legal limits. The enzymatic activities revealed a strict link between the microbiota and the quality of the BSG vermicompost, as the higher abundance of fungi and yeast in the BSG vermicompost was accompanied by increased dehydrogenase activity.

Safety issues

As BSG has been proposed as a component of human and animal nutrition, and also plant nutrition, it is necessary to consider some of the microbiological parameters that influence the quality of this raw material, such as the presence of pathogenic microorganisms and mycotoxins.

Pathogenic microorganisms

Most potential contaminants of beer originate from the raw materials and/or unclean brewing equipment. The raw materials used in brewing include malt, hops, and occasionally brewing water, and these can be contaminated by microorganisms that must be killed during the brewing process, to prevent wort and beer spoilage (Hill 2009). Correct hygienic standards and regular maintenance must be observed, so that the entire beer production process is not microbiologically compromised (Obi 2017). Water must be free of pathogenic microorganisms, such as coliform bacteria, *E. coli*, *Aerobacter aerogenes*, *Salmonella/Shigella* spp., and *Vibrio cholerae* (Hill 2009). However, to date, no known human pathogens have been found to survive in BSG after the brewing process.

Mycotoxins

Mycotoxins are secondary metabolites that are produced by fungi and that can affect human and animal health, due to their neurotoxic, immunosuppressive, teratogenic, and carcinogenic effects (Varga et al. 2015). Contamination of food and beverages by

Table 3 Worldwide mycotoxin regulations: limits and guideline for unprocessed and processed cereal in food

Mycotoxin	Regulation level (μg kg^{-1})*											
	European Union		USA (FDA)			Korea	Japan		China	Indonesia	Malaysia	Brazil
	European Commission (EC) No. 1881/2006 (2006)	Recommendation 2013/165/EU (2013)	Federal Food, Drug, and Cosmetic Act (FFDC Act 409) Compliance Program for Mycotoxins in Foods Sec. 7307.001			Codex Alimentarius Commission CAC/RCP 51-2003 Revised 2017 (2017)	Food Safety Commission (FSC No. 48, 2003/12/15) (2003)		China Food and Drug Administration (CFDA, GB 2761-2017) (2017)	Indonesian National Standardization Agency (SNI) 7385-2009	Codex Alimentarius Commission CAC/RCP 51-2003 Revised 2017 (2017)	Brazilian Surveillance Agency (ANVISA) RDC No. 138, 2019 (2019)
	Maximum limit	Guidance level	FDA action level	FDA advisory level	FDA guidance level		Maximum limit	Guidance level	Maximum limit	Maximum limit	Maximum limit	Maximum limit
Aflatoxin B1	2					10	10		5	15	0.1	
Aflatoxin total (B1, B2, G1, G2)	4		20			15				20	5	5
Ochratoxin A	3					5			5	5	0.5	20
Deoxynivalenol	500			1000		1000		1000	1000	1000	1000	750
Zearalenone	50					200			60			400
Fumonisins (B1, B2)	500				4000	1000				1000		300
T-2/HT-2		50										

*1 μg/kg = 1 ppb (parts per billion)

Table 4 Worldwide mycotoxin regulations: limits and guideline for cereal products for feed and compound feed

Mycotoxin	Regulation level (μg kg^{-1})*											
	European Union		USA (FDA)			Korea	Japan		China	Indonesia	Malaysia	Brazil
	European Commission (EC) No. 1881/ 2006 (2006)	Recommendation EU Reg. 576/2006 (2006)	Compliance Policy Guide (CPG) Sec. 683.100-2019 (2019)			(CAC CODEX STAN 193-1995) Revised 2017 (2017)	Minister of Agriculture, Forestry and Fisheries (MAFF) Act on Safety Assurance and Quality Improvement of Feeds (Law No. 35 of 1953 Revised in 2018) (2018)		USDA Hygienic Standard for Feeds GB 13078-2017	(CAC CODEX STAN 193-1995) Revised 2017 (2017)	–	Brazilian Surveillance Agency (ANVISA) RDC No. 138, 2017 (2017) MERCOSUR 2003
	Maximum limit	Guidance level	FDA action level	FDA advisory level	FDA guidance level		Maximum limit	Advisory level	Maximum limit	Maximum limit		Guidance level
Aflatoxin B1						10	10		20		–	
Aflatoxin total (B1, B2, G1, G2)	5		20					20		50	–	50
Ochratoxin A		50							100		–	
Deoxynivalenol		2		5000				1000	1000		–	
Zearalenone		500				3000		1000	500		–	
Fumonisin (B1 B2)		5000	2000		5000			4000	50,000		–	
T-2/HT-2		250							500		–	

*1 μg/kg = 1 ppb (parts per billion)

mycotoxins is a serious and recurrent problem worldwide, which results in economic losses and health concerns. Several studies have highlighted the need to monitor and determine the exact types and amounts of mycotoxins in cereals and their end-products and by-products, to ensure food and feed safety (Mastanjević et al. 2019; Habschied et al. 2014). Therefore, worldwide legal limits of mycotoxins have been established (Tables 3 and 4).

The contamination of malt by mycotoxigenic fungi and mycotoxins can occur throughout the entire brewer production chain, from the field to the malting and brewing (Bianco et al. 2019, 2018; Mastanjević et al. 2018). Mycotoxigenic fungal species that belong to the genera *Aspergillus* spp. (e.g., aflatoxin, ochratoxin A), *Fusarium* spp. (e.g., T-2 toxin, deoxynivalenol, fumonisins), *Penicillium* (e.g., ochratoxin A, patulin), and *Alternaria* spp. (e.g., alternariol) have been frequently isolated from barley kernels, malted barley, and BSG (Gonzalez Pereyra et al. 2011). Consequently, deoxynivalenol, aflatoxins, fumonisins, trichothecenes, ochratoxin A, and zearalenone are the most significant mycotoxins in the beer chain (Pascari et al. 2018; Habler et al. 2017; Bertuzzi et al. 2011).

Although the concentrations of mycotoxins decrease significantly during the production process (e.g., deoxynivalenol, zearalenone) (Piacentini et al. 2019), BSG can be contaminated by fungi after its production, which will result in increased levels of mycotoxins. Members of the genus *Fusarium* have been frequently isolated in high numbers in BSG, including *F. verticilloides* (50%), *F. proliferatum* (25%), *F. equiseti* (12.5%), and *F. oxysporum* (12.5%) (Mastanjević et al. 2019). *Fusarium* spp. produce the large group of mycotoxins known as the trichotecenes. Further, fumonisins B1 and AFB1 have been detected in malt barley and BSG, which appear to be produced during storage (Gonzalez Pereyra et al. 2011; Kensler et al. 2011). Eight mycotoxins were reported to be produced by *F. culmorum* (i.e., fusarenone-X, 3-acetyldeoxynivalenol, diacetoxyscirpenol, T-2, HT-2, deoxynivalenol, nivalenol, zearalenone), and these have been studied during the production of beer and for its various by-products (i.e., wastewater, spent yeast, BSG) (Mastanjević et al. 2018; Habschied et al. 2014). Malting and brewing by-products can also be contaminated by combinations of mycotoxins and multi-toxins (Mastanjević et al. 2019, 2018; Krstanović et al. 2015; Habschied et al. 2014, 2011). Lotaustraline and tryptophol have been reported at high levels in cereals and in yeast starters used for brewing (Mastanjević et al. 2018). Other mycotoxins and multi-toxins that have been detected in malt and brewing by-products include aurofusarin, beauvericin, brevianamide F, chrysogine, culmorine, 5-hydroxyculmorine, 15-hydroxyculmorine, deoxynivalenol, deoxynivalenol-3-glucoside, linamarin, tentoxine, and zearalenone (Mastanjević et al. 2019).

Conclusions

Brewers' spent grain is the main by-product of the brewing industry, and it is regularly available in large amounts at a low market price. Moreover, it is an interesting raw material due to its richness in valuable compounds and nutrients, and the availability of processes for its stabilization. These are all key factors for development of a plethora of different applications that span from the bio/technological production of added-value goods, functional foods, and animal feed, to the generation of other goods of interest for the pharmaceutical and agricultural sectors. Accordingly, there is great interest on BSG exploitation, as indicated by the number of patents concerning BSG preservation, use as a source of useful compounds, and bioconversion (Table 2).

A better understanding of the potential of microorganisms as biocatalysts for BSG transformation is essential for its recycling and exploitation, and also with a view to reduction of its carbon footprint under the concept of a circular economy. Therefore, we might see further interesting new BSG-based applications in the future. In particular, given the biotechnological and health importance of the various microbial groups that have been isolated from BSG, it is essential to study the metabolic relationships among the different microbial communities, and their influences on the final transformation of BSG, to exclude, or on the contrary, to favor, these new processes.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflicts of interest.

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Chapter 2

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Comparative analysis of vermicompost quality produced from brewers' spent grain and cow manure by the red earthworm *Eisenia fetida*

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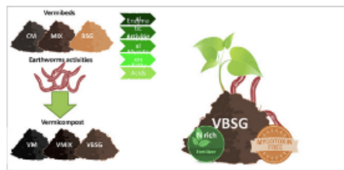
GRAPHICAL ABSTRACT



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ABSTRACT

Brewers' spent grain (BSG) is a by-product of brewing that is usually used as low-value animal feed, although it can be better exploited in biotechnological processes, such as vermicomposting. Here, the chemical, biochemical and microbiological qualities of vermicomposts produced by the earthworm *Eisenia fetida* were evaluated using three substrates: BSG; cow manure (CM); BSG plus cow manure (1:1; BSG/CM). Over after 5 months of bioconversion by earthworms and microorganisms (thereafter vermicomposting), BSG and BSG/CM showed reduced total organic carbon, and increased total nitrogen and total humic substances like (HSl), suggesting enhanced mineralisation and stabilisation. Suitability of BSG as substrate for earthworms was confirmed by the earthworm fatty acid profile, characterised by prevalence of C:17, C18:1, C18:2 and C18:3 fatty acids. Higher fungi and yeast abundance in BSG vermicompost was accompanied by higher dehydrogenase activity. *E. coli, Salmonella* spp. and Ochratoxin A levels were below the legal limits.

1. Introduction

Environmental and economic sustainability is an important aspect that can give beer production added value. In this respect, recovery of the potential value of by-products, such as yeast biomass, waste waters and spent grain, represents an exciting opportunity. Brewers' spent grain (BSG) is the main residue of the brewing process, as it represents 85% of the total by-products (Lynch et al, 2016). Around 20 kg of BSG are produced per 100 l of beer made. The global production of BSG has been estimated at 39 million tonnes per year, with 3.4 million tonnes produced in the European Union. Although a large proportion of this BSG is usually reused as low-value animal feed, which has a market value of €35 per tonne, it is also used in human foods or in biotechnological processes, such as energy production, paper manufacture, and enzyme or microbial biomass production, and also as a source of fine or bulk chemicals.

The main component of BSG is fibre (30%-50%; w/w), which includes the lignocellulose fraction, protein (19%-30%), hydrolysates of proteins, arabinoxylans and phenolic compounds. For these reasons, BSG represents a nutritionally rich by-product that requires the

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appropriate procedures for its recovery and re-use. One of the main problems in the re-use of BSG is its high moisture content, which results in its rapid deterioration and logistic difficulties for its storage and transportation. BSG can also be contaminated with mycotoxins, which can arise along the entire production chain, from cultivation of the barley in the field, to its storage and malt production. During all of these phases, contamination by mycotoxigenic fungi represents a high risk, with the consequent release of mycotoxins.

Stabilisation of BSG might be achieved by vermicomposting it, to recycle the nutrients in agriculture and to maintain soil fertility. Indeed, composting and vermicomposting are two of the best-known processes for biological degradation and stabilisation of organic wastes.

Vermicomposting is a non-thermophilic bio-oxidative decomposition process for organic waste that involves earthworms and their associated microbial communities (Sharmaa and Garga, 2018). In vermicomposting, earthworms have a crucial role, as they influence the activity of microorganism through fragmentation and ingestion of the organic matter (Dominguez et al., 2010). In addition, mesophilic vermicomposting can stimulate the microbial communities, and thus the extent of decomposition of the organic matter (Lazcano et al., 2008).

The close relationship between earthworms and their associated microbiota has also been investigated in terms of their phospholipid fatty acids (Gunya et al., 2016). Further, it has been shown that earthworms have a diverse pool of digestive enzymes that can also digest specific microorganisms, thus reducing microbial populations (Castillo et al, 2013). The use of the epigeic earthworm *Eisenia fetida* in vermicomposting is well documented in the literature for industrial waste (Sharmaa and Garga, 2018; Singh and Surindra, 2012). Indeed, *E. fetida* is the favoured earthworm species for laboratory experiments on vermicomposting, due to its tolerance to environmental variables (e.g., pH, moisture content, temperature). *E. fetida* is small in size and has a uniformly pigmented body, and it characterised by a short life cycle and a high reproductive rate. This earthworm is an efficient biodegrader and nutrient releaser, and an efficient compost producer, and therefore it aids in litter comminution and earlier decomposition.

The importance of earthworm microbial communities is well documented in the vermicomposting of lignocellulosic materials. The decomposition of such raw materials, including BSG, is a particularly difficult process due to the high content of complex heteropolymers, which confers different characteristics and can inhibit the cellulase enzymes (DeAngelis et al., 2011).

Proteobacteria and Actinobacteria are the two major taxa involved in lignin decomposition, where the α-proteobacteria and γ-proteobacteria classes are the most important degraders (DeAngelis et al., 2011). Bacteria from the class Actinobacteria are fundamental in lignin and polyphenol degradation (Kirby, 2005), as well as in the production of antibiotics and enzymes such as chitinases, which can degrade fungal cell membranes (Jayasinghe and Parkinson, 2009). The final product of vermicomposting, the vermicompost itself, is a finely broken up, peat-like material with high porosity, and good aeration, drainage, water holding capacity and microbial activity, and with excellent nutrient status and buffering capacity.

Most studies on vermicomposting have focused on changes in its physicochemical properties and biochemical (i.e., enzymatic) parameters (Singh and Surindra, 2012). These parameters reflect the earthworm and microbial activities. Hydrolytic enzymes involved in the carbon (C), nitrogen (N) and phosphorous (P) cycles, such as dehydrogenases, β-glucosidase, urease, and phosphatases, and also the phenol oxidases involved in lignin degradation, have been studied previously, but their relationships with different microbial taxa through the vermicomposting process has not been extensively studied (Sen and Chandra, 2009). Indeed, limited information is available on the abundance and structure of microbial taxa in vermicomposts.

The aim of the present study was to compare the physiochemical and microbiological quality and safety of vermicompost from both BSG and cow manure (hereafter CM) by the earthworm *E. fetida*.

2. Materials and methods

2.1. Preparation of earthworm beds

Red earthworms (*Eisenia fetida*, Savigny, 1826) were placed in three plastic containers for vermicomposting in the different substrates (hereafter referred to as 'beds'; size, 60 × 40 × 13 cm^3). These had perforated bases, to facilitate the water flow that is necessary to maintain moisture around 80–85% levels. The tops of the beds were covered with thin mesh, to allow gaseous exchange. Three different types of organic substrates were placed inside the beds: BSG, CM, and a 1:1 (v/v) mix of BSG plus CM (hereafter referred to as BSG/CM). Then, 300 g of *E. fetida* earthworms were added to each bed.

In the experimental trial, the organic substrates were left into the beds for 3 months after the completion of earthworms' digestion, to complete their transformation by microbial activities into potential organic fertilisers (i.e., the 'vermicompost'). The earthworms were left undisturbed to survive to the best of their abilities, and to reproduce. Throughout the experiment, some of the main variables that can influence the biological cycle of these earthworms were monitored twice a week (i.e., temperature, moisture, pH) and when necessary, adjustments were made.

2.2. Treatment and analysis of earthworm beds

Samples of the three organic matrices used as bed for vermicomposting were taken and therefore analysed at two different times during the experimental trial: (i) before their bioconversion by the earthworms and micro-organisms; and (ii) after 5 months from the beginning of the experiment, after the earthworms (2 months) and micro-organisms (3 months) completed their activities. Three replicates for each sample of the six substrates considered were initially dried at 45 °C for 48 h, then ground and sieved to 2 mm, for use in the subsequent chemical analyses, which were performed in triple and according to the methods reported by Chefetz et al. (1996).

A high-efficiency elemental combustion analyser (CHN 628; Leco, St. Joseph, Michigan, USA) was used to determine the total organic carbon (TOC) and total nitrogen (TN) levels, with the references for calibration of oat meal (Leco, 502-276) and soils (Soil LCMR Leco, 502-697; Soil Calibration sample for CSN Leco, 502-814; Soil LRM Leco, 502-062).

The total extractable carbon (TEC) and the total Humic Substances like (HSl) were also extracted. In particular, to determine TEC content 0.5 g of each substrate were treated in triplicate with 25 mL 0.1 N $NaOH/Na_4P_2O_7$ at 65 °C for 48 h. The supernatant was centrifuged, filtered (589/2; pore size, 0.70 mm; Whatman, Darmstadt, Germany), and then stored under N_2 at 4 °C (Ciavatta et al., 1990). All samples were immediately frozen at −80 °C, and then later lyophilised (Lyophilizer LyoLab 3000, Heto Lyolab, Switzerland) to complete dehydration.

The HSl was prepared as described for TEC determination in four replicates. The supernatants extracted were merged and then used to immediately fractionate the humic and fulvic substances like content in HSl. From the whole extract, three replicates of 25 mL each were collected, acidified with 50% H_2SO_4 (pH < 2) and centrifuged, to separate the humic substances-like portion (precipitated) from the fulvic substances like (remaining in solution with non humic fraction). Subsequently, the *para*-fulvic fraction was processed using a polyvinylpyrrolidone column, with 0.1 N NaOH for elution. From each column, the *para*-fulvic substances obtained were collected and combined with the corresponding *para*-humic fraction from the same replicate thus immediately frozen at −80 °C, and later freeze dried. Following their lyophilisation, the C contents of total HSl were determined using an elemental analyser (CHN 628; Leco), using the oat meal and soil standards for calibration. pH was also measured for all of the six samples, in aqueous solution (1:20; v/v) using a glass electrode

(XS sensor 250A; Orion, Boston, USA). The C/N ratios were calculated from the TOC and TN.

2.3. FAME of earthworms

Total fatty acids were extracted from 1.0 g samples of the earthworms taken after 5 months of the experimental trial and then lyophilized. Here, 0.2 g Na_2SO_4 was added to each sample, with extraction with 5 mL hexane (reagent grade; Sigma-Aldrich, Milan, Italy). After shaking, the solvent was removed under reduced pressure and ~0.1 mL 2 M methanolic potassium hydroxide solution was added. After vigorously shaking for 1 min, 200 μL isooctane (reagent grade; Sigma-Aldrich, Milan, Italy) was added, and 3 μL was injected into the system for gas chromatography–flame ionisation detection analysis.

The gas chromatography–flame ionisation detection analysis was performed on a gas chromatograph system (GC 2010 Plus; Shimadzu Italia, Milan, Italy) equipped with a split-splitless injector and a flame ionisation detector. Hydrogen was used as the carrier gas, at a flow rate of 1.0 mL min^{-1}. Data acquisition was performed using the GC Solution software (Shimadzu Italia, Milan, Italy). Analyses were performed with a stationary phase column (polyethylene glycol; Supelcowax 10; 30 m × 0.25 mm, 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). The oven temperature programme was 45 °C (held for 1 min), increased to 140 °C at a rate of 20 °C min^{-1}, then increased to 250 °C at a rate of 4 °C min^{-1} (held for 10 min). The injector temperature was 260 °C, and the split injector mode (1:5) was used. The detector temperature was 280 °C. To identify the fatty acids, retention times were compared to those obtained for the standard 37-Component Fame Mix (Supelco, Bellefonte, PA, USA).

2.4. Microbial analysis of beds and vermicomposts

The beds of BSG, CM and BSG/CM and the resulting vermicomposts (after 5 months) were analysed according to Grantina-Ievina et al. (2013). The total number of bacteria was estimated after 24 h incubation on nutrient agar medium (Biolife Italiana S.r.l., Milan, Italy) with 0.01% cycloheximide. The *Lactobacilli* were estimated after 24 h incubation on Man, Rogosa and Sharpe agar (Biolife Italiana S.r.l., Milan, Italy) with 0.01% cycloheximide, at 30 ± 2 °C. The total numbers of cultivable filamentous fungi and yeast were estimated after 48 h incubation on potato destrose agar (Biolife Italiana S.r.l., Milan, Italy) with 0.1% chloramphenicol, at 24 ± 2 °C. The numbers of *E. coli* and coliforms were estimated after 48 h incubation on Mac Conkey agar (Biolife, Milan, Italy), at 37 ± 2 °C. These data are expressed as colony forming units (CFU) × dilution factor × sample weight (g^{-1}). Three replicates were performed for each sample.

For detection of *Salmonella* spp., 25 g samples were enriched in peptone water for 1 day at 37 ± 2 °C, followed by isolation on Salmonella–Shigella agar (Bio-Rad, Hercules, CA, USA), at 37 ± 2 °C for 1 day.

2.5. Mycotoxins determination of beds and vermicomposts

Determination of the BSG mycotoxins was performed using quantitative analysis for the mycotoxins deoxynivalenol, T-2 and HT-2, fumonisins, aflatoxin and ochratoxin A. This was carried out using a 'rapid one-step assay' system (Charm Lateral Flow R.O.S.A., Foss A/S, Hillerod, Denmark), as based on ELISA, and following the protocol provided by the manufacturer. The limit of detection of each mycotoxin with this method was 100 ppb for deoxynivalenol, 10 ppb for T-2 and HT-2, 250 ppb for fumonisins, 2 ppb for aflatoxins, and 2 ppb for ochratoxins. Three replicates were performed for each sample. Similar analyses were carried out for the mycotoxins from the beds and the vermicomposts, for ochratoxin A, fumonisins and T-2 and HT-2 using ELISA in 48-well plates (Bio-Shield fumonisin 0.15– – 6 ppm; Bio-Shield ochratoxin 2.5–40 ppb; Bio-Shield T-2/HT-2 10–500 ppb; Prognosis-Biotech, Or-Sell, Modena, Italy,). This method used samples that were homogenised, weighed and then extracted with methanol: water (70:30; v/v). After filtration, the samples were ready for the ELISA test at 450 nm absorbance, and the data were analysed using the spreadsheet provided with the test.

2.6. Enzymatic activities of beds and vermicomposts

The enzymatic activities for dehydrogenase, urease and β-glucosidase were determined according to Alef and Nannipieri (1995). Dehydrogenase activity was measured in 10 g of each sample, by estimation of rate of reduction of triphenyltetrazolium chloride to triphenylformazan, after incubation at 37 °C for 24 h, and is expressed as μg triphenyltetrazolium formed $g^{-1}h^{-1}$. The urease activity was determined as ammonia released from 5 g samples treated with urea and incubated for 2 h at 37 °C. The urease activity is given as μg NH_4–N released $g^{-1}h^{-1}$. The β-glucosidase activity is given by the p-nitrophenol released from 1 g sample after incubation for 1 h at 37 °C with p-nitrophenylglucoside, and is expressed as μg p-nitrophenol $g^{-1}h^{-1}$. Each lyophilised sample for analysis of the dehydrogenase, urease and β-glucosidase activities was rehydrated for 3 h before analysis. Enzymatic activities were determined in triplicate samples, and all products were read in LVis plates using a microplate reader (SpectroStar Nano; BMG Labtech, Ortenberg, Germany), at 480 nm for dehydrogenase, 690 nm for urease, and 400 nm for β-glucosidase.

2.7. Data analysis

Total organic carbon (TOC), total nitrogen (TN) and total extractable carbon (TEC) levels before and after the transformation of the three substrates (i.e. BSG, CM and BSG/CM) were compared using the Student's *t*-test. Two-way ANOVA was carried out for HS levels and the Tukey test was used for *post-hoc* comparisons. Enzymatic activities were analysed in triplicate, with the mean values given. One-way ANOVA was carried out to compare the means from different treatments, and when significance was obtained ($p < 0.05$), the differences between the individual means were compared using *post-hoc* Fisher's least significance difference ($p < 0.05$) or Student's t-tests ($p < 0.05$) when appropriate, using the NCSS software (Keysville, Utah).

3. Results and discussion

3.1. Vermicompost BSG is a stabilised fertiliser that is rich in nitrogen

The two main variables that influence the biological cycle of red earthworms are temperature and moisture, and these were both kept constant throughout the vermicomposting process over 5 months. In particular, the temperature was maintained around 20 °C to 22 °C, and the moisture around 80% to 85%. For pH, this was higher at the end of this experimental period (i.e., in the vermicomposts) for all of the substrates, as it increased from 3.8 to 5.6 for BSG, from 7.8 to 8.1 for CM, and from 5.9 to 7.2 for BSG/CM.

In general, earthworms avoid substrates with pH < 4.5, as prolonged exposure to such pHs can be lethal for them (Dominguez, 2004). Since earthworms have a natural tendency to shift the pH towards values closer to neutrality, the present trial was also used to test their survival capacity and their ability to modify pH values.

The TOC content of these substrates before and after the vermicomposting process decreased significantly for BSG and BSG/CM substrates (Fig. 1; 3.3%, 4.8% respectively), but showed no change for CM. This was paralleled by the before and after organic matter, which also decreased significantly for the BSG and BSG/CM substrates (from 65.0 ± 0.2% to 59.3 ± 0.1% and from 51.6 ± 0.4% to 43.4 ± 0.2%, respectively), again with no difference seen for CM (that remained unaltered to 38.4 ± 0.8%). Some studies have reported that relatively large amounts of TOC are lost in the form of CO_2 (20%–45%)

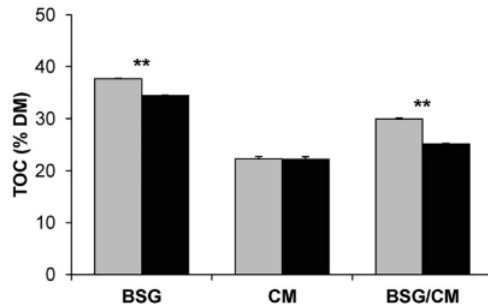

Fig. 1. Total organic carbon (TOC) in the experimental substrates of BSG, CM and BSG/CM before (beds; grey) and after (vermicompost; black) the 5-months of vermicomposting. Data are means ± standard deviation. **, $p < 0.01$.

due to the feeding of earthworms on the organic matter and due to microbial degradation (Kaushik and Garg, 2003). These modifications promote C loss through microbial respiration, in the form of CO_2, and through mineralisation of organic matter. As partial confirmation of this hypothesis, different dynamics were seen for the CM experimental unit, where the earthworms lived under suitable environmental and balanced biochemical conditions.

Conversely, there were significant increases in TN for the BSG and BSG/CM substrates (from 3.64 ± 0.03% to 5.10 ± 0.02% and from 3.18 ± 0.03% to 3.38 ± 0.01%, respectively), with a significant decrease in CM (from 2.90 ± 0.08% to 2.54 ± 0.07%; Fig. 2). The TN of the final substrates (after the removal of the earthworm from the beds) might be the result of greater withdraw of nitrogen by the earthworms for reproductive purposes. Indeed, earthworms cultured in the presence of the spent grain here (i.e., BSG, BSG/CM) will almost certainly have undergone stress caused by the low pH of these substrates, and especially in the early stages of this vermicomposting. Thus, the larger earthworm populations in CM might have used more nitrogen to produce substances required for individual growth and reproduction, thus lowering the N content in the CM substrate. Using wheat straw as bed, Cortez et al. (1989) demonstrated that earthworms could assimilate the 9.4% of the total N ingested. Furthermore, the observed decreases in the levels of N could be related to the leaching of N for addition of constant water to keep the bed at 80–85% of moisture. Finally, small decreases of Nitrogen could be related to nitrification and denitrification phenomena leading to N_2 and N_2O volatilisation (Plaza et al., 2008).

The TN increases in BSG may be related to the higher rates of earthworms' death and decomposition, before vermicompost was collected, due to the harsh environmental condition in this substrate. Indeed, it has been observed that the N content of the compost depends on the extent of the decomposition (Gaur and Singh, 1995). Also, the action of N-fixing bacteria could be hypothesized (Plaza et al., 2008). As demonstrated by Hand et al. (1988), *E. fetida* in cow dung slurry increases the nitrate-N content. Also, the organic C decreases might be involved in this dynamic, as they can cause N increases that are linked to mucus nitrogenous excretory substances, growth stimulatory hormones, and enzymes from the gut of earthworms (Tripathi and Bhardwaj, 2004). However, to better evaluate the N dynamics during the vermicomposting of BSG, the different chemical forms of N in the organic matrices would need to be evaluated before and after vermicomposting.

The C/N ratio represents one of the most widely expressed indices for the maturity of organic matter, as this reflects the mineralisation and stabilisation level (Suthar, 2008). While the C/N ratio for CM before and after vermicomposting processes here were balanced (from 7.68 to 8.75; indicating an equilibrated substrate), the lower values for BSG and BSG/CM after vermicomposting (from 10.34 to 6.76 and from 9.40 to 7.45, respectively) indicate an advanced degree of organic matter stabilisation throughout this vermicomposting (as shown by Zhang et al., 2015).

It has often been reported that the C/N ratio decreases sharply during vermicomposting (Suthar, 2008). This reduction is mainly due to an absolute decrease of Carbon by mineralization and respiration processes (CO_3^- and CO_2) (Nayak et al., 2013), while Nitrogen varies much less because it is biologically reused. The amount of Nitrogen not used by microorganisms remains into vermicompost and it is therefore available. Also the production of mucus and nitrogenous excreta by earthworms will enhance the levels of N, reducing the C/N ratio at the same time (Senapati et al., 1980).

The TEC provides a measurement of the total C in total humic substances like (HSl), and this also significantly increased in the BSG and CM substrates during the vermicomposting (from 21.0 ± 1.2% to 25.2 ± 0.5% and from 17.2 ± 0.8% to 20.6 ± 0.7%, respectively) while no differences were seen for BSG/CM (from 19.1 ± 1.2% to 21.0 ± 1.5%; Fig. 3). HSl expressed as proportions of the TEC varied considerably before and after vermicomposting. HSl increased from 11.2 ± 2.3% to 31.3 ± 1.2% for BSG, 10.2 ± 1.8% to 18.6 ± 0.7% for CM, and 10.5 ± 0.9% to 23.6 ± 1.0% for BSG/CM (Fig. 4). The results of the two-way ANOVA for the effects of substrate (BSG, CM, BSG/CM, before and after vermicomposting) on the HSl substances showed significant differences for both factors and for their interaction (Table 1). In particular, the Tukey *post-hoc* comparison test showed that HSl contents after the transformation of the substrates were significantly higher in BSG and BSG/CM than in CM. The large TEC contents in all of the substrates after vermicomposting would indicate the achievement of a high degree of maturity and stability of the organic matter (Ngo et al., 2011).

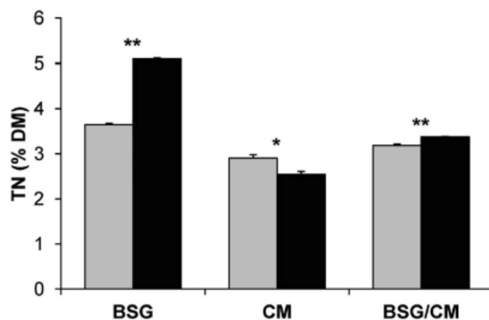

Fig. 2. Total nitrogen (TN) in the experimental substrates of BSG, CM and BSG/CM before (beds; grey) and after (vermicompost; black) the 5-months of vermicomposting. Data are means ± standard deviation. *, $p < 0.05$; **, $p < 0.01$.

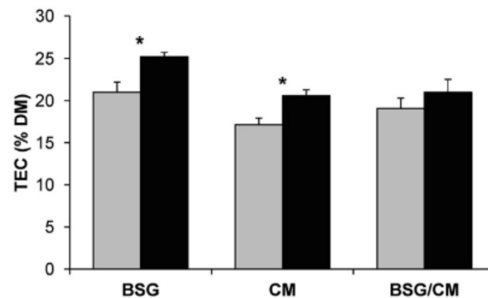

Fig. 3. Total extractable carbon (TEC) in the experimental substrates of BSG, CM and BSG/CM before (beds; grey) and after (vermicompost; black) the 5-months of vermicomposting. Data are means ± standard deviation. *, $p < 0.05$.

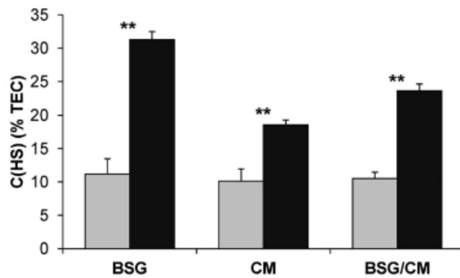

Fig. 4. Total humic-substances like carbon [C (HS)] as a proportion of the total extractable carbon (TEC) in the experimental substrates of BSG, CM and BSG/CM before (beds; grey) and after (vermicompost; black) the 5-months of vermicomposting. Data are means ± standard deviation. **, $p < 0.01$.

Table 1
Two-way ANOVA for the effects of substrate (BSG, CM, BSG/CM) and time (before and after vermicomposting) on total humic-substances like (HS).

Source	df	MS	F	p
Substrate (*S*)	2	143.49	72.93	< 0.001
Time (*T*)	1	1727.12	877.84	< 0.001
S × *T*	2	103.21	52.46	< 0.001
Residual	30	1.97		

Along with the TEC increase after the vermicomposting, the total levels of C in HSl also increased. In particular, the high levels of C in HSl for BSG are almost certainly linked to the large amount of organic C in the unprocessed spent grain (Fig. 1). Both the high TEC and HSl in all of the substrates after vermicomposting would indicate the extended synthesis of organic components recalcitrant to microbial degradation (Plaza et al., 2008).

3.2. Fatty-acids profile provides a biomarker of the health status of E. fetida in BSG

To determine whether BSG is a good substrate for the growth and reproduction of these earthworms, we analysed the fatty-acid profile of *E. fetida* (Table 2). The fatty acids in the whole body or gut of earthworms can be used as a biomarker and as an index of responses to environmental stress (Crockett et al., 2001). In addition, the elevated fat content of *E. fetida* (7.8%) makes this species a good alternative source of protein and fatty acids for animal feed (Gunya et al., 2016). Under the tested conditions here, the pattern of the fatty acids was characterised by the absence of evaluable data for fatty acids with carbon chains shorter than C11 and longer than C20. Only saturated C12, C14, C17 and C20 fatty acids (with prevalence for C17) and unsaturated C18:1, C18:2, C18:3 fatty acids were detected from these earthworms. A comparison of the different growth and reproduction substrates shows that CM had a low content of saturated and unsaturated fatty acids with long and short chains. Linoleic and palmitic oleic acids were the most abundant in *E. fetida* in BSG. Small amounts of linolenic and stearic acids and high contents of myristic, margaric, linolenic, omega 6 and omega 3, arachidonic fatty acids were also found, in agreement with Almeida et al. (2017). The fatty-acid content of the earthworms from the BSG/CM substrate reflects the contribution of BSG. The composition of fatty acids in the earthworm body depends on both species (Paoletti et al., 2003) and diet (Sampedro et al., 2006). Different studies have reported that *E. fetida* contains large amounts of omega 3 fatty acids (Gunya et al., 2016). The highest levels seen for the 17:0 fatty acid, followed by 18:2-cis, is in partial agreement with the fatty-acid data of Antisari et al. (2015). Indeed, they identified fatty acids with C chains of > 15, but no further information is currently

Table 2
Fatty acids identified and quantified in *Eisenia fetida* after the 5-months of vermicomposting in the experimental substrates of BSG, cow manure (CM) and BSG/CM.

Fatty acids	CM	BSG	BSG/CM
(C4:0)	< 0.3	< 0.3	< 0.3
(C6:0)	< 0.3	< 0.3	< 0.3
(C8:0)	< 0.3	< 0.3	< 0.3
(C10:0)	< 0.3	< 0.3	< 0.3
(C11:0)	< 0.3	< 0.3	< 0.3
(C12:0)	14.8 ± 0.3	1.5 ± 0.2	9.2 ± 0.3
(C13:0)	< 0.3	1.6 ± 0.2	< 0.3
(C14:0)	4.2 ± 0.2	4.8 ± 0.2	4.5 ± 0.2
(C14:1)	< 0.3	< 0.3	< 0.3
(C15:0)	< 0.3	0.5 ± 0.2	< 0.3
(C15:1)	< 0.3	< 0.3	< 0.3
(C16:0)	< 0.3	1.6 ± 0.2	< 0.3
(C16:1)	< 0.3	1.2 ± 0.2	< 0.3
(C17:0)	18.3 ± 0.3	29.1 ± 0.3	15.4 ± 0.3
(C17:1)	< 0.3	< 0.3	< 0.3
(C18:0)	17.5 ± 0.2	< 0.3	16.3 ± 0.3
(C18:1-trans)	< 0.3	< 0.3	< 0.3
(C18:1-cis)	< 0.3	3.8 ± 0.2	< 0.3
(C18:2-trans)	< 0.3	< 0.3	< 0.3
(C18:2-cis)	< 0.3	27.7 ± 0.3	14.7 ± 0.3
(C18:3) (omega-6)	32.4 ± 0.2	13.2 ± 0.3	19.4 ± 0.3
(C18:3) (omega-3)	< 0.3	7.8 ± 0.3	10.8 ± 0.2
(C20:0)	12.8 ± 0.3	7.2 ± 0.3	9.7 ± 0.3
(C20:1)	< 0.3	< 0.3	< 0.3
(C20:2)	< 0.3	< 0.3	< 0.3
(C20:3) (omega-3)	< 0.3	< 0.3	< 0.3
(C20:3) (omega-6)	< 0.3	< 0.3	< 0.3
(C20:4) (omega-6)	< 0.3	< 0.3	< 0.3
(C20:5) (omega 3)	< 0.3	< 0.3	< 0.3
(C21:0)	< 0.3	< 0.3	< 0.3
(C22:0)	< 0.3	< 0.3	< 0.3
(C22:1) (omega-9)	< 0.3	< 0.3	< 0.3
(C22:2)	< 0.3	< 0.3	< 0.3
(C22:6n3) (omega-3)	< 0.3	< 0.3	< 0.3
(C23:0)	< 0.3	< 0.3	< 0.3
(C24:0)	< 0.3	< 0.3	< 0.3
(C24:1)	< 0.3	< 0.3	< 0.3

Data are mean ± standard deviation of three independent replicates.

available on fatty acids in earthworms.

3.3. Vermicompost from BSG respects the safety law parameters

To assess the quality of raw materials and end products, the main microbial groups of the BSG and CM used for the beds and of the resulting vermicomposts were determined (Fig. 5). The quantity of fungi and yeast significantly increased during the 5 months of vermicomposting of BSG, and thus earthworm activity positively affected the development of these microbial taxa. The opposite was seen for the vermicomposting of cow manure and BSG/CM, where the high levels of contamination by fungi and yeast in the beds was significantly reduced ($p < 0.01$) during the processing (Fig. 5). The bacterial counts were higher in BSG/CM, which suggests that these two components of this growth and reproduction substrate, namely BSG and cow manure, brought together specific microbial groups that coexist in the mixture, such as *Lactobacilli* from BSG and *Coliforms* from cow manure. In addition, the BSG/CM microflora might have influenced the *Escherichia coli* dynamics during vermicomposting. Indeed, during this process, the levels of *E. coli* were not significantly reduced in BSG/CM, while these were significantly decreased in cow manure ($p < 0.001$). Notwithstanding these differences, the *E. coli* levels were below the legal limits in all of these samples. Similarly, *Salmonella* spp. which are used as a safety indicator, were not found in any of the substrate samples. European Community Regulation N° 1069/2009 defines the health standards related to animal by-products that are not intended for human consumption, where manure is defined as follows: "excrements and/or

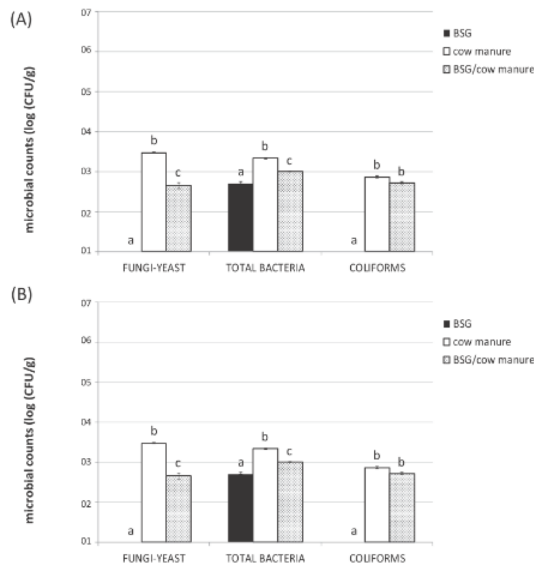

Fig. 5. Total microbial counts in the experimental substrates beds of BSG, cow manure (CM) and BSG/CM before (A; beds) and after (B; vermicompost) the 5-months of vermicomposting. Data are means ± standard deviation. Different letters indicate statistical differences ($p < 0.05$) as determined by ANOVA followed by Tukey-HSD test.

urine from animals of breeding, other than farmed fish, with or without litter". According to Italian legislation (Legislative Decree N° 75/2010, annex 2, point 11 and following), vermicompost refers to worm and insect ejections. In Italy, the microbial quality of vermicompost is regulated by Legislative Decree N° 75/2010. In particular, *Salmonella* spp. should be absent in 25 g of sample, while *E. coli* must not exceed 5×10^3 CFU/g in vermicompost. This decree classifies vermicompost as a soil improver, and it also establishes the parameters for the nitrogen and organic carbon content. If vermicompost is intended to be used in organic farming, the annexes to the legislative decree provide for additional parameters.

There is also a difference between vermicompost from manure and vermicompost obtained from organic waste: only the first can be placed on the market, while the second can only be used for self-consumption. From the regulatory point of view, when the humid matrix is used, this does not result in an earthworm vermicompost; this provides instead a mixed composted soil conditioner that has a commercial value one-fifth to one-tenth of earthworm vermicompost produced from manure. Furthermore, the norms established by EC Regulation N° 1069/2009 must be respected. Thus, from the regulatory point of view, the final product that results from the processing of BSG by earthworms should be more accurately defined as a mixed composted soil conditioner. The vermicompost producer must also be registered in the register of fertiliser manufacturers by submitting an application to the Ministry of Agriculture.

3.4. *Mycotoxins are degraded during vermicomposting*

Preliminary characterisation has shown that BSGs from local breweries are contaminated by ochratoxin A, fumonisins and T-2 and HT-2, while aflatoxins and deoxynivalenol have not been detected (A. Bianco, personal communication). On the basis of this information, ochratoxin A, fumonisins and T-2 plus HT-2 mycotoxins were determined for the BSG and cow manure used for the beds, and for these

Table 3
Mycotoxin contents of the experimental substrates of BSG, cow manure (CM) and BSG/CM before (beds) and after (vermicomposts) the 5-months of vermicomposting.

Substrate	Analysis	Ochratoxin A (ppb)	Fumonisins (ppm)	T-2 + HT-2 (ppb)
BSG	Before	7.5 ± 1.00	< LOQ	338 ± 67.5
	After	< LOQ	0.1 ± 0	16 ± 6.2
Cow manure	Before	< LOQ	< LOQ	< LOQ
	After	< LOQ	< LOQ	< LOQ
BSG/CM	Before	< LOQ	< LOQ	80 ± 10.6
	After	< LOQ	< LOQ	21.3 ± 15.8

Data are means ± standard deviation.
ppb, μg/kg; ppm, mg/kg.
LOQ, limit of quantification; ochratoxin A, 1.5 $\mu g\,kg^{-1}$; fuminisins, 0.1 $mg\,kg^{-1}$; T-2 + HT-2, 10 $\mu g\,kg^{-1}$.

vermicomposts (Table 3). For the cow manure before and after this vermicomposting, none of these mycotoxins studied here were above the detection thresholds.

Ochratoxin A levels were 7.5 ppb in BSG, thus exceeding the limit of 5 $\mu g\,kg^{-1}$ for unprocessed cereals, as defined by CE Regulation N° 1881/2006. Interestingly, the ochratoxin A levels were below the detection threshold after the vermicomposting. This can be compared to the threshold set by EC Regulation N° 1881/2006 of 3 $\mu g\,kg^{-1}$ for ochratoxin A levels in all products derived from cereals and intended for direct human consumption.

Also, the 338 ppb of T-2 plus HT-2 in the BSG here is above the limit suggested by EC Recommendation 2013/165/EU, although, again, this was significantly reduced to 16 ppb after the BSG alone vermicomposting. The same trend was seen for the BSG/CM substrate, where the initial contamination of 80 ppb T-2 plus HT-2 was reduced to 21 ppb after the BSG/CM vermicomposting. The recommendation (2013/165/EU) indicates T-2 and HT-2 levels < 200 ppb (i.e., $\mu g\ kg^{-1}$) in unprocessed cereals, such as maize and barley (including beer barley), and 100 ppb and 50 ppb for cereal products for wheat or other grain-milling products for direct human consumption.

Fumonisines were not detected in any of the beds or vermicomposts studied here. However, the analysis carried out on the lyophilised earthworms showed that *E. fetida* can bioaccumulate this mycotoxin. In particular, the earthworm growth and reproduction in BSG, cow manure and BSG/CM showed contamination of fumonisins of 0.40 ppm, 0.31 ppm and 1.04 ppm. respectively. The low levels of ochratoxin A and T-2 and HT-2 in the earthworms here were not sufficient to explain the strong reduction in these mycotoxins during the vermicomposting. Thus, it can be hypothesised that partial detoxification of ochratoxin A and T-2 and HT-2 was carried out by *E. fetida* and its associated microbiota.

Eisenia fetida is considered a representative species of earthworms, with a wide literature available on its ecology and its use in ecotoxicological experiments (OECD 207; ISO No.11268-1:2012; ISO No. 1268-2:2014; ISO 11268-3:2015). Generally, the substances analysed in acute toxicity tests with earthworms have mainly been chemical and pharmaceutical soil contaminants; only recently have studies been conducted on the effects of mycotoxins on earthworms. Yang et al. (2015) evaluated the multiple toxic endpoints of naturally occurring mycotoxins in the nematode *Caenorhabditis elegans* model (aflatoxin B1, deoxynivalenol, fumonisin B1, T-2, zearalenone). Delgado (2014) evaluated the potential ecotoxicological risk of fumonisin B1 on terrestrial invertebrates, with their study conducted under controlled laboratory conditions by exposing *E. fetida* to fumonisin B1 in an artificial soil. Szabó-Fodor et al. (2017) studied the possible serious risks of aflatoxin B1 on the earthworm *E. fetida*. The results of these studies confirmed that the tests based on EC Regulation N° 1907/2006 with *E. fetida* are applicable and useful in research on toxicities of mycotoxins.

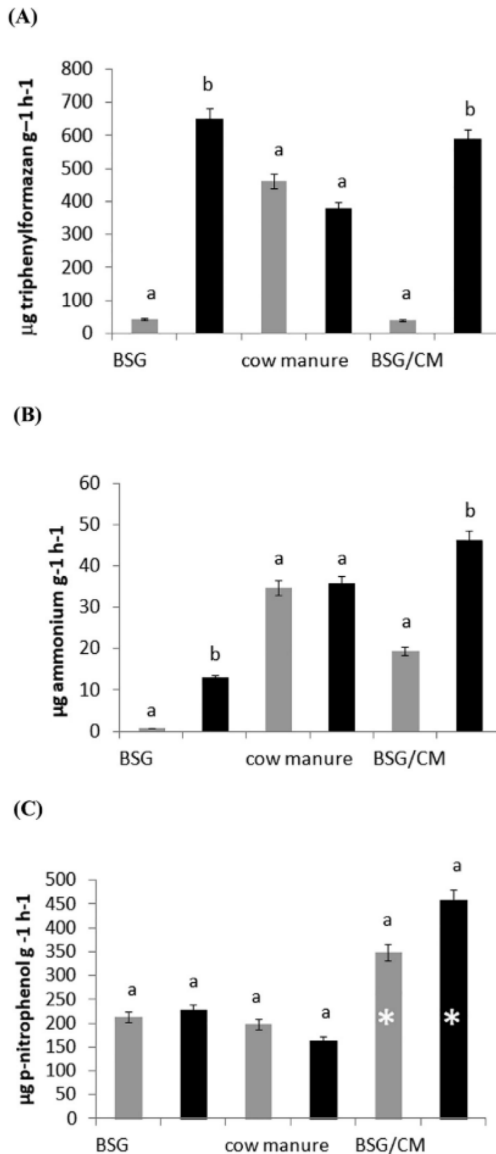

Fig. 6. Enzyme activities for dehydrogenase (A), urease (B) and β-glucosidase (C) in the experimental substrates of BSG, cow manure (CM) and BSG/CM before (beds; grey) and after (vermicompost; black) the 5-months of vermicomposting. Data are means ± standard deviation. Data indicated with different letters indicate statistically significant differences (before and after vermicomposting), data indicated with asterisks indicate statistically significant differences among the different treatment ($P < 0.05$; Fisher's least significant difference tests).

This has provided information on possible acute and sub-acute toxic effects, and the effects of mycotoxins on soil invertebrates.

3.5. Enzymatic activities reveal a strict link between microbiota and quality of BSG vermicompost

Microbial enzyme activities are indicators of the biological properties of the stabilized substrates. The vermicomposting of BSG and BSG/CM resulted in a significant increase in the dehydrogenase activities, which were 15.3-fold and 14.9-fold higher than those in the unprocessed substrates (Fig. 6). These large increases are related to the limited enzymatic activities in the raw materials. On the contrary, the high dehydrogenase activity of CM did not significantly change after its vermicomposting. Dehydrogenase activities are known to be representative of the oxidative activities of active microbial populations, as this intracellular enzyme is found only in living cells (Pankhurst et al., 1997). Hence, the dehydrogenase activity can provide information on the microbial community stress induced in the substrate. Benitez et al. (2005) reported that extracellular dehydrogenase activity can increase due to continuous accumulation of cells releasing extracellular enzymes in humic-like substances during the initial phases of vermicomposting. Lazcano et al. (2008) associated low dehydrogenase activity in non-stabilised substrates.

β-Glucosidase activity did not vary significantly before and after vermicomposting in any of these samples. Before vermicomposting, the β-glucosidase activity of the BSG/CM substrate was 1.7-fold those for BSG and CM. Also, for the BSG/CM substrate after the 5 months of vermicomposting, the β-glucosidase activity was 2.79-fold and 2.01-fold those of the BSG and CM vermicomposts, respectively. β-Glucosidase is an extracellular enzyme that is involved in the C cycle (Alvarenga et al., 2008) and can be used as an indicator for microbial ability to degrade organic matter (Pankhurst et al.,1997). β-Glucosidase degrades glucosides to glucose during cellulose degradation (Esen, 1993). It is believed that β-glucosidase is mainly produced by the fungi in soils (Hayano and Tubaki, 1985). The high β-glucosidase activities seen here might be caused by the greater abundance of fungi, according to a study conducted by Lazcano et al. (2008), where they reported significant correlation between presence of ergosterol in their substrates and β-glucosidase activity, as also seen in previous studies (Aira et al., 2006).

Several studies have shown that urease activity is influenced by the type of substrate used for vermicomposting (Pramanik et al., 2007; Castillo et al.,2013; Yadav et al., 2015). In particular, the urease activity in the present study was probably favoured by the substrates that were particularly rich in nitrogen (Pramanik et al., 2007), as for those obtained from BSG and BSG/CM. In these samples, the urease activity increased 17.46-fold and 2.37-fold compared to the beds before the 5 months of vermicomposting. The main nitrogenous compounds in BSG are proteins, and high levels of urea might have been generated through their degradation by the microbiota. Thus, increased urea would have led to corresponding increases in the urease activities. On the contrary, the urease activities did not change in the manure after vermicomposting. According to Castaldi et al. (2008), it can be postulated that the major nitrogen compound in cow manure was already stored as ammonium, such that the urease activity during vermicomposting remained constantly high. Urease is an extracellular enzyme that is involved in the N cycle, and it can catalyse the hydrolysis of urea-type substrates to CO_2 and NH_3 (Alvarenga et al., 2008). Urease activity has been used as an environmental stress indicator, in particular in substrates with different levels of nitrogen (Pankhurst et al.,1997; Bhattacharyya et al., 2008).

In general, the increased enzyme activities during the vermicomposting of BSG and BSG/CM in particular probably related to increased microbial populations (Fig. 5). The higher enzyme activities in the vermicomposts with respect to the raw materials used for the beds might be due to stimulation of microbial activities during the

bioconversion period (Pramanik et al., 2007; Yadav et al., 2015).

4. Conclusions

BSG are organic by-products that support *E. fetida* healthy growth, as confirmed by the fatty-acids profile of this earthworms' species. Following the activity of earthworms and their associated microbiota, BSG resulted in a vermicompost rich in Nitrogen and that could be safely used as soil-improver. Indeed, vermicompost from BSG respects biological and microbiological safety law parameters, while unprocessed BSG showed ochratoxin A levels exceeding law thresholds. Finally, the enzymatic activities revealed a strict link between microbial populations and the quality of the vermicompost.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 3

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Microbial and chemical dynamics of brewers' spent grain during a low-input pre-vermicomposting treatment

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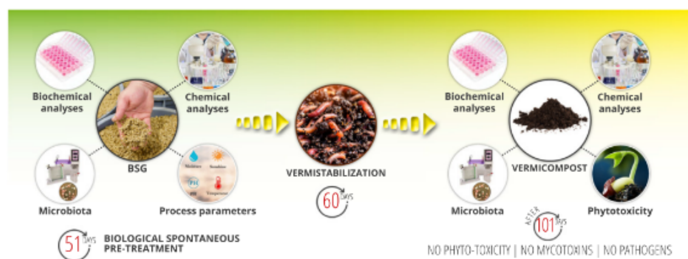
HIGHLIGHTS

- Low-input pre-treatment of brewers' spent grain is eco-sustainable.
- Low-input pre-treatment of brewers' spent grain shows accelerated decomposition.
- Vermicompost from pre-treated brewers' spent grain has good indicators of maturity.
- Vermicompost from pre-treated brewers' spent grain respects the legal requirements.

GRAPHICAL ABSTRACT



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ABSTRACT

The eco-sustainability of industrial processes relies on the proper exploitation of by-products and wastes. Recently, brewers' spent grain (BSG), the main by-product of brewing, was successfully recycled through vermicomposting to produce an organic soil conditioner. However, the pre-processing step there applied (oven-drying) resulted in high costs and the suppression of microbial species beneficial for soil fertility. To overcome these limitations, a low-input pre-processing step was here applied to better exploit BSG microbiota and to make BSG suitable for vermicomposting. During 51 days of pre-treatment, the bacterial and fungal communities of BSG were monitored by denaturing gradient gel electrophoresis (DGGE). Chemical (carbon, nitrogen, ammonium, nitrate content, dissolved organic carbon) and biochemical (dehydrogenase activity) parameters were also evaluated. Mature vermicompost obtained from pre-processed BSG was characterized considering its legal requirements (e.g., absence of pathogens and mycotoxins, lack of phytotoxicity on seeds), microbiota composition, and chemical properties. Results obtained showed that throughout the pre-process, the BSG microbiota was enriched in bacterial and fungal species of significant biotechnological and agronomic potential, including lactic acid bacteria (*Weissella*, *Pediococcus*), plant growth-promoting bacteria (*Bacillus*, *Pseudomonas*, *Pseudoxhantomonas*), and biostimulant yeasts (*Pichia fermentans*, *Trichoderma reesei*, *Beauveria bassiana*). Pre-processing increased the suitability of BSG for earthworms' activity to produce high-quality mature vermicompost.

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1. Introduction

Sustainable agriculture promotes soil health and productivity by favoring the use of organic fertilizers, and by reducing the dependence on non-renewable resources, such as petroleum-based fertilizers (Wang et al., 2020). Suitable substrates to produce organic fertilizers include

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lemon prunings and cereal straw and bran, and urban, horticultural, olive, palm, grape, cotton, and brewery wastes (Assandri et al., 2021a, b; Bianco et al., 2020; Budroni et al., 2020; Mtui, 2009; Saba et al., 2019).

In particular, there has been a great deal of interest in the use of lignocellulosic waste due to its abundance and renewability (Wang et al., 2020). Brewers' spent grain (BSG) is the most abundant by-product of brewing. It consists of the outer shells of barley kernels (i.e., glume, pericarp, integuments), and is particularly rich in cellulose, hemicellulose, lignin, and nitrogen substances (Mada, 2020). As a protein concentrate and fiber source, BSG is one of the most valued agro-industrial by-products for animal feed. Its use can reduce animal diet costs and the need to use grain in animal feed that is edible for humans. It also increases the bioactive compounds in animal products.

Brewers' spent grain has been added directly to the soil to increase organic content, stability of aggregates, and water retention, and to favor nutrient mineralization (Aboukila et al., 2018). However, like many other wastes, fresh BSG is microbiologically unstable and perishable, and its correct disposal and use require stabilizing procedures, such as composting and vermicomposting.

In composting, indigenous microorganisms transform organic matter through mesophilic and thermophilic phases. The latter phase, carried out at around 70 °C, is responsible for the sanitization of the composts, but severely reduces the microbial activity of the final product (Assandri et al., 2021a; Assandri et al., 2021b).

Vermicomposting is an eco-sustainable process in which the complex interactions between microorganisms and earthworms are the basis of fragmentation, bio-oxidation, and stabilization of organic wastes (Lazcano et al., 2008). In contrast to composting, vermicomposting is carried out at lower temperatures (25-40 °C), neutral pH, and higher humidity (70-90%) to allow earthworms' growth (Tognetti et al., 2005). In addition, these temperatures preserve the activity of mesophilic microorganisms fundamental for organic matter decomposition and soil fertility, such as Gammaproteobacteria, Actinobacteria, and Actinomycetes. Thus, vermicompost has a greater market acceptance and higher value than compost due to higher nutrient content and microbial activity (Tognetti et al., 2005). Many genera of Actinobacteria produce antibiotics, which inhibit or eliminate pathogenic bacteria such as *Salmonella* sp. and *E. coli*. However, the absence of a thermophilic phase makes it mandatory to guarantee the safety of the vermicompost by specific microbiological analyses.

In recent years vermicomposting has received much attention as an efficient and low-cost means for the processing of organic waste of various origins. Indeed, earthworms are highly adaptable to a broad range of environmental conditions, and they can consume organic materials with pH from 5 to 8, moisture content between 65% and 70%, and an initial C/N ratio of around 30. Many different lignocellulosic wastes can be converted into high-value humic-like substances through vermicomposting. These have included tree prunings, disposable paper cups, brewers' spent grains, crop residues, medicinal herbal residues, and leaf litter (Saba et al., 2019; Sharma & Garg, 2019; Singh et al., 2021). Contrary to animal wastes, crop and plant wastes are better suited to sub-mesophilic treatments as they are less subjected to contamination by human and animal pathogens.

However, not all organic waste lies within these chemical/ physical parameters. Therefore, additional pre-treatments are commonly required before vermicomposting, to make the organic waste more suitable for earthworm growth and activity. Indeed, some organic residues contain substances that are toxic for earthworms, such as acidic compounds, and can require pre-treatment.

To make lignocellulosic raw materials more palatable for earthworms, pre-treatments are usually carried out over about 3 weeks (Sharma & Garg, 2019). This pre-treatment provides sanitization of the waste, elimination of volatile gases (that are toxic for earthworms), reduction of high moisture content, and improvement in the initial C/N ratio to optimal values. Pre-treatment also promotes initial microbial degradation and softening of the waste (Sharma & Garg, 2019).

Pre-treatments with physical methods have also been proposed to reduce the spontaneous microbial load of wastes (Rahmati et al., 2020). In particular, drying of BSG at 45 °C for 48 h has been shown to make it suitable for direct vermicomposting (Saba et al., 2019). Also, inoculation of BSG with specific microbial taxa has been proposed, which can include aerobic and anaerobic cellulolytic bacteria of the genera *Cellulomonas*, *Pseudomonas*, and *Streptomyces* (Wei et al., 2019). Similarly, lignocellulolytic fungi such as white-, brown-, and soft-rot Basidiomycetes (Kucharska et al., 2018) have been used to reduce the resistance of the substrate to biodegradation and to improve the composting process.

However, the economic feasibility of pre-treatment methods that require high or low temperatures, or the need to add microbial starters to enhance the performance of composting and vermicomposting, is currently questioned due to the high associated costs.

Alternatively, to increase the sustainability of the process, the pre-treatment of wastes could rely on the proper exploitation of their naturally associated microflora. Indeed, the indigenous microbial communities of specific wastes are intrinsically diverse and well adapted to the substrate, and they can be composed of taxa with relevant metabolic traits for the agricultural sector (Oljira et al., 2018).

In this context, the microbiota of fresh BSG remains largely unknown, and only a few reports have described it in terms of broad microbial categories (i.e., aerobic mesophilic bacteria, yeasts and molds, strictly anaerobic bacteria, etc.) without providing any further details on the genera and species present (Robertson et al., 2010). Brewers' spent grain results from the brewing process that is characterized by high temperatures, addition of natural antimicrobials (e.g. hop isoα-acids), and a sub-acidic pH. Thus, no human pathogens have been found to survive in BSG after the brewing process (Bianco et al., 2020). However, BSG can support the growth of mycotoxigenic fungi, such as *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp., as these can already contaminate the raw materials (Bianco et al., 2018, 2019).

Within this frame of reference, this work aimed to evaluate, from a microbiological and chemical perspective, a low-input pre-treatment process designed to make BSG palatable for earthworms. This process was carried out without expensive thermal treatments or chemical and microbiological additives to fully exploit the microflora naturally associated with raw BSG. Finally, the mature vermicompost from pre-treated BSG was evaluated through microbiological and chemical analysis, as well as germination tests, to assess its suitability as a soil conditioner and fertilizer.

2. Materials and methods

2.1. Sampling and pre-treatment of brewers' spent grain

Brewers' spent grain was collected from four microbreweries (MB1, MB2, MB3, MB4) in Sardinia (Italy), as 15 kg from each microbrewery. These BSG samples were filtered, divided into sterile plastic bags, and keep at 4 °C until further processing. From these BSG samples, 500 g aliquots were placed into sterile plastic containers (23 × 15 × 3 cm) at 22 °C in a thermostatic room, with the humidity kept constant at ~70% by periodic turning and addition of sterile distilled water (every 3 days). Each BSG sample was pre-treated in triplicate containers. During this period, temperature, pH, and humidity were monitored daily by averaging three measurements taken at three different points (at the center, and at the two ends of the containers) at 2 cm from the top layer of BSG. Samples were taken in triplicate at 17-day intervals, as T0 (6 h after microbrewery production), T1 (17 days), T2 (34 days), and T3 (51 days). These samples were subjected to chemical, biochemical, and microbiological characterization. After 51 days of pre-treatment, determined by the stabilization of pH around the neutrality (pH 7), pre-treated BSG was further processed by vermicomposting.

2.2. Vermicomposting of pre-treated brewers' spent grain

Vermicomposting was carried out according to Saba et al. (2019). Briefly, plastic containers (bed) were filled with 500 g pre-treated BSG mixed with 500 g cow manure (1:1), to which 15 earthworms (*Eisenia fetida*, Savigny, 1826) were added. After completion of worm-cast production (30 days) and curing (30 days), triplicate vermicompost samples were analyzed for their chemical, biochemical, and microbiological characteristics.

2.3. Chemical and biochemical characterization of brewers' spent grain and vermicompost

Brewers' spent grain was analyzed as detailed by Correddu et al. (2019), as dry matter (DM) content (oven-drying, 105 °C for 24 h), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) (Ankom 220 fiber analyzer; Ankom Technology, Fairport, NY, USA), using the Association of Official Analytical Chemists (AOAC) method 973.18 (AOAC Official Method of Analysis, 1990), according to Robertson & Van Soest (1981). Crude protein (CP) content was determined using the Kjeldahl method (AOAC Official Method of Analysis, 2000; method 988.05), extract ether (EE) using the Soxhlet method (AOAC Official Method of Analysis, 2005; method 920.39), and ash using a muffle at 550 °C (AOAC Official Method of Analysis, 2000; method 942.05). NDF was measured using heat stable amylase, and is expressed as exclusive of residual ash, and ADL was measured using solubilization of cellulose with sulphuric acid. Non-fiber carbohydrates (NFC) were estimated using Eq. (1), as reported by Weiss (1999):

$$\mathrm{NFC}\ (\%\mathrm{DM}) = 100-(\mathrm{NDF}+\mathrm{CP}+\mathrm{ash}+\mathrm{EE}) \quad (1)$$

In BSG and vermicompost samples total carbon and total nitrogen contents were determined by combustion of dried samples in an elemental analyzer (CHN 628; Leco, St. Joseph, Michigan, USA) with the calibration references of oatmeal (502-276; Leco) and soils (Soil LCMR, 502-697; Soil calibration sample for CSN, 502-814; Soil LRM, 502-062; Leco). N-NH_4^+ and N-NO_3^- nitrogen were determined following the official method of soil chemical analysis, as the standard protocols defined by D.M. 13/09/1999, G.U. n° 248, 21/10/99.

Dehydrogenase activity was measured for 10 g samples after incubation at 37 °C for 24 h (Alef & Nannipieri, 1995), and is expressed as μg triphenyltetrazolium formed/g/h. Dissolved organic carbon (DOC) was quantified after 24 h agitation of a 1:10 (*w*/*v*) sample in suspension in deionized water. The liquid phase was then filtered off, and its absorbance at 254 nm was determined. The mycotoxins deoxynivalenol, T-2, HT-2, fumonisins, aflatoxin, and ochratoxin A, were quantified using commercial ELISA kits (Bio-Shield: fumonisin, 0-6 ppm; ochratoxin, 0-40 ppb; T-2/ HT-2, 0-500 ppb; total ES, 0-10 ppb; deoxynivalenol M.E., 0-5 ppm; Prognosis-Biotech, Or-Sell, Modena, Italy).

2.4. Culture-dependent microbiological analysis of brewers' spent grain and vermicompost

The main microbiological groups of fresh BSG samples (within 6 h of collection) and the vermicompost were determined to assess the compliance with legal requirements (Legislative Decree no. 75, 2010). In particular, the occurrence of pathogenic and nonpathogenic fungi, yeast, mesophilic bacteria, lactic bacteria, enterococci, enterobacteria, Actinomycetes, *Pseudomonas* spp., and aerobic and anaerobic spore-forming bacteria was evaluated according to Grantina-Ievina et al. (2013). Enumeration of *Escherichia coli* was according to ISO 16649-2 (2001). Incidence of *Salmonella* spp. was determined as described in ISO 6579 (2002).

2.5. Culture-independent microbiological analysis of brewers' spent grain and vermicompost

Total DNA from BSG sampled during the pre-treatment, and from the mature vermicompost, was extracted according to Ahmed et al. (2009). DNA concentration, integrity, and purity were assessed by spectrophotometric analysis (LvisPLATE SpectroSTAR Nano; BMG Labtech, Ortenberg, Germany) and using 1.5% agarose gel electrophoresis. The 16S rRNA (for bacteria) and internal transcribed spacer (ITS) rRNA (for fungi) genes were amplified from 25 ng DNA in a final volume of 50 μL, using 0.5 μM of each primer (Table S1), 2.5 mM $MgCl_2$, 0.5 mM dNTPs, and 1 U Taq DNA polymerase (Eurx). The specificity of the PCR reactions was confirmed by electrophoresis on 1.5% agarose gels. The amplified DNA fragments were analyzed by denaturing gradient gel electrophoresis (DGGE; DCode Universal Mutation Detection System; BioRad, Carlsbad, CA, USA). DGGE was carried out by loading 200 ng PCR products directly onto polyacrylamide gels under different denaturing conditions, as polyacrylamide 40% to 60% denaturing gradient for bacteria, and 30% to 60% denaturing gradient for fungi (where 100% is considered as 7 M urea, 40% deionized formamide). The PCR products were separated by electrophoresis in TAE buffer, as reported by Correddu et al. (2019). The gels were stained in TE buffer (pH 8) containing SYBR safe (Life Technology, Hercules, CA, USA), with the images acquired (ChemiDoc XRS system; Bio-Rad, Carlsbad, CA, US). The most intense PCR amplicons were excised from the DGGE gels and 1 μL of solution was used as the DNA template for amplification of the 357f and 519r regions for bacteria, and the ITS1f and ITS2 regions for fungi (Table S1). The specificity of the PCR products was assessed by electrophoresis on 1.5% agarose gels stained with 0.001% SyberSafe (Thermo Fisher Scientific, USA). The PCR products were sequenced at an external facility (Macrogen, Amsterdam, The Netherlands), and the sequences obtained were identified by BLAST analysis against the NCBI nucleotide sequence database, and using the Classifier and Sequence Match programs of Ribosomal Database Project II (http://www.rdp.cme.msu.edu).

2.6. Germination index of vermicompost

Germination tests were carried out using cress (*Lepidium sativum*, L.) seeds, according to the method described by Zucconi (1981). After 3 days of incubation, the proportions (%) of germinated seeds and the root lengths were recorded. The germination index (GI) was calculated using the mean value from three biological replicates, as given by Eq. (2):

$$\mathrm{GI}\ (\%) = [(\mathrm{Gt}\%)/(\mathrm{Gc}\%)] \times [\mathrm{Lt}/\mathrm{Lc}] \times 100 \quad (2)$$

where Gt% and Gc% are percentages of germination for treated and the control samples, respectively, and Lt and Lc are mean root lengths for treated and control samples, respectively.

Phytotoxicity tests were carried out with the vermicompost extracts diluted at 75%, 50%, and 30% in sterile distilled water.

2.7. Data analysis

The DGGE images were acquired and analyzed using the Infoquest FP V 4.5 software (Bio-Rad Laboratories, Hercules, CA, USA). The binary matrix (presence/absence of DGGE bands) constructed using the *Band matching* function of the PAST software (version 4.01, https://folk.uio.no/ohammer/past/) was converted into a distance matrix (Bray-Curtis method). The intensity of every single band (i.e., peak density) was used to derive quantitative values for subsequent analysis. The number of OTUs (bands), the Shannon–Wiener index, and the Simpson (or Dominance) index were calculated using the PAST software. To evaluate the effects of the sampling period (T0, T1, T2, T3) and the variation in the biodiversity indices, analysis of variance (ANOVA) followed by

Table 1
Chemical analysis of the BSG samples during the pre-treatment at T0 (6 h after microbrewery production), T1 (17 days), T2 (34 days), and T3 (51 days).

Parameter	Units	Sampling time			
		T0	T1	T2	T3
Temperature	°C	7.00 ± 0.00	19.50 ± 1.29	31.25 ± 3.30	22.50 ± 1.73
pH	–	5.38 ± 0.51	6.07 ± 0.72	8.00 ± 0.71	7.33 ± 0.24
Carbon/nitrogen	–	17.75 ± 4.11	15.00 ± 2.45	11.75 ± 0.96	14.25 ± 0.50
Total carbon	g/kg	603.08 ± 33.43	615.50 ± 26.53	600.23 ± 33.36	601.80 ± 19.45
Total nitrogen	g/kg	34.28 ± 6.60	40.58 ± 6.46	49.38 ± 3.85	43.43 ± 1.15
N-NH_4^+ nitrogen	g/kg	0.08 ± 0.04	1.22 ± 0.77	5.40 ± 3.87	9.00 ± 3.41
N-NO_3^- nitrogen	mg/kg	6.18 ± 4.75	10.28 ± 5.94	14.5 ± 11.46	39.70 ± 40.32
Dehydrogenase	μg/TPF	169.35 ± 115.25	393.33 ± 390.39	nd	663.03 ± 12.92

Data are means ± standard deviation across the four breweries.
nd, not detected; TPF, Triphenylformazan.

Tukey tests ($p < 0.05$) were carried out using the SPSS software, v19 (SPSS Inc., Chicago, IL, USA).

The influence on the composition of the microbial communities of the time of collection during the pre-treatment process was evaluated by permutational multivariate ANOVA (PERMANOVA) and visualized graphically by multivariate nonmetric multi-dimensional scaling analysis.

3. Results

3.1. Evolution of brewers' spent grain chemical and biochemical parameters during the pre-treatment

The four BSG samples differed considerably based on their microbreweries of origin (Table S2). The structural carbohydrates (i.e., NDF, including hemicellulose as NDF-ADF, cellulose as ADF-ADL, and lignin as ADL; Table S1) accounted for more than 50% of BSG dry matter composition for MB1, MB3, and MB4, and 38% for MB2. Crude protein content and NFC ranged from 18% to 25% and from 5.53% to 37.25%, respectively. Large differences were also seen for ADL content, with MB1 and MB2 ~ 40% lower than MB3 and MB4. Fresh BSG samples did not support the growth of earthworms due to pH values below 5 and above 8, after 1 and 5 days from the beginning of their uncontrolled spoilage (Figure Supplementary Fig. S1). Thus, a low-input pre-treatment of BSG was here carried out and monitored for 51 days according to a range of chemical and biochemical parameters (Table 1).

During the pre-treatment, the differences in the BSG samples across the breweries were greatly reduced. This resulted in standardization of the final product due to the almost identical dynamics of the evolution of the chemical parameters. The acidic pH values at T0 increased during the first 29 days (to T2) and eventually converged to values near neutrality at T3 (7.32 ± 0.24). The C/N ratio decreased from T0 to T2, and then stabilized at T3 (14.25 ± 0.50) across all of the samples. The changes in the C/N ratio were related to significant variations in the nitrogen contents of the samples, as the total carbon content of BSG showed no significant variations during pre-treatment. In particular, the total nitrogen content increased significantly from T0 to T2, and then decreased to T3. Instead, there was an increase in ammonium nitrogen content of the BSG throughout the pre-treatment period. Modification in these chemical parameters was accompanied by changes in the temperature of the BSG, which increased from T0 to T2 and then stabilized at 22 °C at T3.

The convergence of the different BSG samples to similar chemical profiles was also accompanied by a related evolution of the dehydrogenase (DHG) activity, which is a representative indicator of the oxidative activity of viable microbial populations. Indeed, the significant differences among the BSG samples in DHG activity during the first days of pre-treatment were almost completely reversed by T3, when all of the BSG samples showed the same activity.

3.2. Evolution of brewers' spent grain microbiota during the pre-treatment

From a microbiological perspective, all of the BSG samples at T0 were within the quality parameters, as there were no *Enterococci* spp., *Enterobacteria* spp., *Pseudomonas aeruginosa*, Coliforms, *E. coli*, *Salmonella* spp., and pathogenic fungi detected (Table S2). The BSG from brewery MB1 showed overall lower contamination, as fungi and yeast were not detected. According to the origins of the BSG, Lactobacilli were strongly represented, as they made up from 20% to 58% of the total bacterial community.

The evolution of the fungal and bacterial communities during the 51 days of pre-treatment was evaluated by the determination of the alpha-diversity indices from the DGGE analysis (Table 2). The richness (Chao 1) of the bacterial communities increased significantly from T0 to T3 ($p < 0.05$). This was accompanied by an increase in the overall biodiversity and the dominance of a few taxa, as shown by the Shannon and Simpson indices, respectively. The fungal communities were more stable, with no significant differences in the three alpha-diversity indices at any of the times considered. To better quantify the differences among the microbial communities during the pre-treatment (i.e., beta-diversity), a dissimilarity matrix was calculated from the DGGE bands using the Bray-Curtis distances, as visualized using nonmetric multidimensional scaling (Fig. 1).

Ordination analysis revealed that the BSG samples across the different times were more similar than those within the same time points, thus suggesting a lack of correlation among the composition of the bacterial and fungal communities and the times of sampling. This was also

Table 2
Alpha-diversity indices of the bacterial and fungal communities during the BSG pre-treatment at T0 (6 h after microbrewery production), T1 (17 days), T2 (34 days), and T3 (51 days).

Sampling	Bacterial community indices			Fungal community indices		
time	Chao1	Shannon	Simpson	Chao1	Shannon	Simpson
T0	20.50 ± 3.12^{a}	2.53 ± 0.24^{a}	0.89 ± 0.02^{a}	18.00 ± 9.83^{a}	2.38 ± 0.61^{a}	0.86 ± 0.07^{a}
T1	28.50 ± 4.80^{ab}	2.93 ± 0.33^{ab}	0.92 ± 0.04^{ab}	25.00 ± 11.27^{a}	2.53 ± 0.71^{a}	0.86 ± 0.12^{a}
T2	27.50 ± 6.66^{ab}	3.05 ± 0.14^{b}	0.94 ± 0.01^{b}	21.50 ± 10.60^{a}	2.33 ± 0.70^{a}	0.84 ± 0.12^{a}
T3	30.00 ± 5.48^{b}	3.03 ± 0.24^{b}	0.94 ± 0.02^{b}	22.75 ± 8.06^{a}	2.57 ± 0.41^{a}	0.89 ± 0.05^{a}

Data are means ± standard deviation, across the four breweries.
Means with different superscript letters are significantly different within columns as determined by ANOVA followed by Tukey HSD test ($p < 0.05$).

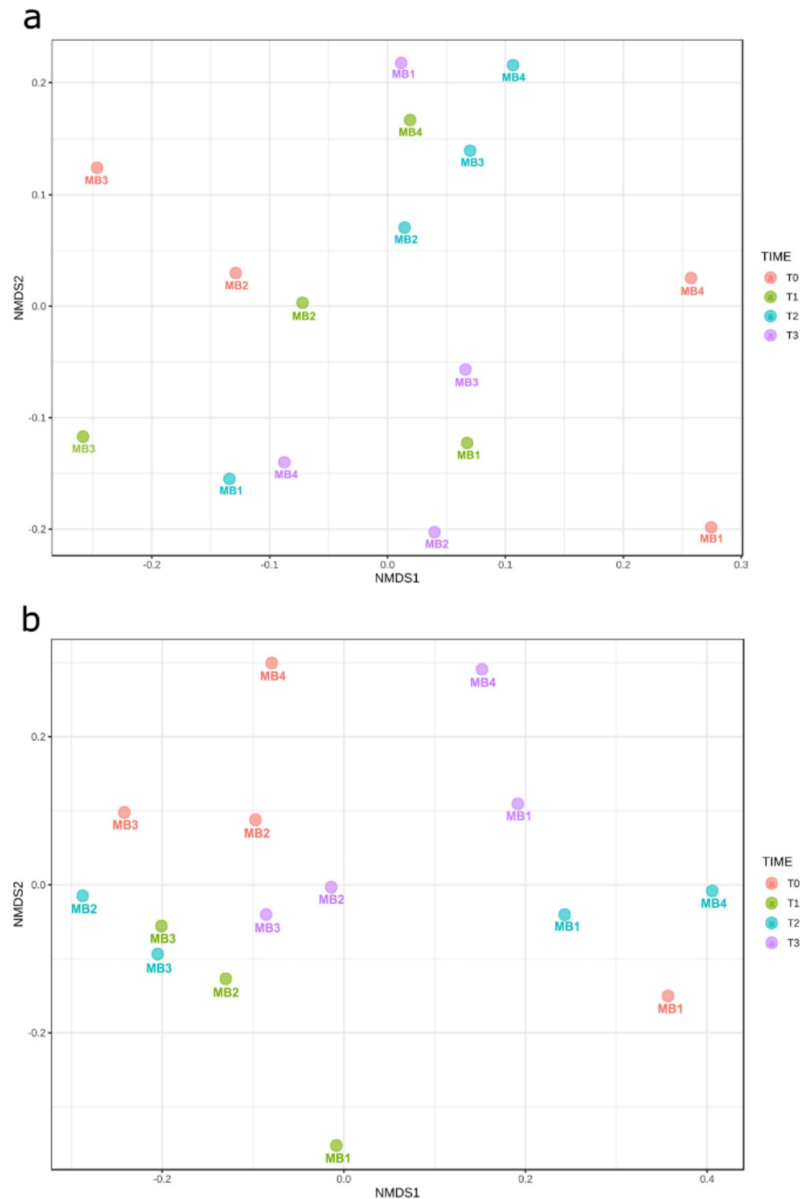

Fig. 1. Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distances for the bacterial (**a**) and fungal (**b**) communities.

confirmed by the lack of significance of the PERMANOVA (experimental factor = time of sampling) (bacteria, $p = 0.712$; fungi, $p = 0.318$). Similarly, the composition of the bacterial community was not associated with the origins (i.e., breweries) of the samples, as they were scattered randomly throughout the ordination space.

To identify the main microbial species involved in the pre-treatment process, 63 bands for the bacterial and fungal communities were extracted, with successful sequencing and identification of 37 DNA fragments from bacteria (190-200 bases) and 26 DNA fragments from fungi (200-300 bases). The relative abundance of the main bacterial and fungal taxa identified through the pre-treatment process is reported in Fig. 2.

3.3. Chemical characterization and microbiota of vermicompost

To assess the quality and maturity of the final vermicompost from pre-treated BSG, chemical, biochemical, and microbiological parameters were determined. These were complemented with the analysis of the pathogenic microorganisms and the index of germinability, as indicated by Italian Legislative Decree 75/2010 (Table 3).

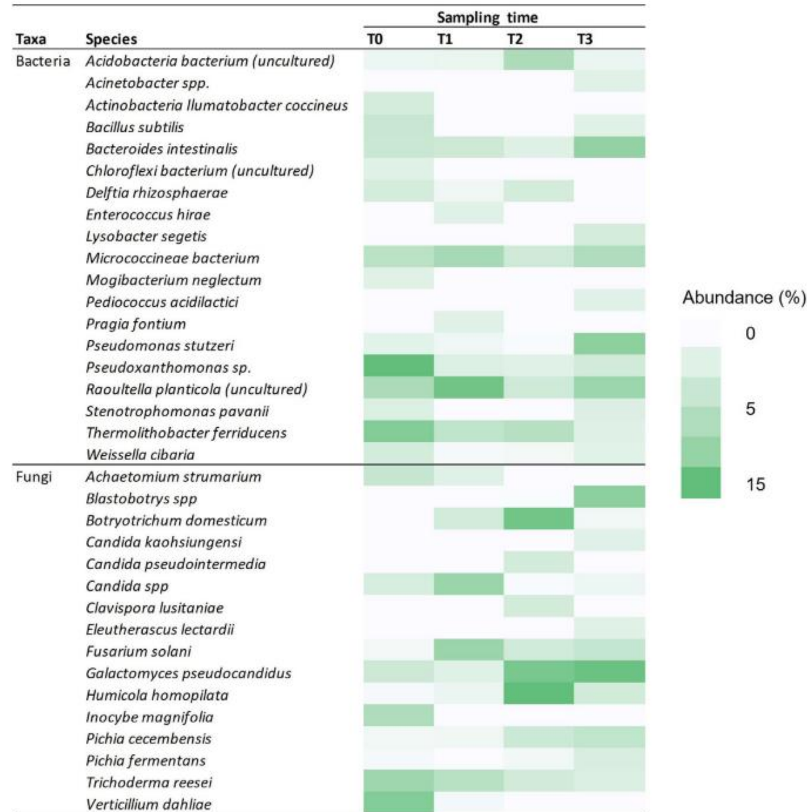

Fig. 2. Relative abundance of fungal and bacterial species identified during the BSG pre-treatment. The intensity of DGGE bands (i.e., peak density) was used to derive quantitative values. T0 (6 h after microbrewery production), T1 (17 days), T2 (34 days), and T3 (51 days).

The vermicompost obtained was not contaminated by mycotoxins. Ochratoxin A, fumonisins, deoxynivalenol, T-2, HT-2, and total aflatoxin were identified at concentrations lower than those recommended by the European Union for food products and animal feed (Bianco et al., 2018; Bianco et al., 2019). Similarly, *Enterococcus* spp., Enterobacteria, Coliforms, *Salmonella* spp., and pathogenic fungi were not detected in the vermicomposts. *Escherichia coli* was only found in a single sample at 60 CFU/g, which was well below the legal limit of 5000 CFU/g (Legislative Decree no. 75, 2010). To further characterize the microbial communities of the vermicompost, 45 DNA fragments from bacteria (190-200 bases) and 36 DNA fragments from fungi (200-300 bases) were extracted from the DGGE gels, sequenced, and classified (Fig. 3).

Culture-independent analyses showed that four of the 27 bacterial taxa identified were common between the BSG and mature vermicompost: *Weissella cibaria, Bacteroides intestinalis, Stenotrophomonas pavanii,* and *Bacillus subtilis*. Similarly, four of the fungal species identified were common between the BSG and mature vermicompost: *Trichoderma reesei, Pichia cecembensis, Verticillium dahliae,* and *Pichia fermentans*.

4. Discussion

Vermicomposting has been suggested as a valuable method to exploit BSG, which is an important by-product of the brewing industry (Saba et al., 2019). During vermicomposting of BSG, the decrease of the total organic carbon was accompanied by an increase in the total nitrogen, indicative of an advanced degree of organic matter stabilization. Vermicomposting also enhanced the amounts of organic components recalcitrant to microbial degradation, given the increases in humic-like substances (Saba et al., 2019). From a microbiological perspective, earthworm activity during vermicompost of BSG led to the enrichment in Firmicutes, Actinobacteria, and Betaproteobacteria involved in cellulose degradation as well as N-fixation, nitrate reduction, and ammonia assimilation (Budroni et al., 2020). Overall, the vermicompost from BSG represented an excellent substrate that could be safely used as a soil improver (Saba et al., 2019). However, the raw BSG had to be pre-treated before vermicomposting as it did not support the growth of earthworms. Saba et al. (2019) used a pre-processing method consisting of the oven-drying of BSG at 45 °C for 48 h. Given the high energy input required, the overall vermicomposting process resulted less attractive from a sustainability perspective. To overcome this limitation and to increase the quality of the final product, the suitability of a more sustainable pre-treatment method that relies on the exploitation of BSG naturally-associated microbiota was here carried out. The advantages of this pre-treatment, over various physical and chemical methods for BSG stabilization, are the potential production of useful microbial by-products, less formation of inhibitory substances (due to milder conditions), minimization of chemical applications, and energy input, and lower costs for waste disposal. In addition, the biological pretreatment of lignocellulosic wastes, such as BSG, is assumed to be the eco-friendliest technique, with mild environmental conditions and low operational costs (De Bhowmick et al., 2018).

Table 3
Chemical, biochemical, microbiological and quality parameters of the vermicompost from the pre-treated BSG.

Parameter/index	Parameter	Units	Mean ± SD
Chemical	Moisture	%	45.31 ± 0.41
	pH	–	7.08 ± 0.21
	Carbon/nitrogen	–	11.00 ± 1.15
	Organic carbon	g/kg	38.63 ± 0.56
	Total nitrogen	g/kg	32.8 ± 0.30
	N-NH_4^+ nitrogen	g/kg	0.02 ± 0.01
	N-NO_3^- nitrogen	g/kg	0.56 ± 0.53
	Dehydrogenase	μg/TPF	117.62 ± 53.38
	Dissolved organic carbon	mg/g	1.09 ± 0.37
Microbial counts	Total bacteria	CFU/g	1.19×10^7 ± 95.4
	Lactobacilli		2.14×10^6 ± 17.1
	Actinomyces		6.32×10^6 ± 50.6
	Aerobic sporigens		1.62×10^8 ± 130
	Anaerobic sporigens		1.00×10^8 ± 801
	Pesudomonas spp.		1.28×10^4 ± 5.13
	Total fungi		4.42×10^4 ± 3.98
	Total yeast		3.44×10^4 ± 3.09
Alpha diversity	Chao1 (bacteria)	–	44.25 ± 3.10
	Shannon (bacteria)	–	3.44 ± 0.10
	Simpson (bacteria)	–	0.96 ± 0.00
	Chao1 (fungi)	–	37.50 ± 5.20
	Shannon (fungi)	–	3.20 ± 0.09
	Simpson (fungi)	–	0.95 ± 0.00
Germination	75%*	%	87.63 ± 5.68
	50%*	%	66.93 ± 1.79
	30%*	%	24.53 ± 7.08

*, **%** vermicompost dilution; TPF, Triphenylformazan; CFU, colony-forming units.

4.1. Chemical dynamics during the pre-treatment

After the 51 days pre-treatment, a proper substrate for vermicomposting was here produced from the four BSG samples, given that the final pH and C/N ratio were suitable for the activity of the earthworm *E. fetida*. Interestingly, a similar period of time (45 days) was required for the pretreatment with fungi (*Bjerkandera adusta*) of lignocellulosic biomass before vermicomposting (Moran-Salazar et al., 2016).

The reduction in the C/N ratio from that of the raw BSG was related to the significant increase in the ammonium nitrogen content. The nitrogen kinetics during the pre-treatment can be attributed to the activity of the microbiota identified on the BSG samples, and in particular to the bacterial species involved in the nitrogen cycle. *Stenotrophomonas pavanii* can fix atmospheric nitrogen (N_2) into ammonia (NH_3), which is rapidly converted into ammonium (Ramos et al., 2011). In contrast, *Pseudomonas stutzeri* is a facultative anaerobic bacterium that favors the release of molecular nitrogen (N_2) through denitrification of nitrate (NO_3^-) through the reduction of organic matter (Lalucat et al., 2006). Given the low levels of nitrate and the high levels of ammonium nitrogen measured during the pre-treatment of BSG, the activity of the nitrogen-fixing bacteria would be predominant. Ammonia production is higher at high aeration rates (De Guardia et al., 2010), temperatures, and pH (Pagans et al., 2006). Accordingly, the higher production of ammonium nitrogen was measured in BSG at temperatures >22 °C and pH >7 (i.e., at T2, T3).

The correlation between ammonification and biodegradation of organic matter has been poorly studied, although peak ammonia emissions are usually seen when biological activity is at its maximum (De Guardia et al., 2010). Accordingly, DHG activity increased during the pre-treatment of BSG and reached its highest at T3. As the DHG enzymes in soil are not in a free form, this represents the activity of live intact cells (Alef & Nannipieri, 1995). Moreover, DHG levels serve as an indicator of microbiological redox systems and can be considered as a good and adequate measure of microbial oxidative activity (Garau et al., 2019). Thus, the high DHG activities measured for BSG at T3 underline an intense microbial activity, which is related to the decomposition of polymeric materials to provide simple and soluble compounds that can be metabolized easily.

This suggests that after this pre-treatment, BSG still requires further maturation (e.g., vermicomposting) to reach adequate stability for its use as a soil amendment. Therefore, pre-treated BSG was mixed with cow manure (1:1) and subjected to vermicomposting according to the protocol described by Saba et al., (2019). This study by Saba et al. (2019) is, to the best of our knowledge, the only vermicompost reported in the literature that was obtained from BSG. With respect to their study, the mature vermicompost here obtained had similar total nitrogen content and pH, while the C/N ratio measured in the present study was a little higher, and like that of organic and mature vermicompost obtained from animal manure (Table S3). Indeed, a C/N ratio of 11 falls within the legal limit required by Italian law for marketed vermicompost (Legislative Decree no. 75, 2010). These data, and the lower DHG activity in the vermicompost from biologically pre-treated BSG, indicated that the pre-treatment suggested here allows a final product to be obtained that is more stable than that reported by Saba et al. (2019).

The stabilization of DHG activity in the mature vermicompost can be attributed to the complete metabolization of the easily degradable organic substances (Garau et al., 2019). Thus, it appears that the microbiota of the BSG vermicompost, which has reached an internal equilibrium, would not significantly modify the dynamics of the microbial communities present in the soil when the vermicompost is used as an amendment. The stability and maturity of vermicompost from the pre-treated BSG are also confirmed by the low DOC. The DOC is a chemical indicator of the molecular transformation of dissolved organic matter that is suitable for the definition of the stabilization of vermicompost (Nigussie et al., 2017). An organic amendment with a high DOC can cause severe damage to crops, as it will continue to consume oxygen, which will hinder root respiration and lead to the production of phytotoxic compounds, such as SH_2. A DOC <10 g/kg indicates stability and maturity of the amendment (Mohanty et al., 2013), and a DOC <4 g/kg is considered safe for plant growth (Gómez-Brandón et al., 2008). Indeed, vermicompost from the pre-treated BSG does not show significant phytotoxicity against the *L. sativum* L. (cress) seeds, according to Regulation (EC) No (2003); Regulation (EU) (2019) and the Italian Legislative Decree n° 75/2010. In particular, the Annex 2 of Legislative Decree n° 75/2010 requires a germination index >60% for a dilution of 30% (30% extract, 70% water). The same minimum requirement is indicated by the Italian Composting ConsorAsd previtium.

4.2. Dynamics of microbial communities during BSG pre-treatment

As previously stated, the rationale of the pre-treatment method here described was to emphasize the activity of the microbiota that is naturally associated with BSG. Indeed, the mild temperature (<32 °C), as well as the high humidity (70%) during the pre-treatment, allows the preservation and the development of microbial species sensitive to high-input processes (i.e., oven-drying or freezing). Both fungal and bacterial communities were monitored during the pre-processing and in the mature vermicompost.

Fungi produce several extracellular enzymes and molecules that play key roles in the biodegradation and hydrolysis of polymeric substances such as cellulose, hemicellulose and lignin (Wagner et al., 2018). The degradation of lignin by fungi is due to the activity of peroxidases and laccases. In addition, fungi can contribute significantly to the bioremediation of contaminated soils (Ferrari et al., 2011). Among fungi, yeasts act as plant growth promoters and biological control agents against soil-borne plant pathogens (Cantrell et al., 2011; Miceli et al., 2011). In this study, an enrichment of "true yeast" belonging to Saccharomycetales (*Blastobotrys* spp., *Galactomyces pseudocandidus*, *Pichia fermentans*, *Pichia cecembensis*, *Candida* spp.) was observed during the pre-treatment of BSG (T2 and T3). *Galactomyces* exhibited significant antagonistic activity against the fungal plant pathogen *Glomerella cingulata* (Fu et al., 2016). *Pichia* spp. isolates were suggested as

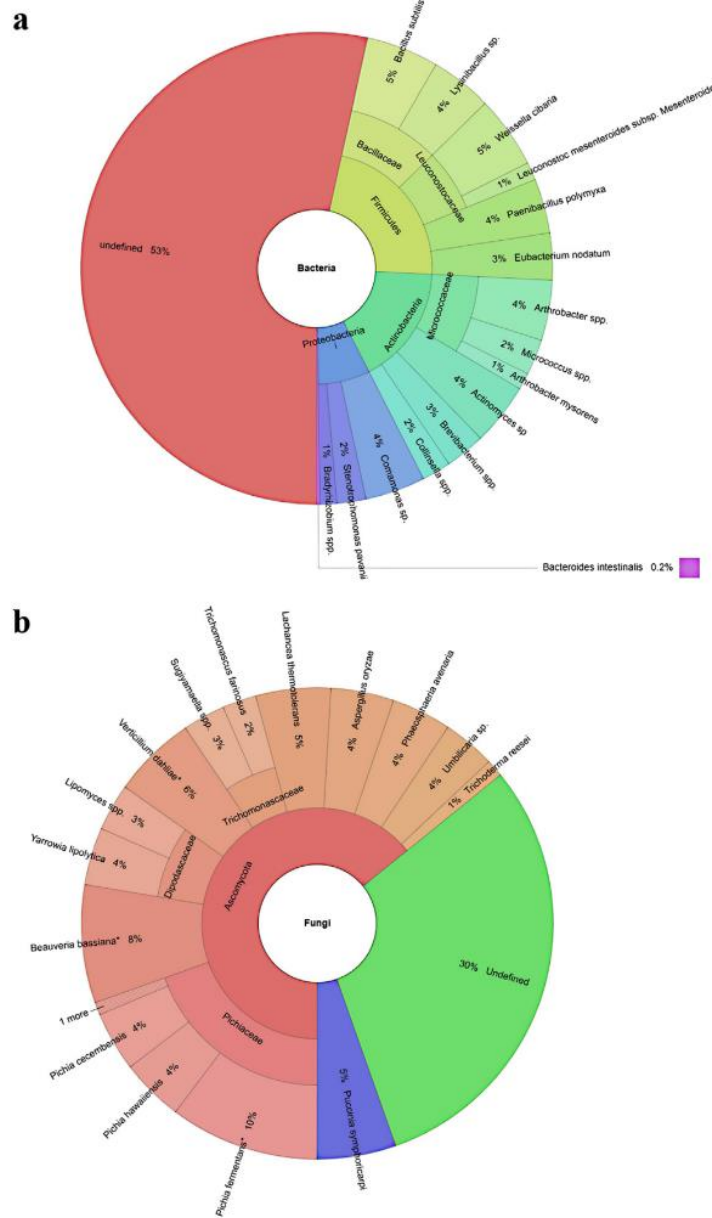

Fig. 3. Bacterial (**a**) and fungal (**b**) communities of mature vermicompost obtained from pre-treated BSG. The intensity of DGGE bands (i.e., peak density) was used to derive quantitative values.

promising candidates for biocontrol of phyllosphere fungi (Gross et al., 2018). *P. cecembensis* showed biocontrol activity against blue mold (*Penicillium expansum*) on apple fruit. Finally, yeast belonging to *Saccharomycetaceae*, might be a good choice for the production of indolacetic acid, the most common naturally occurring plant hormone of the auxin class (Giri & Sharma, 2020).

Similar to what was observed for Saccharomycetales, the pre-treatment of BSG resulted in the enrichment of Sordariales (*Botryotrichum domesticum and Humicola homopilata*) at T2 and T3. Sordariales are involved in the decomposition of decaying plant organic matter because of their high enzymatic activity (Marín-Guirao et al., 2019). Sordariales were found positively correlated to increased maize yields. Particularly, *Botryotrichum* was associated with increases in the fertility of soils with high organic matter (Marín-Guirao et al., 2019).

Conversely, the abundance of different fungal species highly represented in raw BSG, such as *Achetomium strumarium*, *Trichoderma reesei*,

and *Verticillium dahliae*, showed a reduction during the pre-treatment. *Verticillium dahliae* is a soil-borne pathogen with a wide host range, comprising over 300 woody and herbaceous plant species. On the contrary, both *Achetomium strumarium* and *Trichoderma reesei* have great biotechnological potential. The application of *Achetomium strumarium* was suggested for remediation processes of wastewater polluted by textile industries, as it can degrade synthetic azole dyes (Bankole et al., 2017). The use of *Trichoderma* spp. as a biofertilizer or a bioremediation and biocontrol agent is well known (Bhandari et al., 2021). In particular, the use of *Trichoderma reesei* in agriculture has increased exponentially in recent years, as it is considered an efficient biotechnological factory for the production of second-generation biofuel from lignocellulosic biomass (Hinterdobler et al., 2021). Zhang et al. (2021) studied the crucial role of *T. reesei* in humic substance formation and production of lignocellulosic crop residues. *T. reesei* promoted the humification process of corn straw and can contribute to the accumulation and occurrence of stable soil organic matter (Zhang et al., 2021). *Trichoderma*, and *Fusarium* genera encompass the main aerobic fungi identified as lignin degraders (Sindhu et al., 2016). Thus, the identification of these fungal genera suggests active degradation of the most stable polymers during the first stages of BSG pre-treatment.

As with fungi, the pre-treatment here described favored the growth of some bacterial species (i.e., *Acidobacteria bacterium, Bacteroides intestinalis, Pseudomonas stutzeri, Pediococcus acidilactici, Weissella cibaria*) while decreased the occurrence of others (*Actinobacteria llumatobacter, Bacillus* spp., *Chloroflexi bacterium, Enterococcus hirae, Pseudoxanthomonas* spp., *Thermolithobacter ferrireducens*, etc.).

Thermolithobacter ferrireducens, identified at higher population during the first stages of the pre-treatment, has the highest iron reduction rate per cell and is thus considered of great interest for the functioning of soil and bioremediation. *Pseudomonas stutzeri* can fix nitrogen, increase metal bioavailability, and degrade compounds such as hydrocarbons and alkanes (Lalucat et al., 2006). It is known that many lactic acid bacteria (LAB) can grow in beer. Thus, *Weissella cibaria* and *Pediococcus acidilactici* could derive directly from the breweries where the BSG samples were produced. LAB have complex nutritional requirements and usually inhabit nutrient-rich environments. However, LAB plays also important role in soil due to the production of antibiotic compounds able to limit the growth of target species, thus shaping the overall soil microflora. For instance, soil isolates of *Enterococcus, Pediococcus*, and *Weissella* spp., produced bacteriocin-like substances and showed antimicrobial activity against Gram-positive bacteria(Chen et al., 2005).

Acidobacteria and Actinobacteria are both involved in organic matter turnover and carbon cycling as they can utilize different carbon sources, ranging from simple sugars to more complex substrates, such as hemicellulose, cellulose, and chitin (Fließbach et al., 2007; Kielak et al., 2016). The abundance of these bacterial phyla is dependent on soil chemical characteristics. Particularly, nitrate had a positive correlation to Acidobacteria and a negative correlation to Actinobacteria (Li et al., 2020). Thus, it is conceivable that the increasing concentrations of nitrate during BSG pre-treatment are responsible for the decline in Actinobacteria and the increase in Acidobacteria populations. The dominance of *Pseudoxanthomonas* spp. and *Bacillus* during the first stages of BSG pre-treatment could have resulted in the degradation of complex organic compounds, thus increasing the concentration of simpler and easily metabolized compounds (Wang et al., 2010; Wang et al., 2011). This could have influenced the occurrence of Chloroflexi, which are less competitive in a rich and diverse community but thrive in stressed environments (Li et al., 2020). On the contrary, the increasing concentrations of nutrients could be related to the dominance of Bacteroidetes during the last stages of BSG pre-treatment. Particularly, Bacteroidetes can colonize many different habitats such as soil, activated sludge, decaying plant material, compost, dairy products, and diseased animals (Thomas et al., 2011).

4.3. Microbiota of the mature vermicompost

After the pre-treatment, BSG was mixed with cow manure and subjected to vermicomposting. Thus, the fungal and bacterial communities in the mature vermicompost are dependent not only on the microbiota of pre-treated BSG but also on that of the cow manure. In addition, earthworms' activity strongly affects the microflora of the vermicompost. Thus, the evaluation of the microbiota in the mature vermicompost can give further insights about the microbiological quality of the vermicompost and the influence of the starting materials in determining the microbial composition of the final product.

The fungal community identified in the mature vermicompost was dominated by Ascomycota, with only one species of Basidiomycota, *Puccinia symphoricarpi*. This agrees with Huang et al. (2014), who identified Ascomycota and Basidiomycota as the most common fungal phyla in the vermicompost from fresh fruit and vegetable wastes. Similarly, Anastasi et al. (2005) reported that the occurrence of Ascomycetes in vermicompost from plant and animal wastes was twice that in the compost obtained from the same wastes. The main fungal species identified were *Pichia fermentans, Beauveria bassiana, Puccinia symphoricarpi, Lachancea thermotolerans*, and *Verticillium dahliae*, each accounting for >5% of the total fungal community. *Pichia fermentans* and *Verticillium dahliae* were already identified in pre-treated BSG. Similarly, it is conceivable that the occurrence of *Pichia cecembensis* (4%) and *Trichoderma reesei* (1%) in mature vermicompost are related to their presence in BSG. Other fungal species were not identified in BSG. *Beauveria bassiana* is well-known as an entomopathogenic and plant-growth-promoting fungus (Mantzoukas et al., 2021). *Lachancea thermotolerans* is also a plant-growth-promoting yeast as it promoted seedling development of *Nicotiana benthamiana* (Fernandez-San Millan et al., 2020). *Puccinia symphoricarpi* is a phytopathogen that seriously threatens wheat production by causing leaf, stem, and stripe rust diseases. *Yarrowia lipolytica* (accounting for 4% of the fungal community in the vermicompost) can grow on different food wastes as it can degrade hydrocarbons, oil, diesel, and lignocellulosic raw materials (Madzak, 2021). *Y. lipolytica* is also able to grow in media with extremely high (up to 10.0) and low (3.0) pH values (Madzak, 2021). Together with other phylamentous fungi, *Aspergillus oryzae* (4% of the fungal community in the vermicompost) is a promising source of protein and carbohydrate for the food and feed industries, as it can grow on food wastes (Serba et al., 2020). Interestingly, BSG treated directly with *A. oryzae* spores was proposed as protein enriched animal feed (Bekatorou et al., 2005). Species of the lichen genera *Umbilicaria* (4%) were able to grow in extremely harsh environments, particularly in regions of higher latitudes or altitudes. The pre-treatment of BSG did not result in the development of mycotoxigenic fungal species, as all of the mycotoxins analyzed were below the detection limits.

At the phylum level, the bacteria identified in mature vermicompost from the pre-treated BSG belonged to Actinobacteria, Proteobacteria, and Firmicutes, which is in agreement with the findings of Srivastava et al. (2021). Actinobacteria and Firmicutes were also identified among the dominant phyla in the vermicompost from medicinal herbal residues (ADL, 8%/14% soluble solids), and together with Proteobacteria, Bacteroidetes, and Chloroflexi, these accounted for 83% to 93% of the bacterial community (Chen et al., 2018). Members of these phyla are generally involved in the degradation of stable organics, such as lignocellulose.

At the species level, the most represented were *Weissella cibaria* and *Bacillus subtilis*, which have been also detected in BSG, thus remarking the importance of the microbiota of raw material in shaping that of the final product. Finally, the vermicompost obtained from pre-treated BSG respected the limits indicated by Italian Legislative Decree 75/2010 regarding the absence of pathogenic bacterial species.

5. Conclusions

This study evaluated an eco-sustainable process for the pre-treatment of BSG before its vermicomposting. The procedure proposed here requires limited or no external inputs as energy or microbial starters, and it favors the stabilization of BSG for further processing. In addition, this pre-treatment of BSG resulted in accelerated decomposition of the waste, with reduced time required to obtain high-quality vermicompost. Indeed, the vermicompost showed good indicators of maturity and respected the law requirements related to pathogenic microorganisms and mycotoxins, as well as phytotoxicity. Finally, the pre-treatment allowed the growth of BSG naturally-associated fungal and bacterial species that eventually shaped the microbiota of the mature vermicompost, thus increasing its biological quality.

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CRediT authorship contribution statement

Angela Bianco: Methodology, Formal analysis, Investigation, Resources, Writing – original draft. **Francesco Fancello:** Investigation, Formal analysis, Writing – original draft. **Matteo Garau:** Investigation, Formal analysis. **Mario Deroma:** Investigation. **Alberto S. Atzori:** Investigation. **Paola Castaldi:** Formal analysis. **Giacomo Zara:** Methodology, Formal analysis, Data curation, Writing – review & editing. **Marilena Budroni:** Methodology, Formal analysis, Writing – original draft, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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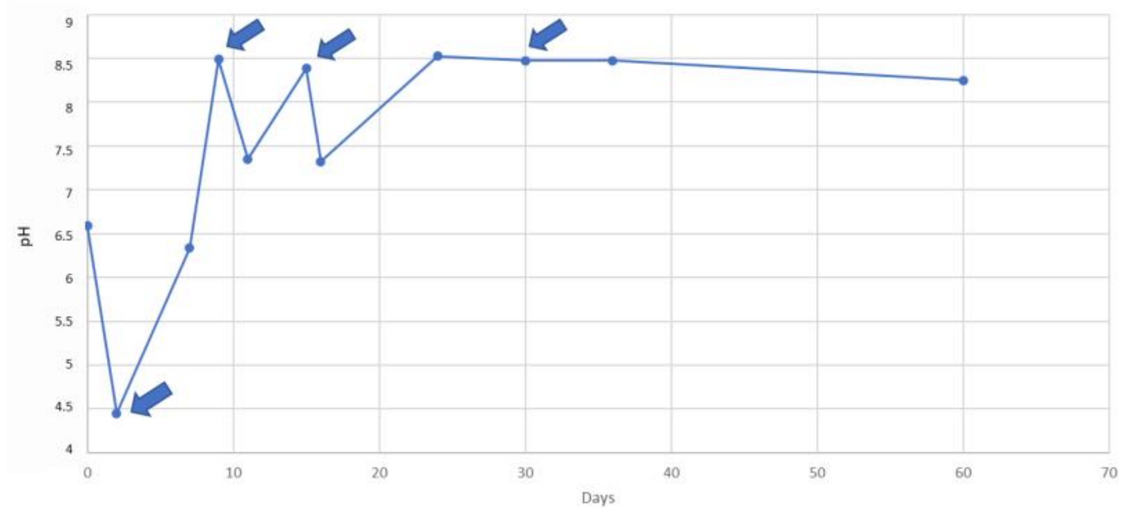
Supplementary data



Supplementary Figure S1. Evolution of pH values during uncontrolled BSG spoilage. Arrows indicate earthworms' addition. Within few hours after their addition, earthworms were unable to survive. Data are means of three independent replicates. Where not visible, standard deviations lie under symbols.

Supplementary Table S1. PCR primers used in this study.

Organism	Primer	Sequence (5'→3')	PCR product (bp)	Reference
Bacteria	GC-357f	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCC CCCGCCCCCCTACGGGAGGCAGCAG	191	Lane, 1991
	519r	ATTACCGCGGCKGCTGG		
	357f	CCTACGGGAGGCAGCAG		
Fungi	ITS1f	TCCGTAGGTGAACCTGCGG	700/900	Gardes and Bruns, 1993
	ITS4	TCCTCCGCTTATTGATATGC		
	ITS1f-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGGGGCTTGTCATTTAGAGGAAGTAA	200/300	Valášková and Baldrian, 2009
	ITS2	GCTGCGTTCTTCATCGATGC		White et al.,1990

Supplementary Table S2. Chemical and microbial characterization of BSG immediately after collection from the four microbreweries (MB1, MB2, MB3, MB4). Data are means ±standard deviation, from three independent samples.

Character	Measure	Units	Microbrewery sample			
			MB1	MB2	MB3	MB4
Chemical	Dry matter	%	25.67 ±0.61	30.25 ±1.59	19.21 ±1.31	19.87 ±0.31
	Ash	%	3.74 ±0.11	2.73 ±0.36	3.29 ±0.43	4.19 ±0.29
	Crude protein	%	20.52 ±0.61	17.83 ±0.54	25.42 ±0.56	22.08 ±0.39
	Neutral detergent fiber	%	54.62 ±0.56	38.47 ±0.95	58.49 ±0.35	53.47 ±0.77
	Acid detergent fiber	%	23.36 ±0.47	17.16 ±0.07	31.10 ±0.90	25.12 ±0.94
	Acid detergent lignin	%	8.00 ±1.04	8.59 ±0.38	15.70 ±0.61	13.41 ±0.23
	Ether extract	%	3.88 ±0.75	3.71 ±0.64	7.27 ±1.04	5.10 ±0.93
	Nonfibrous carbohydrates	%	17.24 ±1.83	37.25 ±1.42	5.53 ±1.03	15.16 ±1.80
Microbial	Total bacteria	Log_{10}	5.74 ±0.30	5.32 ±1.76	3.57 ±0.78	5.38 ±0.48
	Lactobacilli	(CFU/g)	4.80 ±0.60	5.05 ±1.11	2.88 ±1.11	5.14 ±0.78
	Actinomyces		nd	3.39 ±0.95	3.18 ±1.04	3.36 ±1.45
	Aerobic sporigens		4.22 ±1.08	4.35 ±1.18	2.99 ±0.48	2.91 ±0.60
	Total fungi		nd	2.90 ±0.85	2.33 ±1.00	2.83 ±0.28
	Total yeast		nd	2.70 ±1.81	2.36 ±0.48	2.94 ±0.01

nd, not detected

Supplementary Table S3. Comparison of the chemical characteristics of fresh and dried BSG as well as vermicomposts obtained in this work and in Saba et al. (2019).

		Saba et al., 2019		This work	
		Dry BSG	**vermicompost**	**Fresh BSG**	**vermicompost**
pH		5.9	7.2	5.38 ±0.51	7.08 ±0.21
Total nitrogen	g/kg	31.8 ±0.3	33.8 ± 0.1	34.28 ±6.60	32.8 ±0.30
Total carbon	g/kg	29.92±0.25	25.18 ±0.14	603.08 ±33.43	38.63 ±0.56
C/N		9.4	7.45	17.75 ±4.11	11.00 ±1.15
DHG	μg/TPF	39.513	588.738	169.35±115.25	117.62 ±53.38
Fungi-yeast	CFU/g	456.67 ±75	36.6 ±11.5	/	/
Total Bacteria	CFU/g	1010 ±26	1.43×10^{6} ±1.10	1.86×10^{5}± 17	11.9×10^{6} ±95.4
Coliforms	CFU/g	526.6 ±40	506.67 ±23.09	/	44200 ±3.98
Total fungi	CFU/g	/	/	419 ±4.7	/
Total yeast	CFU/g	/	/	400 ±17.25	34400 ±3.09
Lactobacilli	CFU/g	/	1.25×10^{4}±0.11	7.85×10^{4} ±9	2.14×10^{6} ±17.1
Actinomyces	CFU/g	/	/	1.56×10^{3} ±12	6.32×10^{6} ±50.6
Aerobic sporigens	CFU/g	/	/	7.85 x104 ±	162×10^{6} ±130
Anaerobic sporigens	CFU/g	/	/	/	100×10^{6} ±801
Pseudomonas spp.	CFU/g	/	/	/	12800 ±5.13

Chapter 4

4. Evaluation of brewers' spent grain as a low-cost substrate for production of beneficial microbial-consortia-based biofertilizer

4.1. Introduction

Recently, the direct and indirect use of beneficial microbial resources such as agricultural biofertilizer has emerged as a sustainable technology for producing environmentally friendly alternatives to synthetic fertilizers (Hellequin et al., 2020). Next generation biofertilizers are rapidly gaining attention as they are capable of improving crop productivity, microbial diversity and soil nutrients and indirectly promoting plant growth and productivity (du Jardin, 2015). They represent a promising and environmentally friendly innovation to meet the current needs of sustainable agriculture (Rocha et al., 2019). In particular, the use of biofertilizers, as an alternative to synthetic agropharmaceuticals, could be a sustainable strategy to improve soil biodiversity (Baldi et al., 2021; Megali et al., 2014; Raja, 2013; Diacono & Montemuro, 2010) and the nutritional status of plants and their growth (Vessey et al., 2003). A "biofertilizer" contains plant-growth-promoting microbes (PGPMs), which are soil microorganisms that can colonize the roots of plants and provide benefits to their hosts, through modulation of the production of phytohormones, increased availability of soil nutrients and resistance to pathogens (Lopes et al., 2021). Furthermore, PGPMs can mitigate biotic and abiotic stresses and increase plant productivity (Etesami et al., 2020). PGPMs include several genera of fungi and bacteria, such as *Azospirillum, Bacillus, Burkholderia, Enterobacter, Flavobacterium, Pseudomonas, Rhizobium, Frankia, Klebsiella, Clostridium, Trichoderma, Beauveria, Serratia* and *Streptomyces* (Gouda et al., 2018).

With increased environmental awareness, different biofertilizer formulations have been generated from the microbiota of foods and agricultural by-products and waste (Puglia et al., 2021). The production of microbial fertilizers includes a number of operations, including the mass production of the target microorganisms in fermentation systems. Usually, in the laboratory, under highly artificial conditions, microbial isolates are screened for their growth-promoting abilities, partly using model host plants. After selection, the microbial strains are tested in controlled greenhouse experiments. However, when applied in field conditions, the growth promoting effects could be highly variable and often lack consistency, which limits the applicability of the microbial bioinoculant

as a biofertilizer (Sessitsch et al., 2019). The current application of microbial inoculants (bioinoculant) in soil faces multiple challenges, where several aspects need to be considered, ranging from the design of an appropriate formulation to new concepts based on an understanding of the complexity and ecological behaviour of the natural microbiota (Sessitsch et al., 2019). Biochemical, molecular and physiological studies of plant-soil interactions have shown the existence of stress response mechanisms in plants, induced by microorganisms, which could activate Induced Systemic Tolerance (IST) and Induced Systemic Resistance (ISR), respectively in abiotic and biotic stress conditions. The mechanisms of action through which PGPMs influence plant metabolic responses and pathways can be classified as follows: hormonal modulation; balancing the oxidative state of cells; improvement of water use efficiency and physiological responses of photosynthesis; improvement of nutrient use efficiency (Ngalimat et al., 2021). Although the use of PGPMs has increased exponentially in recent years, their application is still hampered by various factors, including the lack of adequate production processes, the poor understanding of the fate of inoculants, and the interrelationships between biofertilizers, the soil/plant microbiome and the plants themselves (Mitter et al., 2016). Particularly, the inoculated microorganisms must compete for nutrients with the well-stabilized and acclimatized native microflora (Baldi et al., 2021). A better understanding of the interactions and production processes of PGPMs would further facilitate their use.

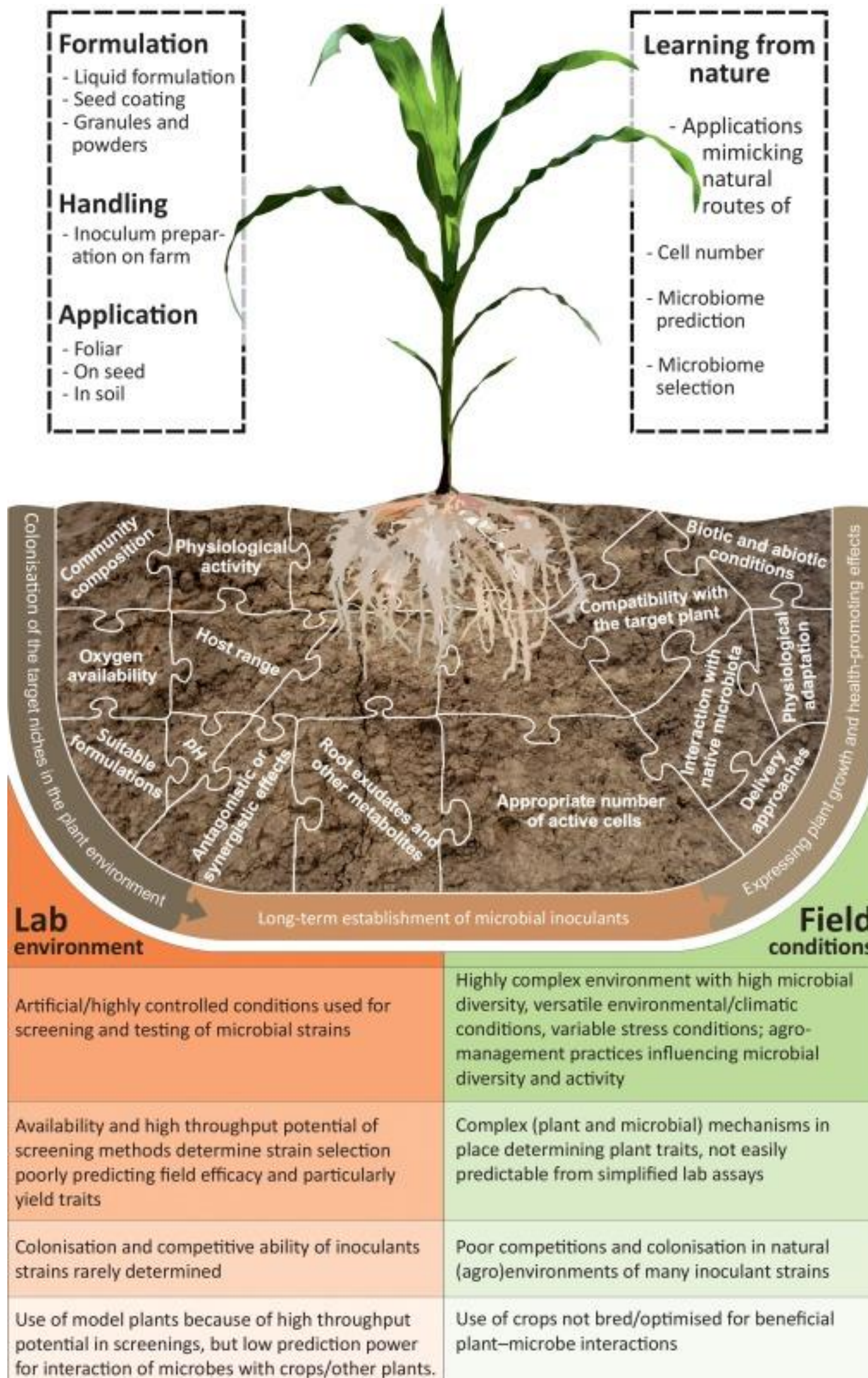


Figure 1: Challenges of microbial inoculation (taken from Sessitsch et al., 2019).

Low-cost materials such as agricultural waste have been used as carbon and nitrogen sources for microbial cell cultures to reduce production-line costs (Elsallam et al.,2021). Recently, many studies have focused on the development and commercialization of microbial bioinoculants based on agricultural wastes (Arumugam et al., 2021). Indeed, the agro-industrial waste recycling process is now accepted as an important tool for the development of sustainable agriculture (Tengerdy and Szakacs 2003). In recent years, there has been a growing trend towards more efficient use of agro-industrial residues based on processes that use solid-state fermentation (SSF).

Solid state fermentation is a process in which microorganisms are grown on solid materials without the presence of free water (Cannel and Moo-Young 1980). SSF includes many bioprocesses, such as bioremediation, biodegradation of hazardous waste, biodetoxification of agro-industrial waste, biotransformation of crops and crop residues for nutritional enrichment, and large-scale production of secondary metabolites, enzymes, antibiotics, organic acids, biopesticides, biosurfactants, biofuels and aromatic compounds. SSF also includes microbial inoculants as biofertilizer production (Vandenberghe et al., 2020; Vassilev et al., 2020; Holker and Lenz 2005; Hölker et al. 2004; Mitchell et al. 2002; Pandey 2003; Tengerby and Szakacs 2003). Solid waste materials act as vectors for microorganisms and spores, and therefore it should not be necessary to develop a sophisticated formulation process (Vassilev et al., 2009, 2015). This is an emerging research field, and a huge number of mixed microbial combinations under SSF conditions can be developed, which could lead to new types of biofertilizers.

As previously indicated, a fundamental advantage of BSG is that it is constantly available in large quantities and at little or no cost. The results obtained previously have shown that the lignocellulosic components of BSG are used by useful microorganisms such as *Trichoderma*, *Bacillus*, and *Pseudomonas* (Bianco et al., 2021). Although the use of microbial inoculants has increased exponentially in recent years, their application is still hampered by adequate, inexpensive, and environmentally sustainable processes.

In this context, the aim of this study was to evaluate the possibility of using raw BSG as a substrate for the growth of PGPMs through SSF by analyzing the changes in the chemical, biochemical and microbial characteristics of the inoculated BSG in respect to raw BSG.

4.2 Material and methods

4.2.1. Experimental set-up

A total of 30 kg BSG was sampled from a Sardinian brewery. Aliquots of 1.5 kg freshly BSG were placed in plastic containers (measuring 25x25x5 cm) at 22 °C in a thermostatic room, with constant humidity at ~70% by periodic turning and addition of sterile distilled water (every 5 days) for 90 days.

Two treatments were set up: raw BSG (B) and BSG inoculated (BM) with a suspension of 3 g in 100 ml sterile water of Bioinoculant M. BM is a commercially available microbial inoculum, wettable powder, for 7.5% it consists of 10.2×10^7 CFU g^{-1}plant growth promoting microorganisms (PGPMs). Each BSG sample was treated in triplicate containers.

During 90 days, pH and humidity were monitored daily by averaging three measurements taken at three different points (at the center, and at the two ends of the containers) at 2 cm from the top of the BSG.

Samples for the chemical, biochemical and microbiological analyses were collected every 7 days from both B and BM over the first month, and for the remaining 2 months, every 15 days, thus obtaining eight sampling points for each experiment, corresponding to t7 (7 days), t14 (14 days), t21 (21 days), t28 (28 days), t42 (42 days), t56 (56 days), t70 (70 days), and t90 (90 days).

4.2.2. Chemical and biochemical characterization of brewers' spent grain

Brewers' spent grain was analyzed within 6 h of production (BSG_{T0}) and at the end of the experiment: BSG_{T90} and BM_{T90} For raw and inoculated BSG, respectively. the fibrous fractions (contents of ash and crude protein [CP]) was determined according to AOAC (2000), neutral detergent fiber [NDF], acidic detergent fiber [ADF] and acidic detergent lignin [ADL] were determined according to Robertson and Van Soest (1981) and Van Soest et al. (1991). The concentration of non-fibrous carbohydrate (NFC) was calculated as [100 - (NDF - NDIP) - CP - EE - ash]. The total carbon content (TC) and the total nitrogen content (TN) were determined by burning dried samples using an elemental analyzer (CHN 628; Leco, St. Joseph, Michigan, USA), with

references for the calibration of the flour d 'oats (Leco, 502-276) and soils (Soil LCMR Leco, 502-697; Soil Calibration sample for CSN Leco, 502-814; Soil LRM Leco, 502-062). N-NH_4^+ and N-NO_3^- were determined according to the official method of chemical soil analysis of the standard protocols defined by D.M. 13/09/1999, Official Gazette n◦248, 21/10/99.

The pH and humidity were measured according to the respective methods ISO 10390/1995 and ISO 11465/1993. Dehydrogenase activity (DHG) was determined by quantifying the triphenilformazan formed in samples treated with triphenyltetrazolium chloride and incubated at 37 °C for 24 h as described by Alef and Nannipieri (1995). The urease (URE) activity was determined as ammonia released in soil samples treated with urea and incubated for 2 h at 37 °C, and the β-glucosidase (GLU) activity was quantified as p-nitrophenol released in the soil samples with p-nitrophenyl glucoside added and incubated for 1 h at 37 °C (Alef and Nannipieri 1995). To determine the activity of phosphomonoesterases (acid and alkaline phosphatases), the activities of acid phosphatase (ACP) and alkaline phosphatase (ALP) were measured according to the method described by Alef et al. (1998).

The dissolved organic carbon (DOC) was determined and quantified by UV absorbance (254 nm) of filtered (45 µm) sample suspensions, as previously described (Brandstetter et al., 1996).

4.2.3. Microbiological characterization of brewers' spent grain

Viable, culture-dependent counts were determined following the protocol described in Bianco et al. (2021). Characterization of the microbial community structure was performed using a next-generation sequencing approach with the Illumina Miseq technology. The DNA of each sample was extracted using GeneMATRIX kits (EURx Molecular Biology Products, Gdańsk, Poland). The composition of the microbiota was determined by sequencing at the BaseClear BV laboratory (Leiden, The Netherlands) of the V3-V4 region (bacteria) and the 5.8S-ITS1 region (fungi). The demultiplexed FASTQ files were analyzed with the QIIME 2 bioinformatics suite (Quantitative Insights into Microbial Ecology; version 2021.4) following the methods and protocols previously described in Budroni et al. (2020).

4.2.4 Statistical analyses

Alpha-diversity was estimated with the Shannon's index. Dissimilarity matrix for beta diversity analysis was calculated with the Bray-Curtis distance and visualized using non-metric multidimensional scaling (NMDS). The significance of the differences in the chemical, biochemical and microbial characteristics of the treatments was assessed by using ANOVA followed by Tukey HSD post-hoc test (FDR adjusted p.value <0.05). All calculations were performed by using the R statistical software (ver 4.0.1).

4.3 Results

4.3.1. Chemical and biochemical dynamics during solid state fermentation of BSG.

BSG samples were analyzed within 6 h from the production (BSG_{TO}) and after 90 days in the two treatments: uninoculated (BSG_{T90}) and inoculated (BM_{T90}) BSG (Figures 2 and 3).

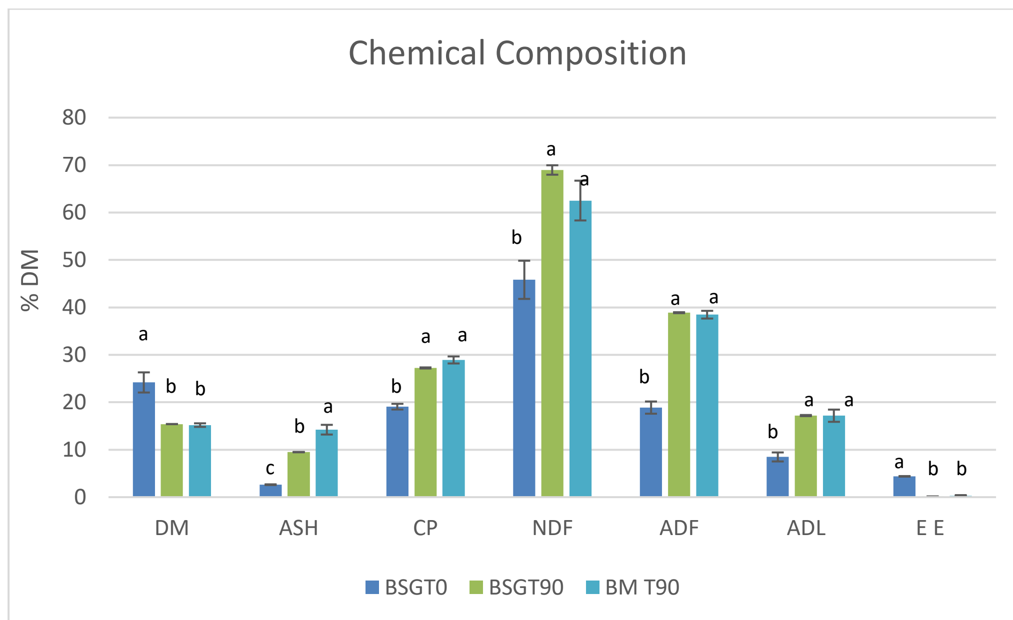


Figure 2: Results of the chemical analysis: BSG_{T0} (start BSG), B_{T90} (uninoculated BSG, end of treatment), BM_{T90} (inoculated BSG, end of treatment). DM, dry matter at 105 °C; PC, crude protein; NDF, neutral detergent fiber (hemicellulose); ADF, acid detergent fiber (cellulose); ADL, acid detergent lignin (lignin); EE, lipids. Different letters indicate statistical differences ($p < 0.05$) as determined by ANOVA followed by Tukey-HSD test.

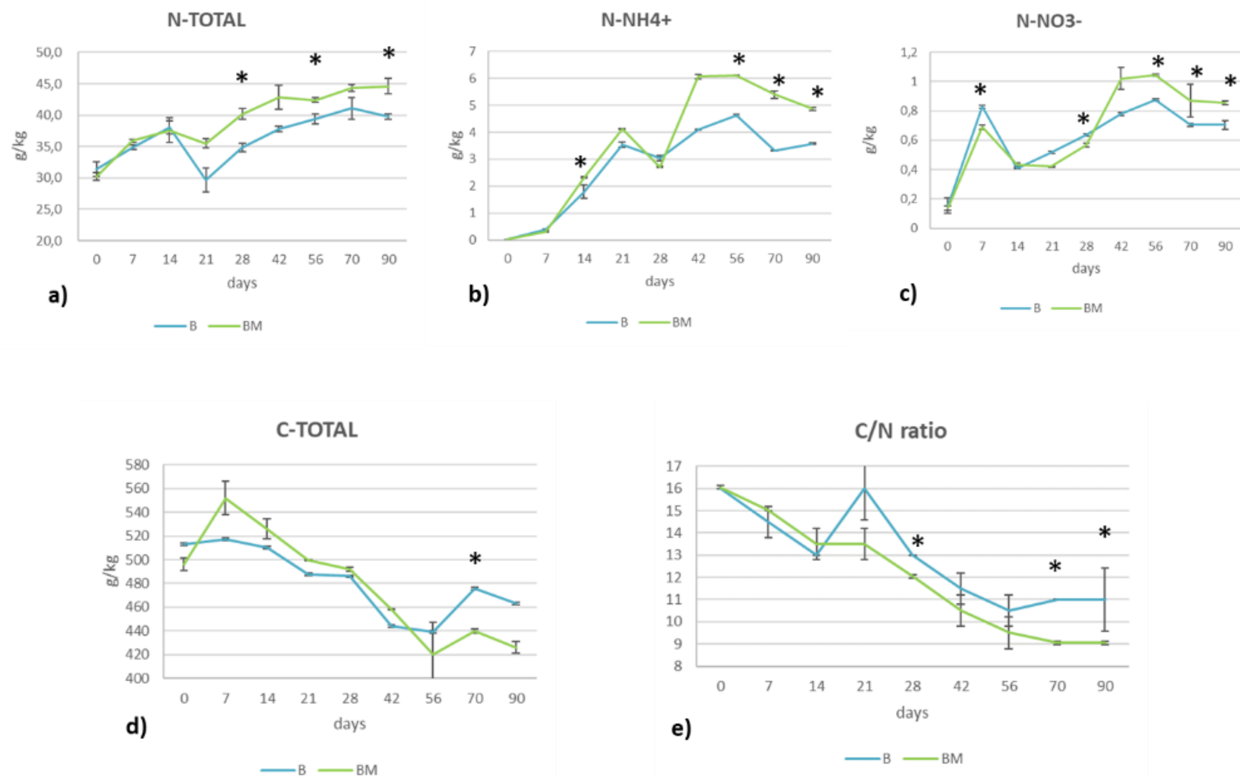


Figure 3: Chemical analysis of the B and BM samples during the 90 days of treatment. **a)** N_total, total nitrogen; **b)** N-$NH4^{+}$, ammonium nitrogen; **c)** N-NO_3^{-}, nitrate nitrogen; **d)** C_total, total carbon; **e)** C/N, carbon: nitrogen ratio. *, p< 0.05.

In both of the treated samples, the ash and the fiber contents (NDF, ADF, ADL) increased during the 90 days of treatments. Conversely, TC and C/N ratio (figure 3d, 3e) were reduced. The lipid content (EE) was reduced from 4% in BSG_{T0} to 0.11% and 0.30% in B_{T90} and BM_{T90}, respectively.

Overall, total N increased during the treatment period, with a marked decrease after 21 days, followed by a gradual increase and stabilization for up to 90 days. From day 21st, the total nitrogen content of BM samples was always higher than that in B samples. After 90 days, TN was 1.27 and 1.48-fold higher, respectively in the uninoculated B and in the inoculated BM samples, compared to raw BSG.

A similar trend was observed for the inorganic N forms (i.e., NH4 + and NO3-) (figure 3b, 3c) that increased over the treatment, reaching the highest values after 56 days. From that point up to the

90 days, the inorganic N content was significantly higher in BM samples of than in B samples.

Total carbon decreased during the treatment period, reaching the lower value after 56 days. After 90 days, total C was 1.11 and 1.16 times lower in sample B and BM, respectively, compared to time 0. After 70 days the total C in BM was 1.08 times lower than in B (p-value < 0.005).

The C / N ratio in the B and BM samples was 1.45 and 1.77 times lower, respectively, compared to that of raw BSG. From day 70 to the end of the treatment period (i.e., 90 days) the C / N ratio in sample BM was significantly lower than in sample B. However, in all the samples starting from the 42nd day, the C / N ratio was always less than 12, which is an indicative value of organic matter stability.

During the two treatments (B and BM), the pH values showed similar trends (Figure 4). Starting from pH 5.8 of the freshly produced BSG, in the first week an acidification of the substrate was observed (B: pH 4.51; BM: pH 4.67). In the second week, the pH reached 8.76 for B and 8.59 for BM, and then stabilized at about neutrality (pH 7.00) from the 42nd to the 90th day of treatment. In general, sample BM did not show significant differences with the uninoculated sample B, the only exception was observed at 7 days, with a p-value of 0.002.

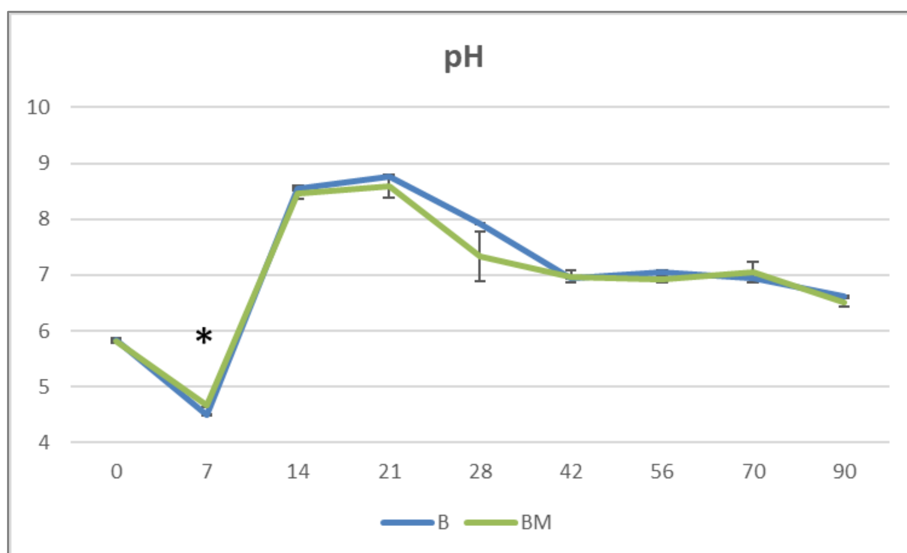


Figure 4: Variation of pH in the uninoculated (B) and inoculated (BM) BSG samples over the 90 days of treatment. *, P< 0.05

From the biochemical perspective, DHG activity in the B and BM samples increased after 7 days and remained stable up to 56 days, at around 800 µg TPF g^{-1} d.m. h^{-1}. After that time, the DHG activity in the BM samples slightly decreased. In contrast, the DHG activity in the B samples rapidly decreased to 78.48 µg TPF g^{-1} d.m. h^{-1} after 70 days of incubation, and then increased to 419 µg TPF g^{-1} d.m. h^{-1} after 90 days of incubation. In particular, the DHG activity of the BM samples was 7.65-fold and 1.75-fold higher than that of the B samples at 70 and 90 days, respectively (Figure 5a).

Variations in the GLU activity were observed for both treatments (Figure 5b). GLU activity increased up to 7 days in uninoculated BSG, and up to 14 days in inoculated BSG. In both the treatments, GLU activity decreased after 21 days and reached a maximum peak at 28 days (614 and 573 µg p-nitrophenol g^{-1} d.m. h^{-1} in B and BM, respectively). At 42 days, the GLU activity rapidly decreased to 124 and 138 µg p-nitrophenol g^{-1} d.m. h^{-1} in B and BM, respectively, stabilizing up to 90 days.

In general, URE activity was the same for both samples of inoculated (BM) and uninoculated (B) samples (Figure 5c). The only exception was after 42 days, where the URE activity was 5.35-fold higher for BM than B. However, the URE activity remained stable for 28 days and then increased to 56 days to their highest values (379 and 381 µg NH_4-N g^{-1} d.m. h^{-1}). URE activity then decreased to 70 days and remained stable for up to 90 days. For the PHA activities in an acidic environment (Figure 5d), during the 90 days of SSF, the PHA activity increased up to 14 days, to reach the highest PHA activities (855 and 1071 µg p-nitrophenol g^{-1} d.m. h^{-1} in B and BM, respectively), and subsequently decreased before a slight increase until it finally stabilized after 70 days. During the SSF period, BM showed a PHA activity between 1.25-fold and 9.93-fold higher than for B. On the contrary, the PHB activities in the alkaline medium (Figure 5e) during the period of SSF did not show any significant differences between B and BM, except for the PHB activities at 28 and 42 days, which were 1.60-fold and 1.23-fold higher in uninoculated BSG compared to inoculated BSG. However, for both the B and BM samples, PHB activity increased to a maximum up to 14 days, (2414 and 2187 µg p-nitrophenol g^{-1} d.m. h^{-1} in B and BM, respectively), and thereafter, the PHB activity rapidly declined at 21 days, after which it remained stable for up to 90 days.

DOC generally increased throughout the SSF period (Figure 6). After 90 days, compared with time 0, the DOC was 14.48-fold and 14.78-fold higher in B and BM, respectively. After 42 days of incubation,

the highest DOC levels were observed for both treatments, followed by a gradual decrease and stabilization for up to 90 days. The BM sample showed higher DOC than the B sample, although the BM treatment showed greater DOC fluctuations during the SSF period.

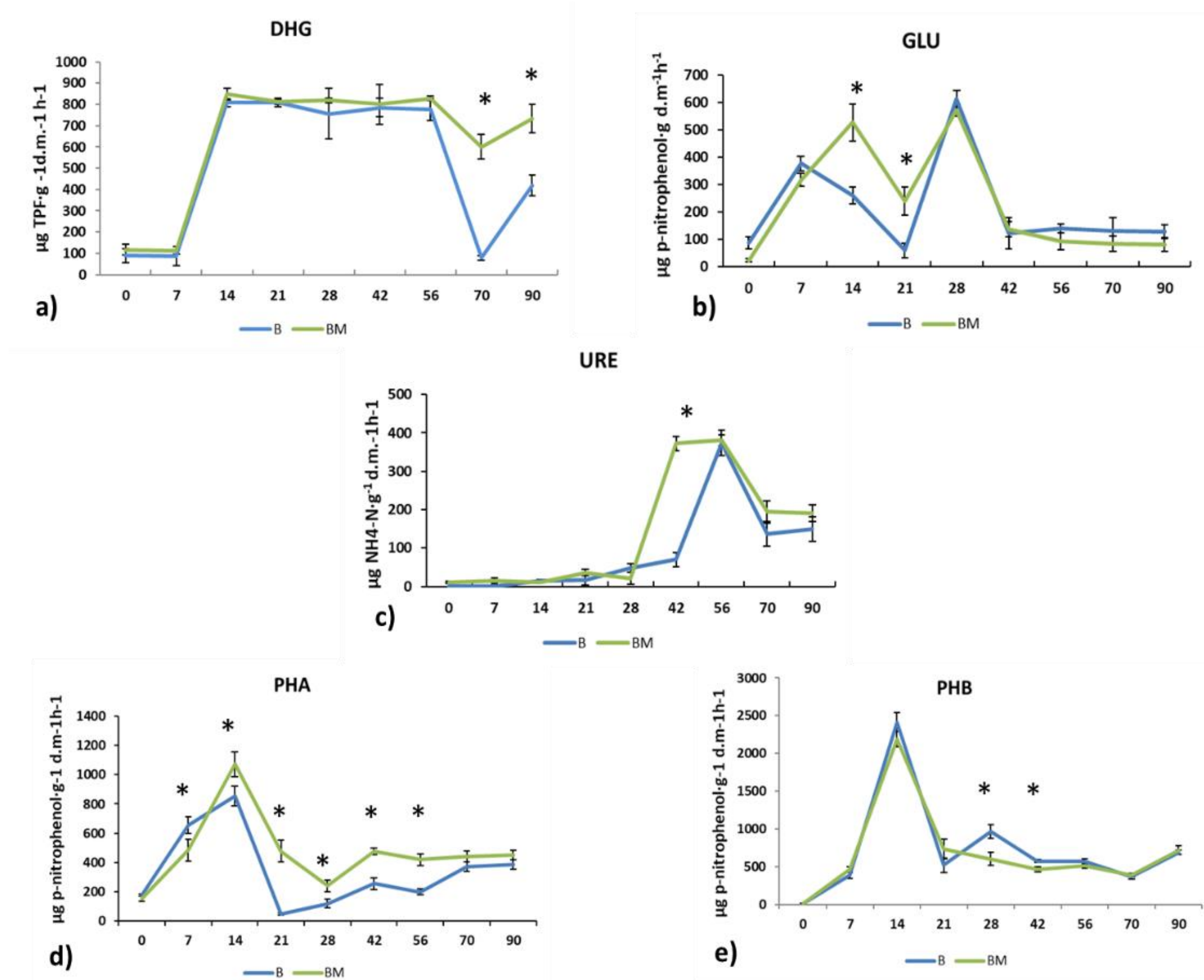


Figure 5: Results of the enzymatic activities detected over 90 days of SSF in the uninoculated B and inoculated BM samples: (a) Dehydrogenase (DHG); (b) β-glucosidase (GLU); (c) Urease (URE); (d) acid phosphatase (PHA); (e) alkaline phosphatase (PHB). Data are means ± SD (n = 3). *, $P < 0.05$; Fisher's least significant difference tests.

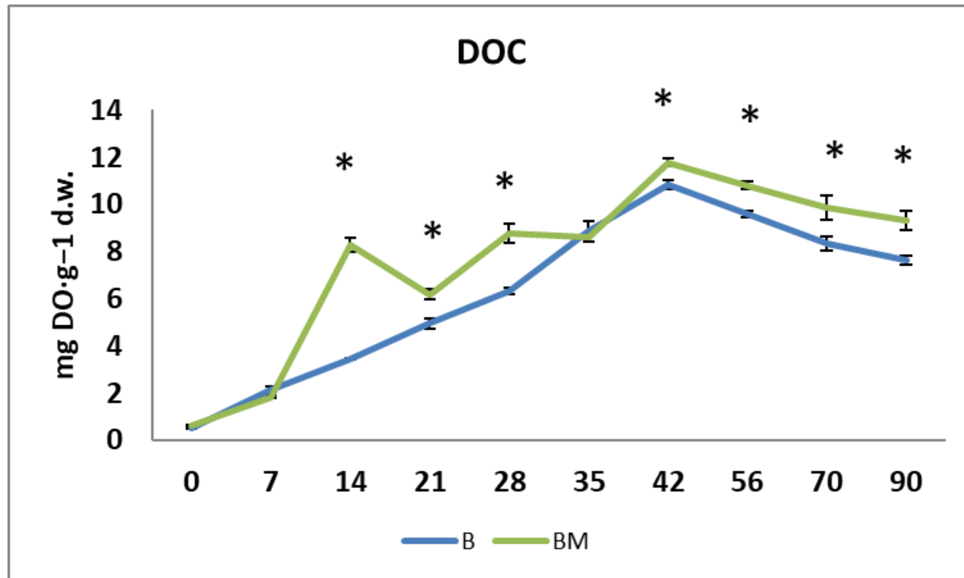
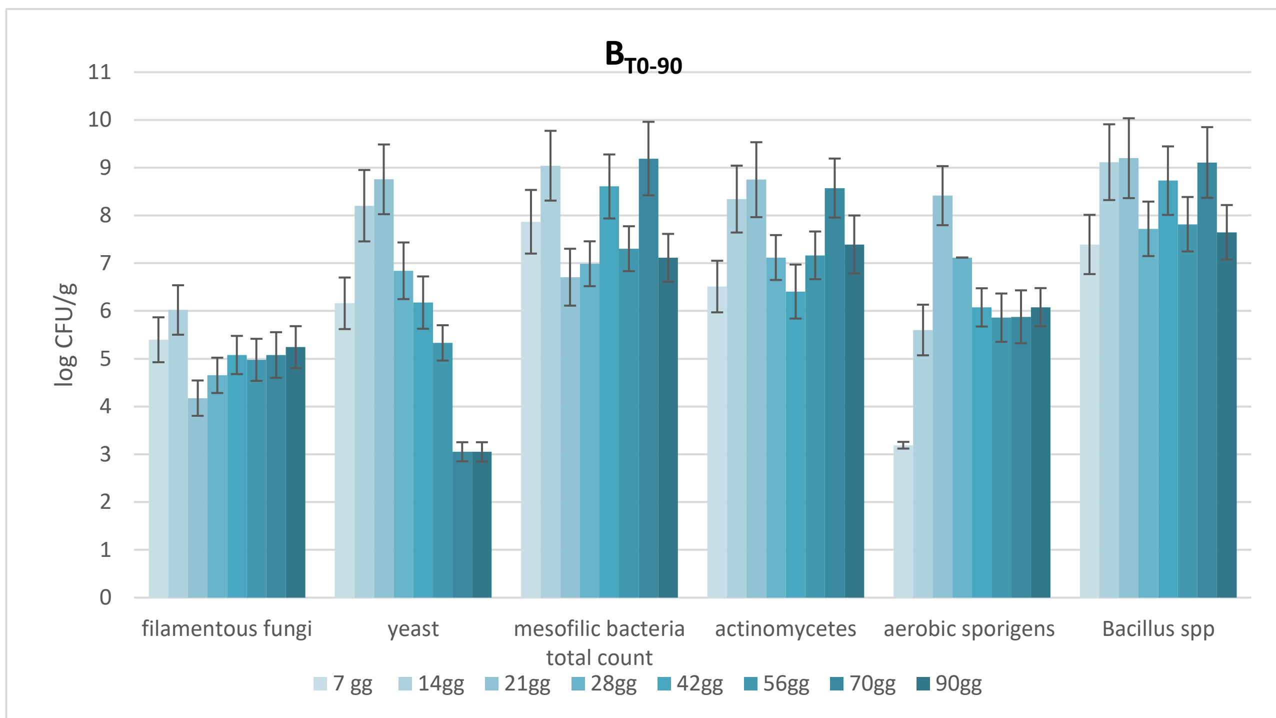


Figure 6: The dissolved organic carbon (DOC) production during the 90 days of SSF in the uninoculated B and inoculated BM samples. Data are means ± SD (n = 3). *, $P < 0.05$; Fisher's least significant difference tests.

4.3.2. Structure and analysis of the microbial community during solid state fermentation of BSG.

To characterize the dynamics of the microbiota in both the uninoculated (B) and in the inoculated (BM) BSG samples during SSF, culture-dependent microbiological analyses were carried out (figure 7). Overall, the viable counts of the main microbiological groups analyzed did not show any significant difference between treatments at each time point. Considering the dynamics during the treatment, yeast counts showed a significant decrease after 56 days. Mesophilic bacteria showed a maximum concentration at t14, t42 and t70 with a microbial population higher than 1x 10^9 cfu / g, that lowered to 1 x 10^7 cfu /g at t90. Actinomycetes rapidly increase in the first 21 days of treatment. Subsequently, the population remained stable, as not significant differences were observed among t28, t56, t70 and t90 samples. The same trend was observed for aerobic spore-forming bacteria, that showed an increasing population in the first day of the treatment, then stabilize at the concentration of 1 x 10^6 cfu/g starting from t42. Similarly, the viable counts of *Bacillus* spp. increased in the first days of SSF, than reached at t90 a population size not different form that a t0.

A)



B)

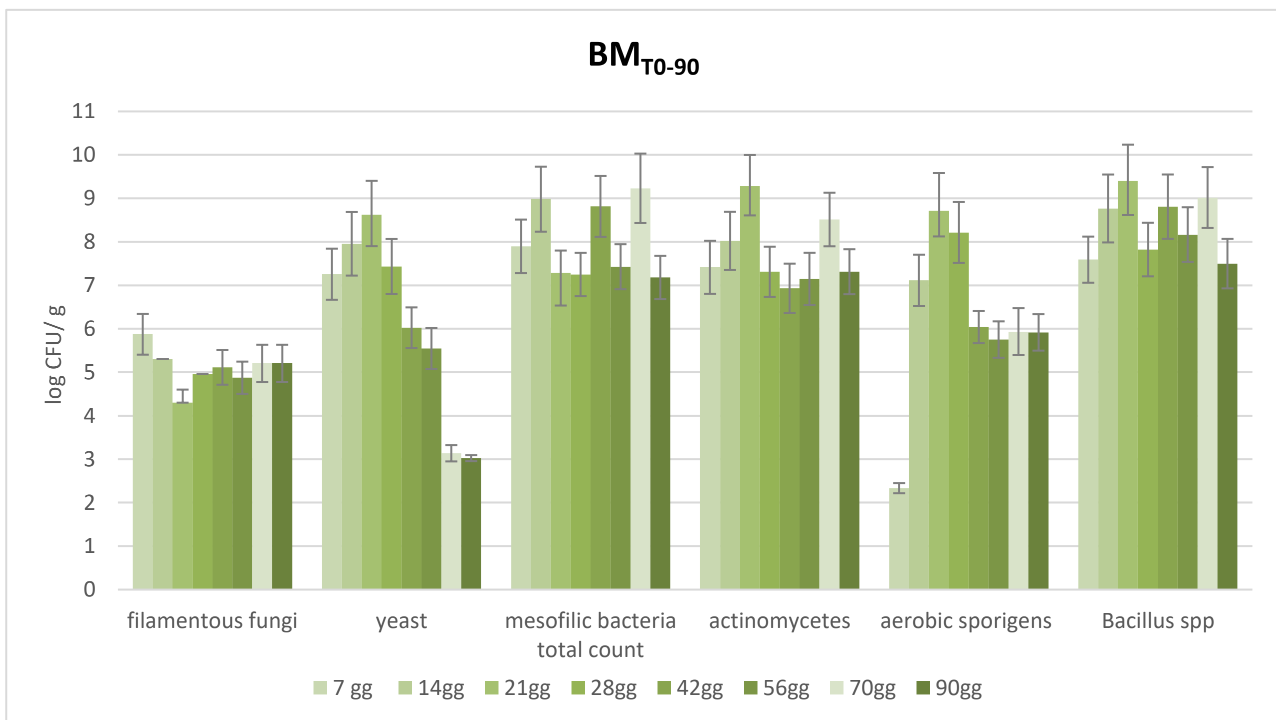


Figure 7: Viable counts in uninoculated B (A) and inoculated BM (B) BSG samples. Data are means ± SD (n = 3).

To get a better picture of the fate of the inoculated PGPMs during the SSF of the BSG, and to evaluate the changes in the natural microbiota of BSG after the inoculum, a high-throughput community sequencing analysis was carried out

4.3.2.1 Microbial characterization of the bioinoculant M

Figures 8 and 9 show the composition of the fungal and bacterial community obtained by next generation sequencing of DNA extracted from the bioinoculant M. *Saccharomycetaceae*, *Debaryomycetaceae, Pichiaceae, Aspergillaceae, Malasseziaceae, Cladosporiaceae and Cordycipitaceae* were detected at higher abundance in the samples. Particulary, *Saccharomyces* was the predominant genus.

Enterococcaceae, Pseudomonadaceae, Leuconostocaceae, Lactobacillaceae, Planococcaceae, Streptococcaceae, Acetobacteraceae, Streptomycetaceae, Staphylococcaceae, Propionibacteriaceae were the predominant bacterial families.

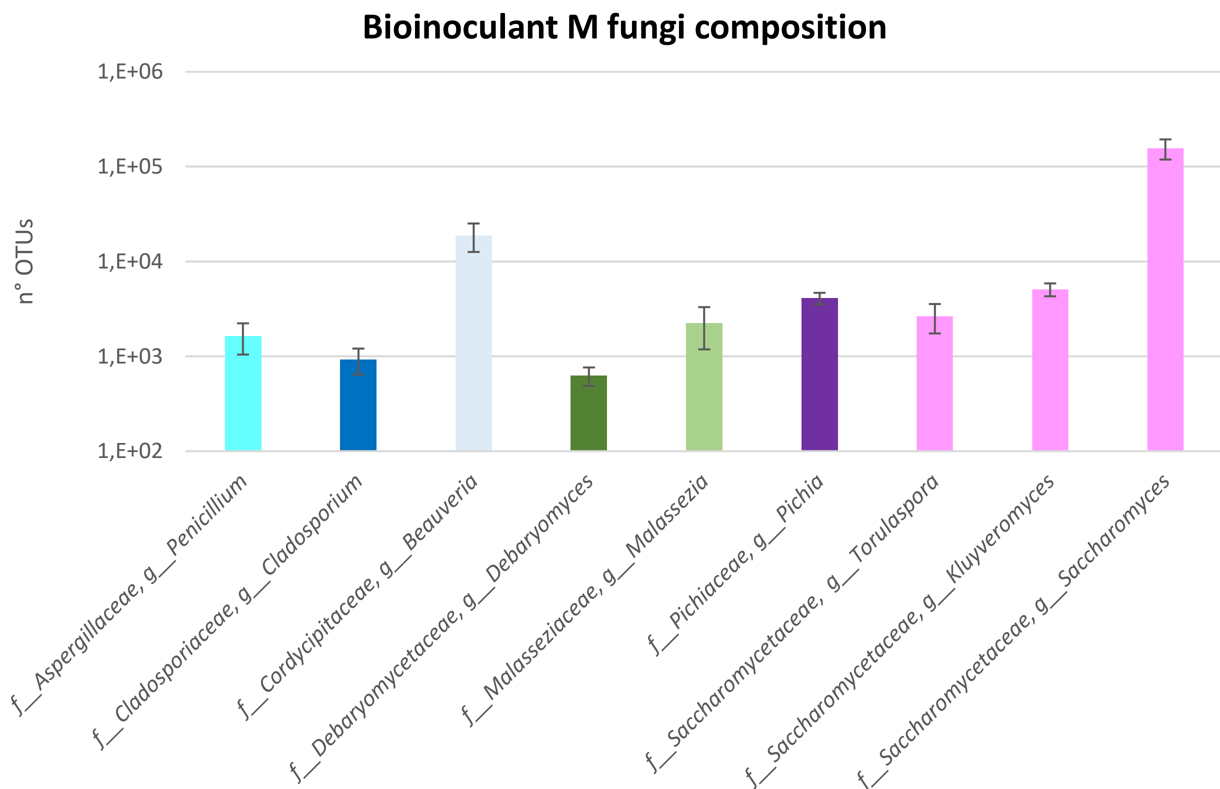


Figure 8: Composition of the specific fungal family of Bioinoculant M used to inoculate BSG. Data are means ± SD.

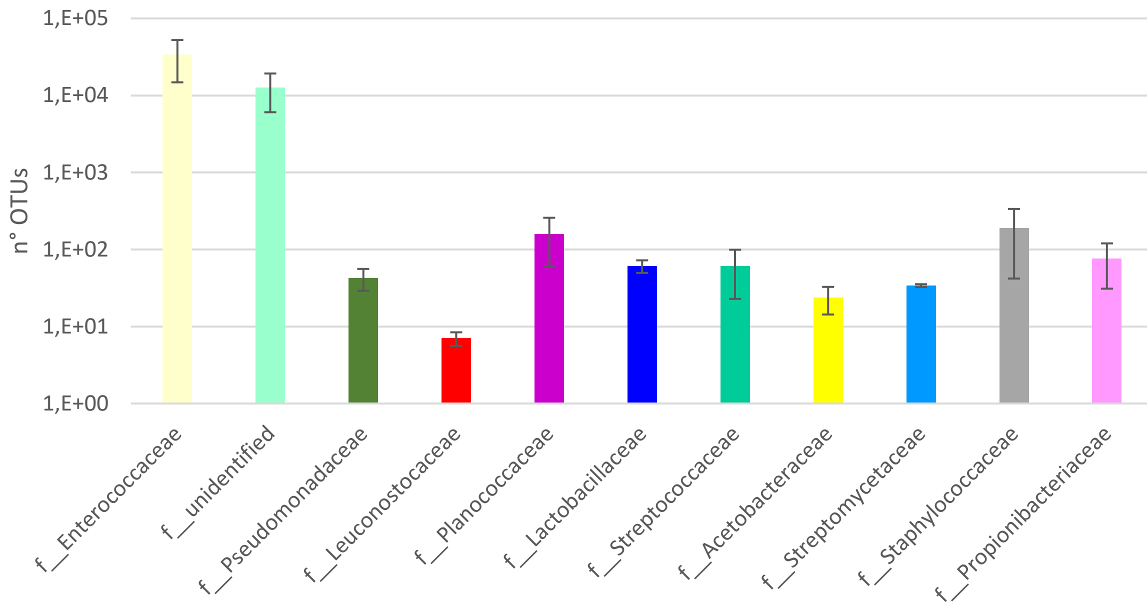


Figure 9: Composition of the specific bacterial family of Bioinoculant M used to inoculate BSG. Data are means ± SD.

4.3.2.2. Evolution of the fungal communities

The evolution of the fungal communities in raw BSG (samples B) and in inoculated BSG (BM) during 90 days SSF was followed by metabarcoding analysis of the ITS sequences. Illumina sequencing (MiSeq) generated a dataset that ranged from 7,000 to 15,000 raw sequences per sample. Alpha diversity analysis suggested an increased biodiversity during the fermentation in the BM samples. The Shannon Index is based on the species richness (the number of species present) and species abundance (the number of individuals per species), and thus it measures the biodiversity of the samples in terms of richness and abundance of OTUs. During the treatment, the Shannon Index reached its highest values after 70 days in BM samples. In the B samples, the biodiversity was higher at B_{T0} and was then reduced, reaching its lowest value after 70 days (Figure 10).

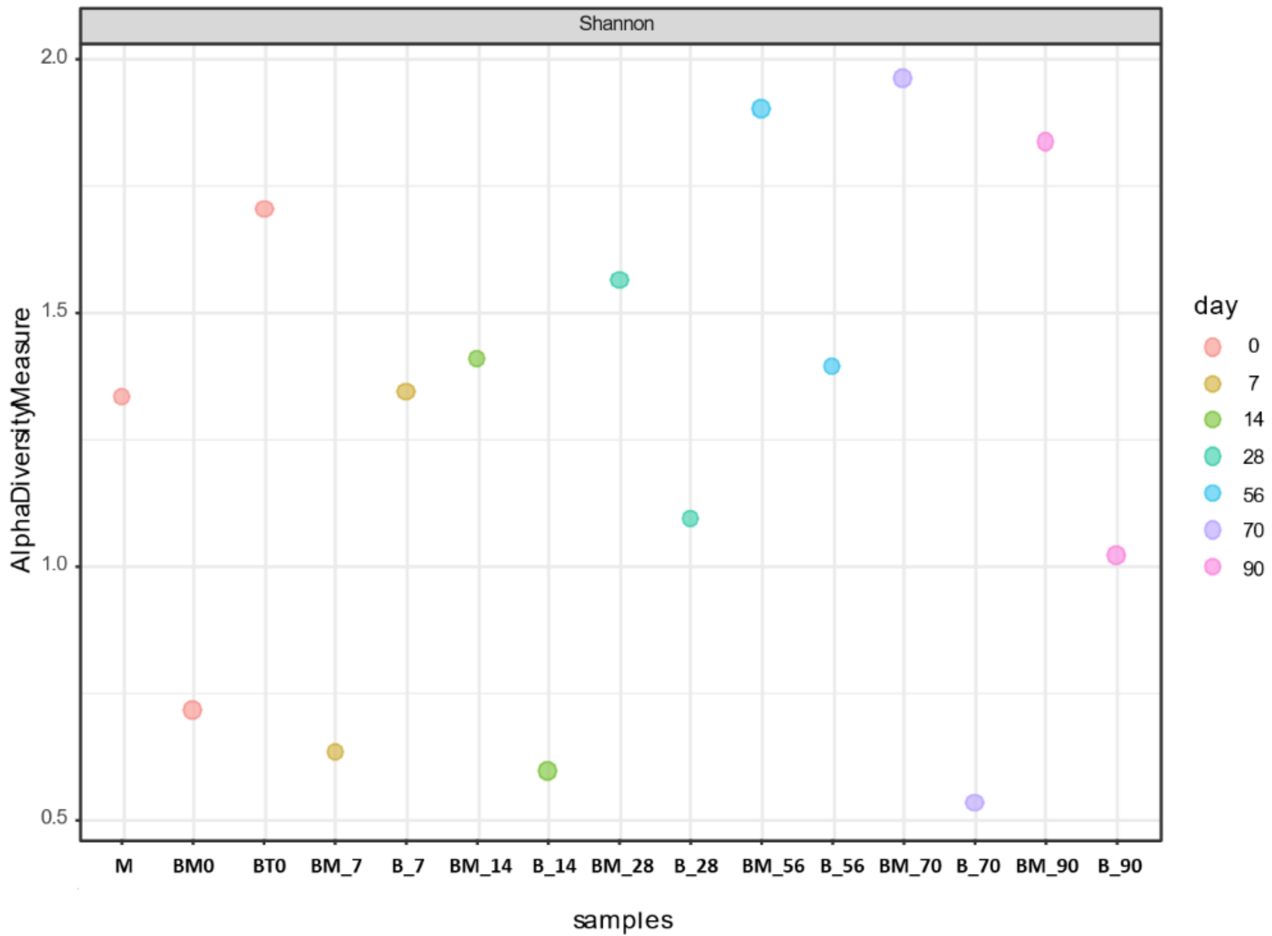


Figure 10: Fungal alpha diversity. M, fungal community present in the microorganism-based preparation used to inoculate the BSG; B, uninoculated BSG; BM, inoculated BSG.

To compare the biodiversity of the two samples, beta biodiversity analysis at the OTU level was carried out (Figure 11). Of note, there were differences between the B and BM samples through the fermentation, with high similarities between the fungal communities of the same treatment after 70 and 90 days. This suggested that the microbial communities reached an equilibrium that was different for the untreated and treated samples.

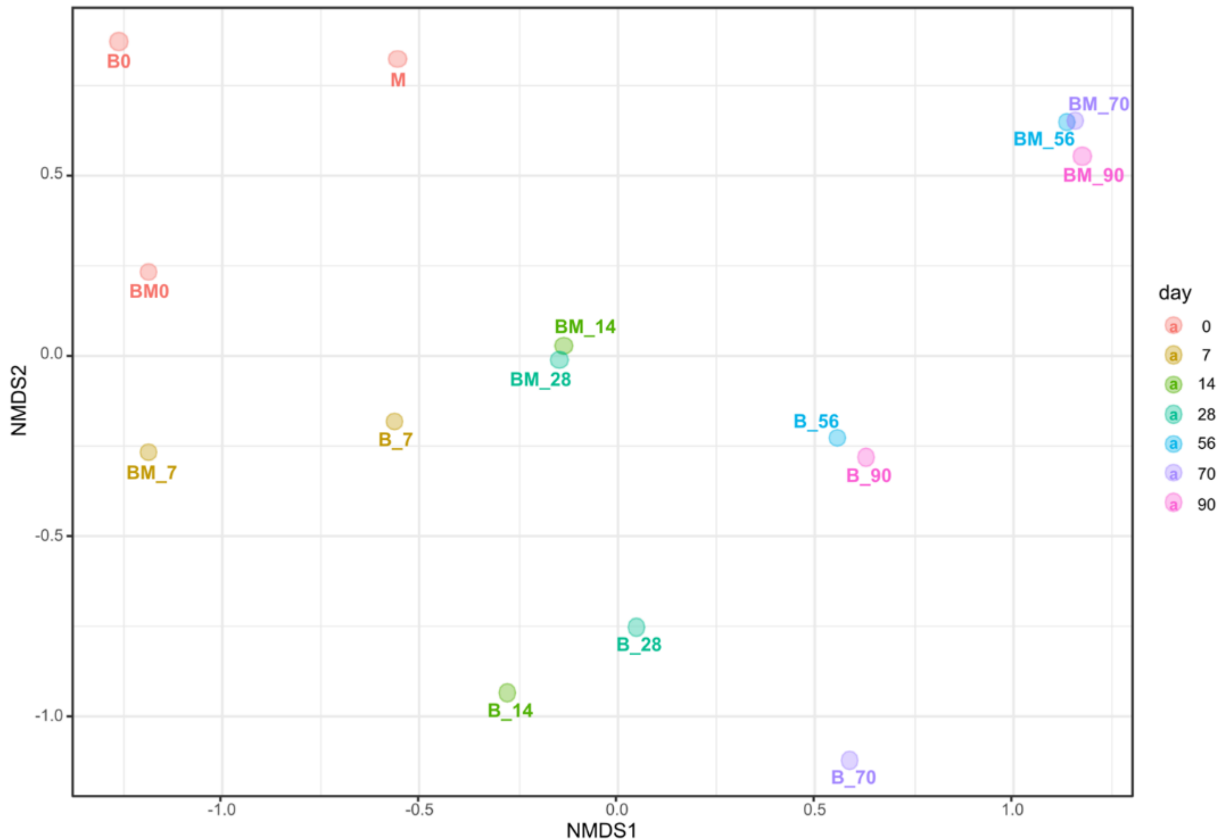


Figure 11: Non-metric multidimensional scaling (NMDS; Bray-Curtis distance) analysis and distribution of samples on the two axes NMDS1 and NMDS2. M, fungal community present in the microorganism-based preparation used to inoculate BSG; B, uninoculated BSG; BM, inoculated BSG.

Figure 12 shows the compositions of the fungal communities under the two treatments at the different sampling times. The fungal community in the B (uninoculated) samples changed over the 90 days: after 7 days, the dominant family were Mucoraceae and Pichiaceae; after 14 days, Mucoraceae were by far the most represented family. Similarly, after 28 days Aspergillaceae and Mucoraceae were the dominant fungal families. After 56 days, OTUs belonging to Ascodesmidaceae and Microascaceae appeared, while family Aspergillaceae decreased. At t70 and t90 the dominance of the Mucoraceae was restored. In accordance with the beta diversity analysis, the compositions of fungal communities at the family level stabilized between 70 and 90 days of spontaneous treatment.

As observed for B samples, the fungal communities in the inoculated BSG samples (BM) at time 0 were characterized by an abundant presence of Pichiaceae and a minor presence of

Saccharomycetaceae and Mucoraceae. However, after only 7 days of fermentation, a significant shift in the composition of the fungal communities in the two treatments was observed. Indeed, the proportion of Pichiaceae was particularly higher at t7, while at t14 at t28 the community was characterized by four dominant families: Aspergillaceae, Mucoraceae, Pichiace and Saccharomycetaceae. From t56, Pichiace and Saccharomycetaceae were no longer detected, the family Chaetomiaceae increased and there was a consistent presence of Ascodesmidaceae and Microascaceae up to t70. At t90 the dominant families were Ascodesmidaceae, Trichosporonaceae and Physalacriaceae.

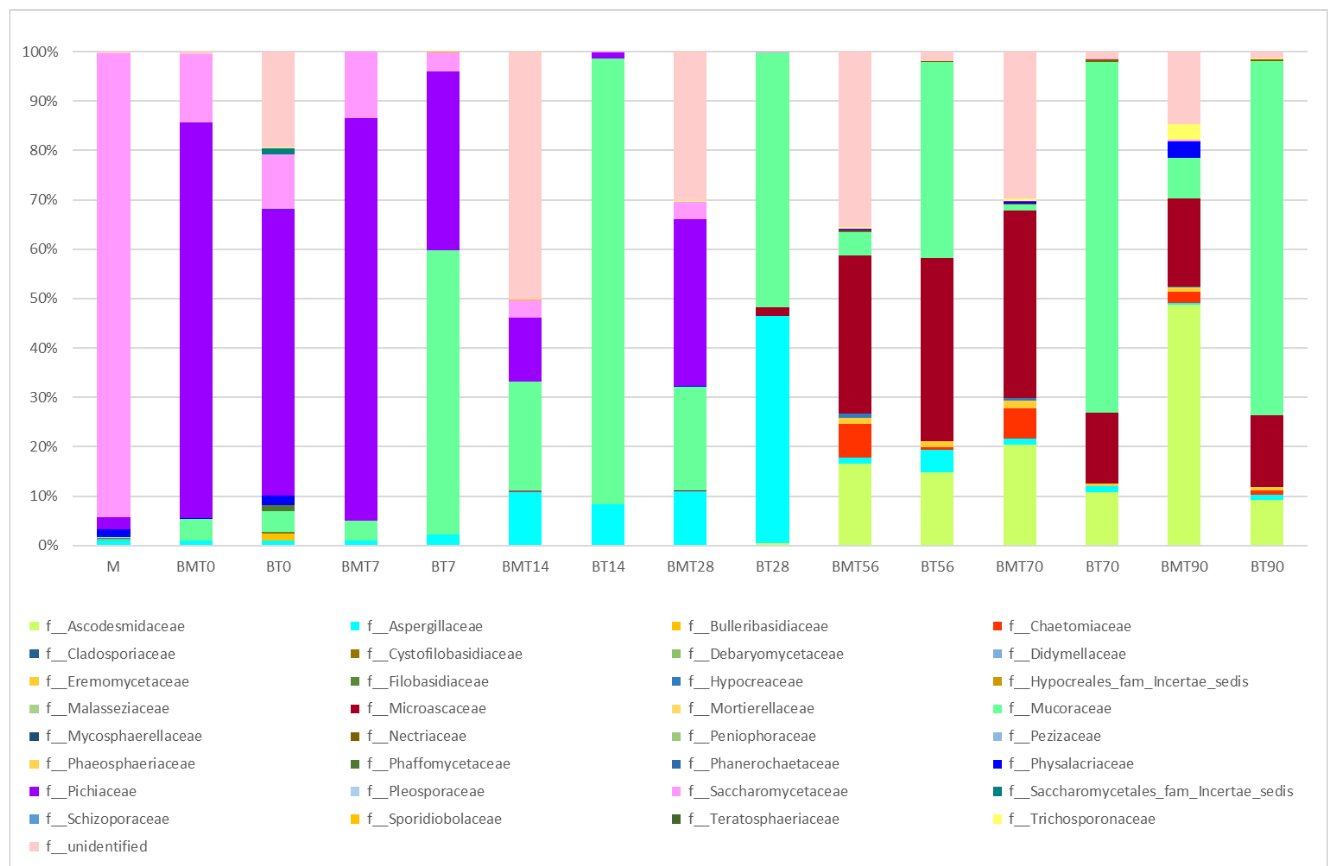


Figure 12: Relative abundance composition of the fungal communities in the uninoculated B and in the inoculated BM treatments. M, fungal community at time zero present in the preparation based on microorganisms used to inoculate the BSG.

Fungal families with significantly different abundance in the B and BM samples, at each time points, are reported in table 1.

Table 1. Fungal families showing significantly different abundance in treated (BM) and untreated (BM) BSG samples.

Sampling point	Familiy	n° OTU			Adjusted P-value
		M	B	BM	
T0	f__Saccharomycetaceae	*164362	1784	5781	0.003
	f__Debaryomycetaceae	625	4	1	0.003
	f__Pichiaceae	4114	9439	*33589	0.004
	f__Aspergillaceae	1875	174	406	0.014
	f__Mucoraceae	163	*490	1823	0.017
	f__Physalacriaceae	2787	324	29	0.019
T7	f__Saccharomycetaceae		1944	8138	0.019
	f__Mucoraceae		*29147	2418	0.040
	f__Pichiaceae		1.720	*49310	0.049
T14	f__Mucoraceae		29284	1742	0.000
T28	f__Saccharomycetaceae		25	748	0.025
	f__Mucoraceae		*33.251	4543	0.015
	f__Aspergillaceae		29711	2.350	0.047
T56	f__Aspergillaceae		3.212	750	0.010
	f__Microascaceae		34	1.797	0.033
	f__Mucoraceae		*28333	013	0.038
	f__Chaetomiaceae		387	4.414	0.048

T70	f__Hypocreaceae	1	361	0.000
	f__Chaetomiaceae	55	3994	0.007
	f__Microascaceae	15	1535	0.013
	f__Mucoraceae	*41898	805	0.024
T90	f__Physalacriaceae	22	1479	0.048
	f__Saccharomycetaceae	6	125	0.048
	f__Mucoraceae	*42399	3724	0.048
	f__Trichosporonaceae	13	1469	0.048
	f__Ascodesmidaceae	5472	*22193	0.048

The higher presence of Saccharomycetaceae in bioinoculant M was reflected in the higher abundance of this family in the inoculated BSG (BM) at t0. The occurrence of Pichiaceae was higher in samples B and BM at t0 compared to M. Mucoraceae family was present at all sampling times and became the dominant family after 90 days of treatment in sample B compared to sample BM. Indeed, Mucoraceae was less represented in the BM samples at the later times and was associated mainly with t14 and t28. Conversely, the dominant family of BM samples at t90 was that of the Ascodesmideaceae. After the SSF, the fungal families showing significant different abundance at t90 in the B and BM samples were: Physalacriaceae, Saccharomycetaceae, Mucoraceae, Trichosporonaceae, and Ascodesmidaceae.

4.3.2.3. Evolution of the bacteria communities

The evolution of the bacterial communities in raw BSG (samples B) and in inoculated BSG (BM) during 90 days SSF was followed by metabarcoding analysis of the 16S region. Illumina sequencing (MiSeq) generated a dataset that ranged from 75,863 to 84,060 raw sequences per sample. Alpha diversity analysis suggested an increased biodiversity in both samples from day 0 to day 90. In particular, the Shannon Index was lower at t0 and then increased, reaching its highest value after

28 days (Figure 13). A Shannon Index between 2 and 3 indicates that in both the B and BM treatments the bacterial communities were very rich in terms of OTU number and distribution.

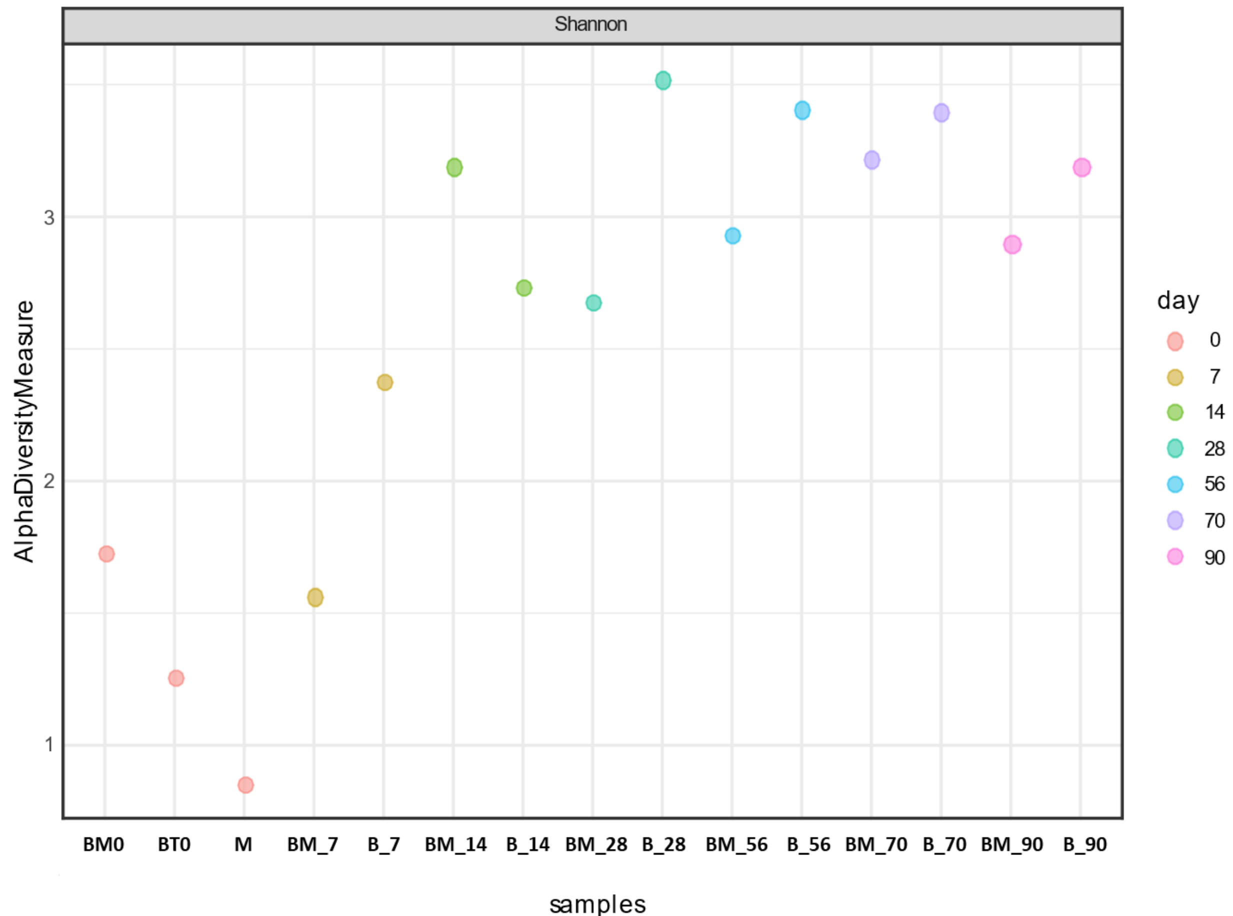


Figure 13: Bacterial alpha diversity. M, bacterial community present in the microorganism-based preparation used to inoculate the BSG; B, uninoculated BSG; BM, inoculated BSG.

The beta biodiversity analysis highlights the differences in the bacterial communities of the samples relating to the two treatments (B and BM) after the 14th day of SSF. It is interesting to note that the bacterial communities of both the B and BM treatments from t56 to t90 were very similar, as they were clustered together.

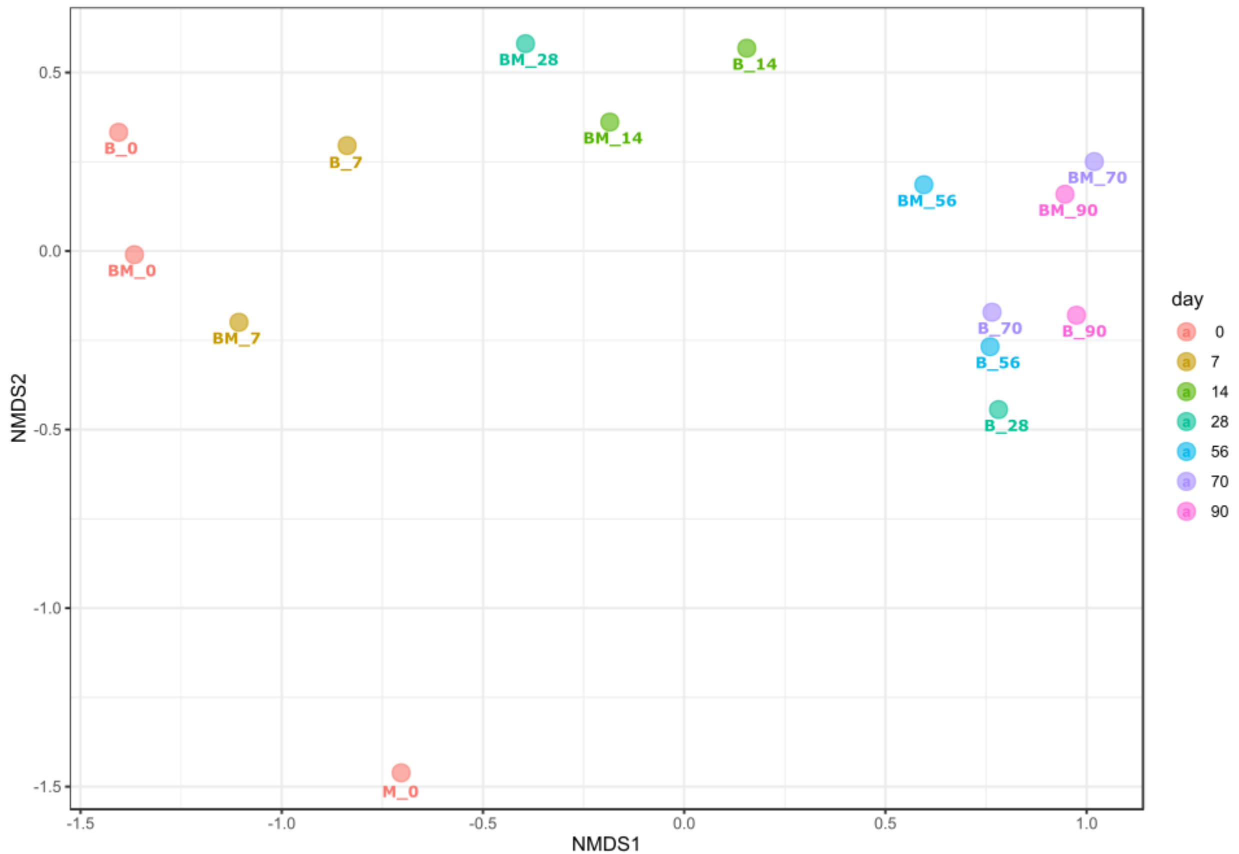


Figure 14: Non-metric multidimensional scaling (NMDS; Bray-Curtis distance) analysis and distribution of the samples on the two axes NMDS1 and NMDS2. M, bacterial community present in the microorganism-based preparation used to inoculate the BSG; B, uninoculated BSG; BM inoculated BSG.

Figure 15 shows the phyla of the bacterial communities present in the B and BM treatments and in the inoculum. The bacterial community was stabilized by t56 for both treatments. In accordance with the results of the beta diversity, the bacterial communities of the B and BM samples were very similar especially in the last few days of the treatments. In the M inoculum the phylum Firmicutes was dominant. In samples B and BM at t0, Proteobacteria, Cyanobacteria and Actinobacteria were observed. At t7 in both treatments the differences in the bacterial composition at the phylum level were not significant, while at t14 Proteobacteria became dominant in samples B and Actinobacteria and Bacteroidetes were present at higher abundances in BM samples. At t28 the phylum Planctomyces was firstly detected in samples B. From t56 to t90, Firmicutes, Proteobacteria,

Actinobacteria, Bacteroidetes, and Planctomyces became the main phyla in both the samples, with not significant differences in their abundance in the two treatments.

Figure 16 shows the families of the bacterial communities present in the B and BM treatments and in the inoculum. Leuconostocaceae was not present in the bacterial community of the inoculum, but it was a dominant family in both B and BM treatments from t0 to t28.

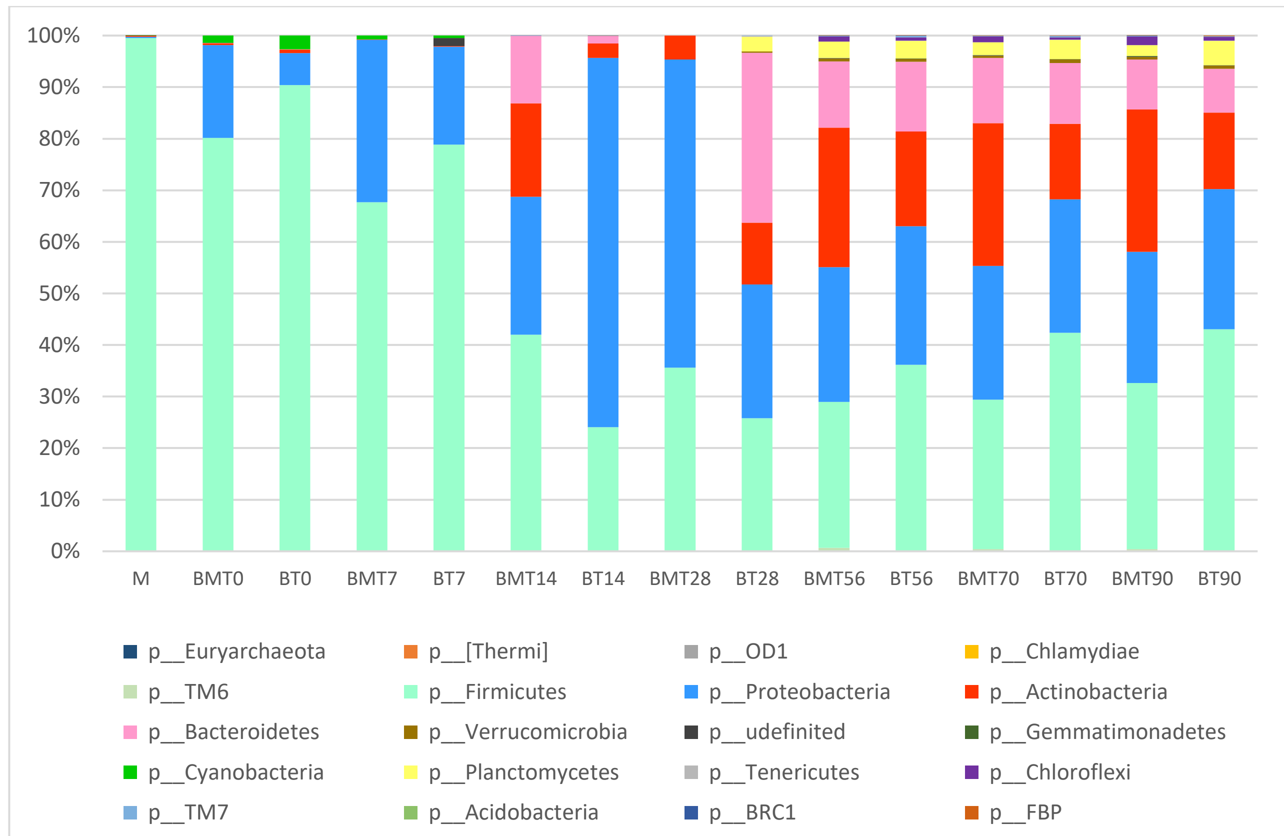


Figure 15: Phylum compositions of the bacterial communities for the B and BM treatments. M, bacterial community at time zero present in the preparation based on microorganisms used to inoculate the BSG.

At t0, the dominant family in B samples was Leuconostocaceae (figure 16); at t7 the occurrence of OTUs belonging to Lactobacillaceae, Paenibacillaceae, Clostridiaceae, Acetobacteraceae and Enterobacteriaceae increased. At t14 the family Xanthomonadaceae prevailed. At t28 Paenibacillaceae were much more abundant than in previous times, with Sphingobacteriaceae, Porphyromonadaceae, Chitinophagaceae and Phyllobacteriaceae. From t56 to t90, the bacterial

communities settled down: the presence of Paenibacillaceae and Planococcaceae, belonging to the Bacillales order, were dominant.

In BM samples the dominant families in the first days after the inoculum were Leuconostocaceae, Lactobacillaceae and Acetobacteraceae. At14, the dominant families at t7 decrease drastically and other families appeared: Enterobacteriaceae, Enterococaceae, Sphingobacteriaceae, Microbacteriaceae, Xanthomonadaceae, Nocardiaceae, Planococcaceae, Clostridiaceae and Brucellaceae. After 28 days, the dominant families were Enterobacteriaceae, Xanthomonadaceae and Leuconostocaceae; from t56 to t90, the bacterial communities abundance (%) was constant and the most prevalent families were Sphingobacteriaceae, Hyphomicrobiaceae, Nocardiaceae, Planococcaceae, Clostridiaceae, Rhizobiaceae, Brucellaceae and Promicromonosporaceae; from t70, families belonging to the Lactobacillales (Enterococcaceae, Leuconostocaceae, Lactobacillaceae and Streptococcaceae) were no longer detected.

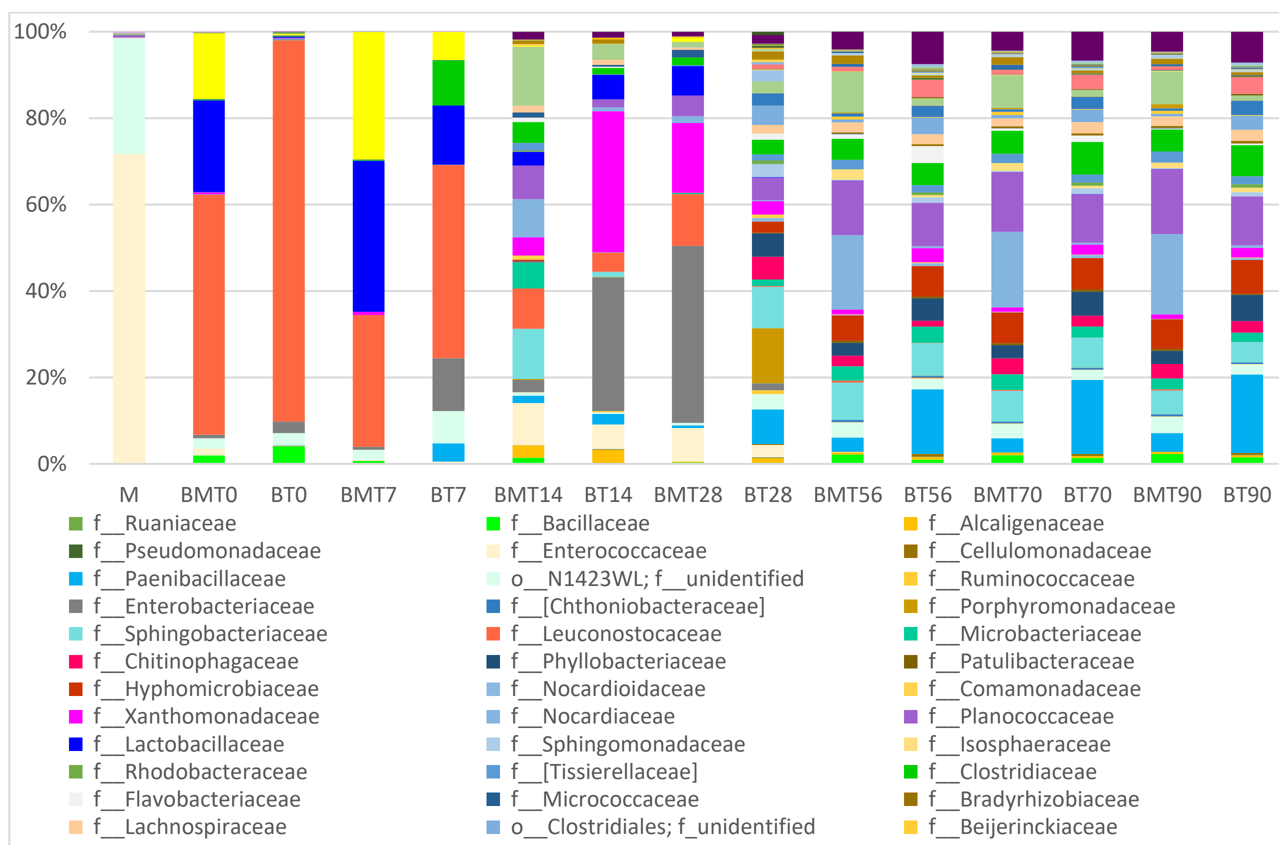


Figure 16: Family compositions of the bacterial communities for the B and BM treatments. M, bacterial community at time zero present in the bioinoculant used to inoculate the BSG.

Bacterial families with significantly different abundance in the B and BM samples, at each time points, are reported in table 2.

Table 2. Bacterial families showing significantly different abundance in treated (BM) and untreated (B) BSG samples

Sampling point	Family	n° OTU			FDR adjusted p value
		M	B	BM	
T0					
	f__Enterobacteriaceae	5	*570	144	0.0057
	f__Pseudomonadaceae	43	9	0	0.0102
	f__Leuconostocaceae	7	*19342	9417	0.0102
	f__Enterococcaceae	*33597	43	272	0.0432
	f__Bacillaceae	0	*895	334	0.0490
	f__Lactobacillaceae	61	43	*3604	0.0500
T7	f__Enterobacteriaceae		2086	138	0.0046
	f__Clostridiaceae		1782	713	0.0072
	f__Xanthomonadaceae		0	823	0.0084
	f__Paenibacillaceae		715	1512	0.0157
	f__Lactobacillaceae		2349	1339	0.0185
T14	f__Xanthomonadaceae		5753	713	0.0012
	f__Clostridiaceae		267	823	0.0016
	f__Nocardiaceae		155	1512	0.0042
	f__Planococcaceae		338	1339	0.0068
	f__Sphingobacteriaceae		197	1998	0.0113
	f__Bacillaceae		3	236	0.0146
	f__Leuconostocaceae		795	1589	0.0178

T28	f__Xanthomonadaceae	557	2703	0.0000
	f__Leuconostocaceae	45	2006	0.0000
	f__Phyllobacteriaceae	971	0	0.0042
	f__Enterobacteriaceae	272	6863	0.0052
	f__Clostridiaceae	615	306	0.0058
	f__Lactobacillaceae	26	1144	0.0122
	f__Paenibacillaceae	1447	101	0.0127
T56	f__Rhizobiaceae	731	179	0.0002
	f__Nocardiaceae	88	3131	0.0056
	f__Planococcaceae	1838	2286	0.0058
	f__Paenibacillaceae	2746	590	0.0074
	f__Phyllobacteriaceae	943	541	0.0112
T70	f__Paenibacillaceae	3152	587	0.0000
	f__Rhizobiaceae	624	191	0.0000
	f__Phyllobacteriaceae	1017	535	0.0008
	f__Nocardiaceae	74	3139	0.0011
	f__Brucellaceae	261	1355	0.0065
T90	f__Bacillaceae	126	*468	0.0068
	f__Nocardiaceae	46	*3968	0.0083
	f__Planococcaceae	979	*3217	0.0152
	f__Brucellaceae	102	*1628	0.0191
	f__Sphingobacteriaceae	405	*1180	0.0208

The bacteria family statistically significant at t90, at the end of the treatment in the B and BM samples were: Bacillaceae, Nocardiaceae, Planococcaceae, Brucellaceae and Sphingobacteriaceae.

Bacillus (Bacillaceae), *Rhodococcus (*Nocardiaceae), *Sporosarcina* (Planococcaceae), *Paenochrobactrum* (Brucellaceae), and *Sphingobacterium* (Sphingobacteriaceae) were the more represented bacterial genera (data not shown).

4.4. Discussion

The objective of this study was to verify the possibility of increasing the agronomical value of an agro-industrial waste product, such as BSG, by adding a bioinoculant. The rationale is that the use of BSG as a growth substrate to produce microbial biofertilizers through SSF, could improve the stabilization of BSG allowing the production of easy degradable organic compounds, favor the bioavailability of nutrients for plants and increasing the biocontrol of pest and pathogens in the long term. Particularly, the analysis of the microbial, chemical, and biochemical data obtained during a 90 days SSF treatment allowed us to identify the effect of the inoculant on the spontaneous microflora and on the chemical characteristics of BSG. Considering the key issues that an adequate formulation of the bioinoculant requires the long-term to have successful establishment, the colonization of target niches in the plant environment, the expression of the growth of relevant plants, and the effects of promoting plant health, a poorly studied fundamental aspect is that the inoculated microorganisms must compete with a very diverse resident microflora (Roy et al. 2021). Also, this study aimed to investigate the dominant microbial communities at the end of the SSF (t90) in the two samples B and BM that can exert key functions in the plant-soil interaction, being involved in the nitrogen fixation processes, carbon and nitrogen cycles, nutrient uptake and soil shaping.
Depending on the size, diversity, and community interactions with the resident populations, a microbial inoculant introduced into the soil environment can establish itself well or badly (Compant, et al. 2010). In addition, the spontaneous microbiota of agro-industrial wastes is not always dangerous or ineffective but can led to several advantages once introduced into soil-plant systems, including enrichment of the soil with organic matter, metabolites, and minerals (e.g., P).

4.4.1 Chemical and biochemical dynamics of BSG during SSF

Enzymatic activities data are important as they can provide information on (i) the metabolic state of the microbial population; (ii) the chemical-physical conditions of the substrate on (iii) the degree of alteration and of the relationships between changes in physico-chemical properties of substrate and microbial biodiversity (van Beelen & Doelman, 1997; Trasar-Cepeda et al., 2000).
In this work the general reduction of total carbon during the treatments period was probably due to its consumption for the metabolism of microorganisms, as also suggested by the high activity of

dehydrogenase (DHG), which indicates that the indigenous microorganisms were able to synthesize this enzyme for the degradation organic matter (Margesin et al., 2006; Castaldi et al. 2008). In fact, DHG is an intracellular enzyme that highlights the metabolic activities of live microorganisms, since it is involved in the respiratory chains of all microorganisms (Ros et al., 2006). Between 70 and 90 days there are statistically significant differences in the microbial communities of BSG and BM both at the metabolic level and in carbon consumption.

The DOC value (small molecular carbohydrates and organic acids) increase in both samples B and BM during the first 42 days was probably due to a synthesis by microorganism and solubilization of simple organic compounds from organic matter greater than their degradation or utilization by microorganisms (Charest et al., 2004; Castaldi et al. 2008).

While the DOC reduction rate from the 42 days up to 90 days in B and BM suggests that the microbial community growth was supported by water-soluble organic carbon rather than more recalcitrant substrates (Charest et al., 2004; Zhu et al., 2021). In support of these hypotheses the GLU activity increases up to the first month of stabilization highlighting that the microorganisms have synthesized this extracellular enzyme to degrade the organic materials to obtain simple organic compounds necessary for their metabolism.

The phosphatase activity (PHAcid and PHB alkaline) in B and BM samples showed how the microbial communities were able to adapt to the changes in the substrate induced during the SSF process (Sharma et al. 2013). In fact, the microorganisms can synthesize different phosphatases (Sharma et al. 2013). During the first 7 days, phosphatases active at low pH were produced, supplemented by increased synthesis of basic phosphatases in the following days in accordance with changes in the pH of the substrate.

The high availability of nitrogen present at time 0 in both B an BM samples, probably may have inhibited the urease synthesis by the microorganisms (García et al., 1992; Castaldi et al. 2008). Therefore, at 21 days, when a decrease in nitrogen content was recorded, a prompt response from the microbial community was observed with an increase in urease activity (URE). This led to a corresponding increase in ammonium nitrogen (NH_4^+) content starting at 28 days, which also correspond with the pH decrease probably due also to the oxidation of ammonium (i.e., nitrification; Castaldi 2008). Therefore, as also highlighted by the increase in nitrate nitrogen (NO_3^-) starting

from 21 days, it is clear that the microbial community during the stabilization process is able to manage and use the various forms of nitrogen present in the substrate, modifying their needs through processes of hydrolysis and nitrification (Nannipieri et al., 1980).

4.4.2. Microbiota composition after solid state fermentation of BSG.

From the vital counts data associated with the chemical and biochemical data obtained, and from the analysis of the microbiota using next-generation sequencing, significant differences were found between the bacterial and fungal microbial communities of two treatments. First of all, it was observed that the microbiome of the bioinoculant M used as inoculum were not able to grow on BSG and to overcome the competition with the BSG spontaneous microbiota. In fact, the OTUs identified in sample B and BM at the beginning of the treatment, were not longer detected in BSG samples after 7 days. However, the addition of the exogenous microbial communities did affect the dynamics of BSG spontaneous microbiota, particularly at t90. In fact, in the BM sample at t90 some significant chemical and biochemical variations were observed in addition to the microbiological ones just described. In BM_{T90} higher levels of total nitrogen were detected, greater consumption of total carbon, with the consequent reduction of the C / N ratio compared to B_{T90}. Furthermore, the BM_{T90} sample showed greater dehydrogenase activity and a greater presence of dissolved organic carbon.

In this study, at the end of the Solid-State Fermentation (SSF, t90) in the two samples B and BM the results obtained have revealed that the microbial OTUs in the bioinoculant did not dominate over the microbiota naturally present in the raw BSG, as they were not detected further in the t7 samples. However, an indirect effect of the bioinoculant was related to the changes in the microbial communities of the BSG samples. Particularly, the fungal genera significantly different at the end of the treatment in the B and BM samples were: *Mucor* (respectively OTUs 42399, 3724 adjusted P-value 0.048), *Cryptomarasmius (respectively OTUs 22, 1479 adjusted P-value 0.048), Cephaliophora (respectively OTUs 22193, 5472 adjusted P-value 0.048), Kluyveromyces (respectively OTUs 6, 125 adjusted P-value 0.048) and Trichosporon (respectively OTUs 13, 1469 adjusted P-value 0.048)*. From the NGS analysis in the B_{T90} sample compared to the BM_{T90} sample, a significant dominance of the *Mucor* genera was found (respectively OTUs 42399, 3724 adjusted P-value 0.048). On the contrary,

in the BM_{T90} sample, was found the dominant genera *Cephaliophora* (respectively OTUs 22193, 5472 adjusted P-value 0.048). The genus *Mucor*, highly dominant in B, untreated, BSG samples at t90, is composed of many ubiquitous species, with about 50 recognized taxa, many of which have widespread occurrence mainly distributed in the soil and atmosphere (Ruano-rosa and Mercado-Blanco, 2015; Walther et al., 2013). *Mucor* grows in a wide range of temperature (from 20 ° C to 40 ° C with optimum at 30 ° C) and pH (from 4 to 12 with optimum at 5.5) and it is known to produce a wide range of enzymes to degrade organic matter in different niches and have a relevant importance in various industrial applications (Voigt et al., 2016; Voigt and Kirk, 2014), thank to for to the production of several enzymes among which cellulases (Morin-Sardin et al., 2017; Alves et al., 2002). Fungi that selectively removes lignin results in the acceleration of BSG decomposition and the release of inorganic elements.

Mucor circinelloides plays an important role in degrading agricultural crop residues rich in lignocellulosic compounds (Baba et al., 2005). It has been reported that *M. circinelloides* can produce a full set of cellulose-degrading enzymes, indicating that this fungus has the potential to be used in biomass conversion (Saha, 2004). *M. circinelloides* was found to be a good producer of cellulases, xylanases and polygalacturonases (Lee et al., 2011, Thakur et al., 2010). Huang (2014) identified an active β-glucosidase in *M. circinelloides* that might be of industrial relevance. The present finding of an active β-glucosidase in *M. circinelloides* demonstrates that enzymes from this group of fungi have a potential for cellulose degradation (Morin-Sardin et al., 2017). *Mucor* sp is gradually gaining attention in biological research because of its ability to produce catalase, proteases, and secondary metabolites such as phytoalexin which are usually activated in stressed environments (Andrade et al., 2002; Simões et al., 2005). Some Mucor sp. produced a variety of lytic enzymes such as β-1, 3- glucanase, catalase, and proteases. These enzymes are highly important in cell-wall degradation to enable easy penetration and colonization of the pathogen, induce defense response and control disease spread, and indirectly promote plant growth and development (Nartey et al., 2022). *Mucor* spp. was shown to be highly tolerant to elevated levels of zinc (Zn), cadmium (Cd) and lead (Pb) and to accelerate plant-host growth under either toxic-metal stress or control conditions. Fungi *Mucor* spp. was reported to upregulate the enzymatic antioxidant system comprising superoxide dismutase (SOD), catalase (CAT), and thus conferring Cd tolerance in

Arabidopsis thaliana plants (Sachdev et al., 2021). A number of species of fungal endophytes have been shown to positively affect the growth and fitness of plants, so attempts have been made to use these microorganisms in agriculture and phytoremediation. *Mucor* sp. they have also been shown to be able to decompose strigolactone (SL) in planta and downregulate the expression of SL biosynthesis genes.

Mucor has, to date, received little scientific exploration for its biocontrol potential as compared to *Trichoderma*. Ziedan et al. (2013) indicated the biological control potential of *M. hiemalis* on inflorescence brown rot of date palm. Microorganisms that show catalase activity are usually known to be highly resistant to environmental stresses by degrading excess H_2O_2 and maintaining reactive oxygen homeostasis during stress activities such as pathogen attack (Geetha et al., 2014). Additionally, the extraction and large-scale production of these enzymes could be useful in the manufacturing of good biocontrol products such as biofertilizer.

In conclusion, the *Mucor* genus is a promising biological control agent primarily through hyperparasitism and the production of effective microbial volatiles. The use of *Mucor* spp may provide an alternative means as an ecological approach to control plant diseases, ultimately helping to reduce the overall use of pesticides.

Cephaliophora was the genera most represented in BM sample, at the end of the SSF. Little is known about the physiological and biochemical characteristics of the genus *Cephaliophora* and family Ascodesmicaceae (Pezizales, Ascomycota). Originally Thaxter (1903) described two coprophilic species for the genus *Cephaliophora: C. tropica* and *C. irregularis* haxt. A third accepted species in the genus is C. uniformis Narayanan (1962), isolated from jute sacks. Three species rotifer-capturing species *C. longispora* and *C. muscicola* and C. navicularis have also been recently described (Ruszkiewicz-Michalska et al., 2017).

C. tropica is a non-pathogenic saprotrophic fungus isolated from soil, litter, manure, residual decaying plants and other materials (Zlotnikov etal., 2007). *Cephaliophora* grows a temperature between 20 ° C and 40 ° C in a pH range from 5 to 9. Rodríguez-Berbel et al., (2022) found that in a soil treated with an organic fertilizer, the most abundant fungal genera was *Cephaliophora*. Tosi et al., (2021) studying the long-term effect of nitrogen (N) fertilized soils, found that the fungal genera positively related to total N input were mainly Ascomycota, one of which was the saprophytic genus

Cephaliophora. This is in accordance with our study, as the high N content in BM samples are related to the higher abundance of *Cephaliophora*. Particularly, from t21 to t90 the BM sample showed a total content of N higher than B.

As regards the bacterial community, the significant different families at the end of the SSF treatment in samples B and BM were: Bacillaceae (*respectively OTUs 126, 468 adjusted P-value 0.0068)*, Nocardiaceae *(respectively OTUs 46, 3968 adjusted P-value 0.0083)*, Planococcaceae *(respectively OTUs 979, 3217 adjusted P-value 0.0152)*, Brucellaceae *(respectively OTUs 102, 1628 adjusted P-value 0.0191)* and Sphingobacteriaceae *(respectively OTUs 405, 1180 adjusted P-value 0.0208).*

The genus most present respectively for each family were: *Bacillus, Rhodococcus, Sporosarcina, Paenochrobactrum* and *Sphingobacterium*.

Members of the Bacillaceae family play key roles in soil ecology and plant health and growth stimulation. Many members of the Bacillaceae are saprophytes that participate in carbon, nitrogen, sulfur and phosphorus cycles in natural habitats, such as soil. Soil- and rhizosphere-inhabiting members of the *Bacillus* genus such as *B. subtilis, B. cereus*, and *B. mycoides* are known for their role as beneficial rhizobacteria promoting plant growth (act as biofertilizers) or protect plants from phytopathogens (function as biopesticides). Many *Bacillus* isolates can break down cellulose, hemicellulose, and pectin which suggests their involvement in the degradation and mineralization of plant and humic materials in the soil (Mandic-Mulec et al 2015). Furthermore, some *Bacillus* sp. can modify lignocellulose and, due to a variety of cellulase and xylanase activities, has shown a high potential for lignocellulosic decomposition (Mandic-Mulec et al 2015). The Bacillaceae family are reported to be the main producers of industrially important enzymes (xylanases) involved in lignocellulose degradation (Nagar at al., 2012). The Bacillaceae family was the best producers of xylanase. The ability of *Bacillus* species to produce extracellular xylanase from various organic residues as carbon sources, such as soybean extract, wheat bran, oat bran and rice bran was evaluated (Rodrigues et al., 2019). Furthermore, chitinase activity, which facilitates the degradation of fungal cells insect walls and exoskeletons, it is also common among many members of the Bacillaceae soil (Mandic-Mulec et al 2015). Moreover, inoculants prepared from endospore-forming Bacillus spp. have proven efficient and environmentally friendly as an alternative to chemical pesticides (Wu et al., 2015). Optimum growth temperature for *Bacillus* spp is 30-37 °C, with a

minimum temperature of 18 °C and a maximum of 43 °C; growth has been demonstrated over the pH range 4.9-9.3. *Bacillus* spp was observed at higher abundance in BM samples at t90.

The Nocardiaceae family is a member of the order Corynebacteriales (Goodfellow and Jones 2012) in the phylum Actinobacteria. They are aerobic, immobile, mesophilic, chemo-organotrophic bacteria with an oxidative metabolism. Microorganisms classified in the Nocardiaceae family have been isolated from different habitats, notably soil, coniferous litter, herbivorous manure, freshwater and marine sediments, and wastewater systems (Goodfellow, 2014). Recently there is growing interest in Nocardiaceae as a source of commercially significant primary and secondary metabolites and as agents of biodegradation and bioremediation (Goodfellow, 2014). One of the genera belonging to the Nocardiaceae family is *Rhodococcus* that received the most attention to the discovery of the production of useful enzymes biocatalysts in industrial processes. *Rhodococci* can utilize a wide range of compounds such as carbon source alone and can degrade a wide range of organic compounds (aliphatic and aromatic hydrocarbons, halogenated, polychlorinated biphenyls, nitroaromatics, heterocyclic, nitriles, sulfuric, steroids, herbicides) (Goodfellow, 2014).

Rhodococcus have immense potential in bioremediation (van der Geize and Dijkhuizen, 2004). The ability of *Rhodococcus* to grow in polluted habitats makes them serious candidates for bioremediation of industrially polluted sites and for the management of agricultural and urban waste (Goodfellow, 2014). Genus *Rhodococcus* is able to maintain significant degradation activities at a wider range pH (from 3 to 11) and to growth at temperatures between 1° and 30 ° C, with optimum at 20° C.

R*hodococcus* spp is a phosphate solubilizing bacterium (PSB) able to produce plant growth promoting substances (PGPs) (Pereira and Castro, 2014). R*hodococcus* has been used successfully as a biofertilizer to improve growth in P-deficient soils, constituting an interesting alternative to the application of chemical P fertilizers. Interestingly, in sample BM, this genus is represented with a significantly higher number of OTUs at times t14, t56, t70 and t90 compared to sample B.

Planococcaceae, a family within the Bacillales order, includes 14 genera to which the genus *Sporosarcina* belongs, consisting of naturally occurring soil-borne bacteria found in this study. Achal et al., (2012) investigated the role of microbial calcite precipitated by *Sporosarcina* spp to clean up soil contaminated with arsenic (As III). The genus *Sporosarcina,* endophytic bacteria, has a high

urease activity. In fact, urease is a key enzyme that leads to the precipitation of calcite. *Sporosarcina* ureolytic bacteria couple calcification to their metabolic assimilation processes by scavenging protons. The presence of ammonium ions and the additional release of CO_2 into the surrounding medium increases the pH which accelerates the precipitation rate of the urease-induced calcite (Achal et al., 2012). *Sporosarcina* spp, able to induce precipitation of calcium carbonate in the environment it can be an environmentally friendly technology to prevent environmental pollution.
Janarthine and Eganathan (2012) observed the activity of promoting root hair growth, indoleacetic acid production (IAA) and nitrogen fixation induced by the endophyte *Sporosarcina* spp. Endophytic *S. aquimarina* promotes plant growth and produces plant growth promoting substances probably by means like plant growth promoting rhizobacteria (PGPR). The optimal growth temperature was 32°C and the optimal pH was 7.0. (Janarthine and Eganathan, 2012).
Given the strong urease activity of *Sporosarcina* spp, the higher total nitrogen concentration and urease activity in BM samples, well correlates with the higher abundance of this genus in BM samples at t14, t56, t90. Particularly, urease activity is considered a good indicator of nitrogen (N) mineralization, generating accessible N for plant growth (Sinsabaugh and Follstad Shah, 2012, Tabatabai, 1994).
The family Sphingobacteriaceae belongs to the phylum Bacteroidetes having representatives in practically all environments including humans, rhizosphere, soils, wastewaters, among others. Some genera of this family have demonstrated great potential as plant growth promoters, bioremediators in soil and producers of some value-added compounds such as carotenoids and antimicrobials. (Figueiredo et al., 2021). Some genera are well-known as plant growth promoters since several strains have been described as promising agents for improving crop yields. For instance, *Sphingobacterium* spp. can grow at 16–37 °C (optimum 32 °C), at pH ranges of 6–8 (optimum growth occurs at pH 7) and in 0–4 % (w/v) NaCl; and can induce plant's antioxidant systems and energy metabolism allowing them to cope with salinity-induced toxicity (Vaishnav et al., 2020; Fan et al., 2020). Puentes-Téllez et al., (2020) used two sources of lignocellulose (sugarcane bagasse and straw) bagasse enrichments, to obtain lignocellulose-degrading bacteria through an enriched process. The one of the abundant taxa with a strong and positive relationship with lignin and cellulose degradation were the Sphingobacteriaceae ($P < 0.05$) (Puentes-Téllez et al., 2020). This family has

been found to carry enzymatic capacities to degrade lignin (Duan et al., 2014) and hemicellulose fractions of wheat straw (Jiménez et al., 2015) but also as acting like “cheaters” during the degradation process, helping to remove the cello-oligosaccharides produced by polymer degraders (Jiménez et al., 2014). In this study the *Sphingobacteriaceae* family was present with significantly higher number of OTUs at times t14 and t90 in the BM sample compared to B. Genus *Sphingobacterium* could be used to degrade the lignocellulosic substance of agricultural waste.

4.5 Conclusion

In this work it was found that the microorganisms present in the bioinoculant M were not able to grow on BSG and to overcome the competition with the BSG spontaneous microbiota. In fact, the OTUs identified in the bioinoculant were no longer detected in inoculated BSG (BM) already after 7 days of SSF. This suggests that it is mandatory to study the natural microbiota of a by-product used as a growth substrate, to understand the compatibility with a specific bioinoculant. On the other hand, results obtained showed that the addition of the inoculant shaped the spontaneous microbiota of BSG, that resulted enriched of microbial genera with multiple potential: from soil fertility to protection from harmful agents (insects, micro and mesofauna), and from fungal diseases to plant biostimulation. In addition, in the BM sample at t90 some significant chemical and biochemical differences were observed in comparison with the uninoculated BSG (B). In BM_{T90} higher levels of total nitrogen were detected, greater consumption of total carbon, with the consequent reduction of the C / N ratio compared to B_{T90}. Furthermore, the BM_{T90} sample showed greater dehydrogenase activity and a greater presence of dissolved organic carbon. Thus, the bioinoculant M influenced the substrate and the microbial community of BSG, and further studies are needed to understand the nature of the interactions between the microbial communities of raw materials and the bioinoculant.

Furthermore, agronomic tests will be set up to determine the possible use of BSG enriched with microorganisms in agriculture, directly as an organic fertilizer in the soil.

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5. General conclusion

5. General conclusions and perspectives

Brewers' spent grain is regularly available in large quantities at a low market price. Furthermore, it is an interesting raw material due to its wealth of valuable compounds and nutrients. These are all key inputs for the development of a multitude of applications that range from biotech production of value-added goods, functional foods and animal feeds, to the generation of other goods of interest to the pharmaceutical and agricultural sectors. A better understanding of the potential of microorganisms as biocatalysts for the transformation of BSG is essential for its recycling and exploitation, particularly in view of the reduction of its carbon footprint under the concept of a circular economy.

In this PhD study, detailed and in-depth bibliographic research was initially carried out on the role of microorganisms in the biotransformation of BSG. The microbiota of fresh and dried BSG were then studied, and a spontaneous microbiological process (low input) was optimized for the pre-treatment of BSG before vermicomposting. The hygienic-sanitary, phytosanitary quality and phytotoxicity of the vermicompost obtained were analyzed. In addition, a study was conducted on the potential use of BSG as a growth medium **as a no-cost substrate for the production of beneficial microbial consortia** based on biofertilizers.

In Saba et al. (2019), oven dried BSG was a valuable feed for earthworms. The vermicompost obtained showed enhanced mineralization and stabilization, with a reduction in particular of the C/N ratio and an increase in total humic substances. Analysis of the vermicompost showed physical, chemical, and microbiological characteristics equivalent to, and for some parameters, more than, vermicompost obtained from cattle manure alone. Oven drying at about 45 °C for 48 h was the most effective stabilization method in Saba et al. (2019), to prolong the shelf-life of BSG. However, oven drying has a very high energy cost, making it inadequate for small breweries. Furthermore, heat treatment standardizes the microbial community. How sustainable is it for microbreweries to dry their BSG before vermicomposting it? How can BSG be enhanced in a sustainable way? Thus, it was decided to use less energy-intensive methods to increase the sustainability of this brewing waste and to reduce gas emissions, through a low input microbiological process of BSG microbiota.

What are the risks and benefits of a biological "spontaneous" pre-treatment of BSG? On the one hand, the "natural" occurring microbiota can be advantageous for the vermicomposting

biotechnology and for the increase in the vermicompost bio-stimulation towards plant growth. On the other hand, it is necessary to avoid BSG contamination by mycotoxigenic fungi that can produce mycotoxins. In Bianco et al. (2021), it was shown that low-input pre-vermicomposting treatment is a practice that allows waste BSG to be managed with zero impact, to allow breweries to cover the costs of disposal.

The pre-treatment allowed the growth of the naturally associated fungal and bacterial species of the BSG that eventually shaped the microbiota of the mature vermicompost. During the pre-treatment, the BSG microbiota was enriched in bacterial and fungal species of significant biotechnological and agronomic potential, including plant-growth-promoting bacteria, antibacterial microorganisms, and biostimulating and entomophathogenic fungi.

The vermicompost obtain from the pre-treated BSG showed good indicators of maturity and respected the legal requirements related to pathogenic microorganisms and mycotoxins, as well as phytotoxicity. This is a basic requirement for a high quality vermicompost that is to be placed on the market.

The methodology proposed here is interesting from an environmental point of view as:

- the microbiota of BSG and the vermicompost were characterized.

-a collection of bacterial, yeast and fungal strains has been created that can be used for the selection of biocatalysts starters for stabilization and transformation processes of BSG and other by-products of barley and malt processing.

- a lignocellulosic production waste can be recycled in an eco-sustainable way. No pathogenic microorganisms were detected in the stabilized BSG. In particular, the vermicompost obtained showed good indicators of maturity and complied with legal requirements. Finally, the identification of the main bacterial and fungal species constitutes a useful database for the further exploitation of BSG.

For the use of BSG as a low-cost growth substrate, BSG is not only a substrate that supports the growth of microorganism-based preparations, but also enriches their composition with beneficial microorganisms for the soil and for plants. All microorganisms grown on agro-industrial by-products and further introduced into soil–plant systems have several advantages, including enrichment of

the soil with organic matter, metabolites, and minerals (P), and they can be incorporated into soil maintenance routines. It should be noted that this type of biotechnological approach that is potentially aimed at increasing the growth and productivity of beneficial plant microorganisms is an emerging field of research.

An important aspect for beer production of the future is the creation of a circular economy based on agriculture and sustainable processing, which allows the production and processing of agricultural products in a profitable, competitive, and efficient way, while responding to the emerging need for protection of the environment and natural resources. In Sardinia, the craft beer sector is booming and is showing exponential growth in economic terms. It can be understood how fundamental it is also for Sardinian craft breweries to try to produce and create a beneficial supply chain from an economic point of view, which can create added value equally distributed along all the sectors of the supply chain. The possibility of recovering and enhancing BSG directly in the company would bring significant savings in production costs. Currently, many breweries have agreements with agricultural companies to which they give their BSG free of charge as livestock feed.

The search for alternative uses for BSG represents a big step forward in the circular economy perspective, not only for breweries, which would benefit from the enhancement of a by-product, but also for other companies that would expand their offers with products that respond to the concept of sustainability. An example is the creation of Business Networks as a privileged tool for the dissemination of system sustainability. Several partners pursue the aim of individually and collectively increasing their innovative capacity and their competitiveness in the market. To this end, based on a common network program, the partners collaborate in predetermined forms and areas relating to the operation of their businesses or to the exchange of information or services of an industrial, commercial, technical, or technological nature, or to exercise one or more of these in common. These represent multiple activities that fall within the scope of their business. A network contract makes it possible to share innovative ideas, know-how and services, rationalize costs, and fill the gaps of a small company size. However, it only makes sense in the hypothesis in which a beneficial organization is established, in which the interactions between the partners creates greater value than what the individuals could produce on their own.

In the case of beer production, the criticality is to dispose of by-products such as BSG, and also of the exhausted yeast and wastewater. Here the opportunity arises to create income through the formulation of new soil improvers or to purify wastewater with the help of microorganisms. In this context, the soil improver produced and the purified water can be used in the local cultivation of hops, or in the cultivation of barley and wheat, with a view to local production, with a short supply chain that is environmentally friendly.

In perspective, we may see more interesting new BSG-based applications in the future. Given the biotechnological and sanitary importance of the various microbial groups that have been isolated from BSG, it is essential to study the metabolic relationships between the different microbial communities and their influences on the final transformation of the BSG, to exclude, or conversely to promote, these new processes. The next step is to evaluate the effectiveness of the vermicompost obtained from the BSG for the production of organic vegetables, sod turf, and ornamental plants. This will be compared with other soil improvers obtained from BSG, such as compost and biochar, and the possibility of replacing peat will be evaluated.

Providing innovative solutions in the valorization of production waste will serve to increase the quality of brewing production, along with the profitability and environmental sustainability of craft microbreweries.