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

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Angela Bianco, Francesco Fancello, Matteo Garau, Mario Deroma, Alberto S. Atzori, Paola Castaldi, Giacomo Zara*, Marilena Budroni.

Department of Agricultural Sciences, University of Sassari, 07100 Sassari, Italy

***Corresponding author: Giacomo Zara**

Department of Agricultural Sciences

Viale Italia, 39

University of Sassari

07100 Sassari, Italy

Email: gzara@uniss.it

Tel: +39-079-229276

ORCID: https://orcid.org/0000-0001-5302-7424

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

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.

Key words: Beer wastes; by-products; microbiota; biological pre-treatment; fertilizer.

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

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

78 responsible for the sanitization of the composts, but severely reduces the microbial
79 activity of the final product (Assandri et al., 2021a; 2021b).
80 Vermicomposting is an eco-sustainable process in which the complex
81 interactions between microorganisms and earthworms are the basis of fragmentation,
82 bio-oxidation, and stabilization of organic wastes (Lazcano et al., 2008). In contrast to
83 composting, vermicomposting is carried out at lower temperatures (25-40 °C), neutral
84 pH, and higher humidity (70-90 %) to allow earthworms' growth (Tognetti et al., 2005).
85 In addition, these temperatures preserve the activity of mesophilic microorganisms
86 fundamental for organic matter decomposition and soil fertility, such as
87 Gammaproteobacteria, Actinobacteria, and Actinomycetes. Thus, vermicompost has a
88 greater market acceptance and higher value than compost due to higher nutrient
89 content and microbial activity (Tognetti et al., 2005). Many genera of Actinobacteria
90 produce antibiotics, which inhibit or eliminate pathogenic bacteria such as *Salmonella*
91 sp. and *Escherichia coli*. However, the absence of a thermophilic phase makes it
92 mandatory to guarantee the safety of the vermicompost by specific microbiological
93 analyses.
94 In recent years vermicomposting has received much attention as an efficient
95 and low-cost means for the processing of organic waste of various origins. Indeed,
96 earthworms are highly adaptable to a broad range of environmental conditions, and
97 they can consume organic materials with pH from 5 to 8, moisture content between
98 65% and 70%, and an initial C/N ratio of around 30. Many different lignocellulosic
99 wastes can be converted into high-value humic-like substances through
100 vermicomposting. These have included tree prunings, disposable paper cups, brewers'

spent grains, crop residues, medicinal herbal residues, and leaf litter (Singh et al., 2021; Saba et al., 2019; Sharma and Garg, 2019). 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 and 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 and 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 hours 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

123 (Kucharska et al., 2018) have been used to reduce the resistance of the substrate to
124 biodegradation and to improve the composting process.
125 However, the economic feasibility of pre-treatment methods that require high
126 or low temperatures, or the need to add microbial starters to enhance the
127 performance of composting and vermicomposting, is currently questioned due to the
128 high associated costs.
129 Alternatively, to increase the sustainability of the process, the pre-treatment of
130 wastes could rely on the proper exploitation of their naturally associated microflora.
131 Indeed, the indigenous microbial communities of specific wastes are intrinsically
132 diverse and well adapted to the substrate, and they can be composed of taxa with
133 relevant metabolic traits for the agricultural sector (Oljira et al., 2018).
134 In this context, the microbiota of fresh BSG remains largely unknown, and only
135 a few reports have described it in terms of broad microbial categories (i.e., aerobic
136 mesophilic bacteria, yeasts and molds, strictly anaerobic bacteria, etc.) without
137 providing any further details on the genera and species present (Robertson et al.,
138 2010). Brewers' spent grain results from the brewing process that is characterized by
139 high temperatures, addition of natural antimicrobials (e.g. hop iso-α-acids), and a sub-
140 acidic pH. Thus, no human pathogens have been found to survive in BSG after the
141 brewing process (Bianco et al., 2020). However, BSG can support the growth of
142 mycotoxigenic fungi, such as *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp., as
143 these can already contaminate the raw materials (Bianco et al., 2019; 2018).
144 Within this frame of reference, this work aimed to evaluate, from a
145 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.

169

170 **2.2. Vermicomposting of pre-treated brewers' spent grain**

171 Vermicomposting was carried out according to Saba et al. (2019). Briefly, plastic
172 containers (bed) were filled with 500 g pre-treated BSG mixed with 500 g cow manure
173 (1:1), to which 15 earthworms (*Eisenia fetida,* Savigny, 1826) were added. After
174 completion of worm-cast production (30 days) and curing (30 days), triplicate
175 vermicompost samples were analyzed for their chemical, biochemical, and
176 microbiological characteristics.

177

178 **2.3. Chemical and biochemical characterization of brewers' spent grain and**
179 **vermicompost**

180 Brewers' spent grain was analyzed as detailed by Correddu et al. (2019), as dry matter
181 (DM) content (oven-drying, 105 °C for 24 h), neutral detergent fiber (NDF), acid
182 detergent fiber (ADF), and acid detergent lignin (ADL) (Ankom 220 fiber analyzer;
183 Ankom Technology, Fairport, NY, USA), using the Association of Official Analytical
184 Chemists (AOAC) method 973.18 (AOAC Official Method of Analysis, 1990), according
185 to Robertson and Van Soest (1981). Crude protein (CP) content was determined using
186 the Kjeldahl method (AOAC Official Method of Analysis, 2000; method 988.05), extract
187 ether (EE) using the Soxhlet method (AOAC Official Method of Analysis, 2005; method
188 920.39), and ash using a muffle at 550 °C (AOAC Official Method of Analysis, 2000;
189 method 942.05). NDF was measured using heat stable amylase, and is expressed as
190 exclusive of residual ash, and ADL was measured using solubilization of cellulose with

191 sulphuric acid. Non-fiber carbohydrate (NFC) was estimated using Equation (1), as
192 reported by Weiss (1999):
193
194 $$NFC\ (\%DM) = 100 - (NDF + CP + ash + EE) \quad (1).$$
195
196 In BSG and vermicompost samples total carbon and total nitrogen contents
197 were determined by combustion of dried samples in an elemental analyzer (CHN 628;
198 Leco, St. Joseph, Michigan, USA) with the calibration references of oatmeal (502-276;
199 Leco) and soils (Soil LCMR, 502-697; Soil calibration sample for CSN, 502-814; Soil LRM,
200 502-062; Leco). $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ nitrogen were determined following the official
201 method of soil chemical analysis, as the standard protocols defined by D.M.
202 13/09/1999, G.U. n° 248, 21/10/99.
203 Dehydrogenase activity was measured for 10 g samples after incubation at 37
204 °C for 24 h (Alef and Nannipieri, 1995), and is expressed as μg triphenyltetrazolium
205 formed/g/h. Dissolved organic carbon (DOC) was quantified after 24 h agitation of a
206 1:10 (w/v) sample in suspension in deionized water. The liquid phase was then filtered
207 off, and its absorbance at 254 nm was determined. The mycotoxins deoxynivalenol, T-
208 2, HT-2, fumonisins, aflatoxin, and ochratoxin A, were quantified using commercial
209 ELISA kits (Bio-Shield: fumonisin, 0-6 ppm; ochratoxin, 0-40 ppb; T-2/ HT-2, 0-500 ppb;
210 total ES, 0-10 ppb; deoxynivalenol M.E., 0-5 ppm; Prognosis-Biotech, Or-Sell, Modena,
211 Italy).
212

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 (Italian Legislative Decree 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-Levina et al. (2013). Enumeration of *Escherichia coli* was according to ISO 16649-2: 2001. Incidence of *Salmonella* spp. was determined as described by 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 et al. (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 Equation (2):

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

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

281 The influence on the composition of the microbial communities of the time of
282 collection during the pre-treatment process was evaluated by permutational
283 multivariate ANOVA (PERMANOVA) and visualized graphically by multivariate
284 nonmetric multi-dimensional scaling analysis.
285
286 **3. Results**
287 **3.1. Evolution of brewers' spent grain chemical and biochemical parameters during**
288 **the pre-treatment**
289 The four BSG samples differed considerably based on their microbreweries of
290 origin (Table S2). The structural carbohydrates (i.e., NDF, including hemicellulose as
291 NDF-ADF, cellulose as ADF-ADL, and lignin as ADL; Table S1) accounted for more than
292 50% of BSG dry matter composition for MB1, MB3, and MB4, and 38% for MB2. Crude
293 protein content and NFC ranged from 18% to 25% and from 5.53% to 37.25%,
294 respectively. Large differences were also seen for ADL content, with MB1 and MB2
295 ~40% lower than MB3 and MB4. Fresh BSG samples did not support the growth of
296 earthworms due to pH values below 5 and above 8, after 1 and 5 days from the
297 beginning of their uncontrolled spoilage (Figure 1S). Thus, a low-input pre-treatment of
298 BSG was here carried out and monitored for 51 days according to a range of chemical
299 and biochemical parameters (Table 1).
300 During the pre-treatment, the differences in the BSG samples across the
301 breweries were greatly reduced. This resulted in standardization of the final product
302 due to the almost identical dynamics of the evolution of the chemical parameters. The
303 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 (Figure 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 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 Figure 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).

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, 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 5,000 CFU/g (L.D. 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 (Figure 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*.

372 Similarly, four of the fungal species identified were common between the BSG and
373 mature vermicompost: *Trichoderma reesei, Pichia cecembensis*, *Verticillium dahliae,*
374 and *Pichia fermentans*.
375
376 **4. Discussion**
377 Vermicomposting has been suggested as a valuable method to exploit BSG, which is an
378 important by-product of the brewing industry (Saba et al., 2019). During
379 vermicomposting of BSG, the decrease of the total organic carbon was accompanied
380 by an increase in the total nitrogen, indicative of an advanced degree of organic matter
381 stabilization. Vermicomposting also enhanced the amounts of organic components
382 recalcitrant to microbial degradation, given the increases in humic-like substances
383 (Saba et al., 2019). From a microbiological perspective, earthworm activity during
384 vermicompost of BSG led to the enrichment in Firmicutes, Actinobacteria, and
385 Betaproteobacteria involved in cellulose degradation as well as N-fixation, nitrate
386 reduction, and ammonia assimilation (Budroni et al, 2020). Overall, the vermicompost
387 from BSG represented an excellent substrate that could be safely used as a soil
388 improver (Saba et al., 2019). However, the raw BSG had to be pre-treated before
389 vermicomposting as it didn't support the growth of earthworms. Saba et al. (2019)
390 used a pre-processing method consisting of the oven-drying of BSG at 45 °C for 48 h.
391 Given the high energy input required, the overall vermicomposting process resulted
392 less attractive from a sustainability perspective. To overcome this limitation and to
393 increase the quality of the final product, the suitability of a more sustainable pre-
394 treatment method that relies on the exploitation of BSG naturally-associated

395 microbiota was here carried out. The advantages of this pre-treatment, over various
396 physical and chemical methods for BSG stabilization, are the potential production of
397 useful microbial by-products, less formation of inhibitory substances (due to milder
398 conditions), minimization of chemical applications, and energy input, and lower costs
399 for waste disposal. In addition, the biological pretreatment of lignocellulosic wastes,
400 such as BSG, is assumed to be the eco-friendliest technique, with mild environmental
401 conditions and low operational costs (De Bhowmick et al., 2018).

402 *Chemical dynamics during the pre-treatment*

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

408 The reduction in the C/N ratio from that of the raw BSG was related to the
409 significant increase in the ammonium nitrogen content. The nitrogen kinetics during
410 the pre-treatment can be attributed to the activity of the microbiota identified on the
411 BSG samples, and in particular to the bacterial species involved in the nitrogen cycle.
412 *Stenotrophomonas pavanii* can fix atmospheric nitrogen (N_2) into ammonia (NH_3),
413 which is rapidly converted into ammonium (Ramos et al., 2011). In contrast,
414 *Pseudomonas stutzeri* is a facultative anaerobic bacterium that favors the release of
415 molecular nitrogen (N_2) through denitrification of nitrate (NO_3^-) through the reduction
416 of organic matter (Lalucat et al., 2006). Given the low levels of nitrate and the high
417 levels of ammonium nitrogen measured during the pre-treatment of BSG, the activity

418 of the nitrogen-fixing bacteria would be predominant. Ammonia production is higher
419 at high aeration rates (De Guardia et al. 2010), temperatures, and pH (Pagans et al.
420 2006). Accordingly, the higher production of ammonium nitrogen was measured in
421 BSG at temperatures >22 °C and pH >7 (i.e., at T2, T3).
422 The correlation between ammonification and biodegradation of organic matter
423 has been poorly studied, although peak ammonia emissions are usually seen when
424 biological activity is at its maximum (De Guardia et al., 2010). Accordingly, DHG activity
425 increased during the pre-treatment of BSG and reached its highest at T3. As the DHG
426 enzymes in soil are not in a free form, this represents the activity of live intact cells
427 (Alef and Nannipieri, 1995). Moreover, DHG levels serve as an indicator of
428 microbiological redox systems and can be considered as a good and adequate measure
429 of microbial oxidative activity (Garau et al., 2019). Thus, the high DHG activities
430 measured for BSG at T3 underline an intense microbial activity, which is related to the
431 decomposition of polymeric materials to provide simple and soluble compounds that
432 can be metabolized easily.
433 This suggests that after this pre-treatment, BSG still requires further maturation
434 (e.g., vermicomposting) to reach adequate stability for its use as a soil amendment.
435 Therefore, pre-treated BSG was mixed with cow manure (1:1) and subjected to
436 vermicomposting according to the protocol described by Saba et al (2019). This study
437 by Saba et al. (2019) is, to the best of our knowledge, the only vermicompost reported
438 in the literature that was obtained from BSG. With respect to their study, the mature
439 vermicompost here obtained had similar total nitrogen content and pH, while the C/N
440 ratio measured in the present study was a little higher, and like that of organic and

441 mature vermicompost obtained from animal manure (Table S3). Indeed, a C/N ratio of
442 11 falls within the legal limit required by Italian law for marketed vermicompost
443 (Legislative Decree 75/2010). These data, and the lower DHG activity in the
444 vermicompost from biologically pre-treated BSG, indicated that the pre-treatment
445 suggested here allows a final product to be obtained that is more stable than that
446 reported by Saba et al. (2019).
447 The stabilization of DHG activity in the mature vermicompost can be attributed
448 to the complete metabolization of the easily degradable organic substances (Garau et
449 al., 2019). Thus, it appears that the microbiota of the BSG vermicompost, which has
450 reached an internal equilibrium, would not significantly modify the dynamics of the
451 microbial communities present in the soil when the vermicompost is used as an
452 amendment. The stability and maturity of vermicompost from the pre-treated BSG are
453 also confirmed by the low DOC. The DOC is a chemical indicator of the molecular
454 transformation of dissolved organic matter that is suitable for the definition of the
455 stabilization of vermicompost (Nigussie et al., 2017). An organic amendment with a
456 high DOC can cause severe damage to crops, as it will continue to consume oxygen,
457 which will hinder root respiration and lead to the production of phytotoxic
458 compounds, such as SH_2. A DOC <10 g/kg indicates stability and maturity of the
459 amendment (Mohanty et al., 2013), and a DOC <4 g/kg is considered safe for plant
460 growth (Gómez-Brandón et al., 2008). Indeed, vermicompost from the pre-treated BSG
461 does not show significant phytotoxicity against the *L. sativum* L. (cress) seeds,
462 according to European Regulation (EC 2003/2003; EC 1009/2019) and Italian
463 Legislative Decree n° 75/2010. In particular, the Annex 2 of Legislative Decree n°

464 75/2010 requires a germination index >60% for a dilution of 30% (30% extract, 70%
465 water). The same minimum requirement is indicated by the Italian Composting
466 ConsorAsd previtium.
467 *Dynamics of microbial communities during BSG pre-treatment*
468 As previously stated, the rationale of the pre-treatment method here described
469 was to emphasize the activity of the microbiota that is naturally associated with BSG.
470 Indeed, the mild temperature (< 32°C), as well as the high humidity (70%) during the
471 pre-treatment, allows the preservation and the development of microbial species
472 sensitive to high-input processes (i.e., oven-drying or freezing). Both fungal and
473 bacterial communities were monitored during the pre-processing and in the mature
474 vermicompost.
475 Fungi produce several extracellular enzymes and molecules that play key roles
476 in the biodegradation and hydrolysis of polymeric substances such as cellulose
477 hemicellulose and lignin (Wagner et al., 2018). The degradation of lignin by fungi is due
478 to the activity of peroxidases and laccases (Rhamati et al., 2020). In addition, fungi can
479 contribute significantly to the bioremediation of contaminated soils (Ferrari et al.
480 2011). Among fungi, yeasts act as plant growth promoters and biological control
481 agents against soil-borne plant pathogens (Cantrell et al. 2011; Miceli et al. 2011). In
482 this study, an enrichment of “true yeast” belonging to Saccharomycetales
483 (*Blastobotrys* spp, *Galactomyces pseudocandidus*, *Pichia fermentans*, *Pichia*
484 *cecembensis*, *Candida* spp.) was observed during the pre-treatment of BSG (T2 and T3).
485 *Galactomyces* exhibited significant antagonistic activity against the fungal plant
486 pathogen *Glomerella cingulata* (Fu et al, 2016). *Pichia* spp. isolates were suggested as

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 and 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

533 to the production of antibiotic compounds able to limit the growth of target species,
534 thus shaping the overall soil microflora. For instance, soil isolates of *Enterococcus*,
535 *Pediococcus*, and *Weissella* spp., produced bacteriocin-like substances and showed
536 antimicrobial activity against Gram-positive bacteria(Chen et al., 2005)**.**
537 Acidobacteria and Actinobacteria are both involved in organic matter turnover
538 and carbon cycling as they can utilize different carbon sources, ranging from simple
539 sugars to more complex substrates, such as hemicellulose, cellulose, and chitin (Kielak
540 et al., 2016; Fließbach et al., 2007). The abundance of these bacterial phyla is
541 dependent on soil chemical characteristics. Particularly, nitrate had a positive
542 correlation to Acidobacteria and a negative correlation to Actinobacteria(Li et al.,
543 2020)**.** Thus, it is conceivable that the increasing concentrations of nitrate during BSG
544 pre-treatment are responsible for the decline in Actinobacteria and the increase in
545 Acidobacteria populations. The dominance of *Pseudoxanthomonas* spp. and *Bacillus*
546 during the first stages of BSG pre-treatment could have resulted in the degradation of
547 complex organic compounds, thus increasing the concentration of simpler and easily
548 metabolized compounds (Wang et al, 2011; 2010). This could have influenced the
549 occurrence of Chloroflexi, which are less competitive in a rich and diverse community
550 but thrive in stressed environments (Li et al., 2020). On the contrary, the increasing
551 concentrations of nutrients could be related to the dominance of Bacteroidetes during
552 the last stages of BSG pre-treatment. Particularly, Bacteroidetes can colonize many
553 different habitats such as soil, activated sludge, decaying plant material, compost,
554 dairy products, and diseased animals (Thomas et al., 2011).
555 *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. (2004) 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 phytopathogens 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 (pH 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 feeds (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.

602 At the species level, the most represented were *Weissella cibaria* and *Bacillus*
603 *subtilis*, which have been also detected in BSG, thus remarking the importance of the
604 microbiota of raw material in shaping that of the final product. Finally, the
605 vermicompost obtained from pre-treated BSG respected the limits indicated by Italian
606 Legislative Decree 75/2010 regarding the absence of pathogenic bacterial species.
607
608 **5. Conclusions**
609 This study evaluated an eco-sustainable process for the pre-treatment of BSG before
610 its vermicomposting. The procedure proposed here requires limited or no external
611 inputs as energy or microbial starters, and it favors the stabilization of BSG for further
612 processing. In addition, this pre-treatment of BSG resulted in accelerated
613 decomposition of the waste, with reduced time required to obtain high-quality
614 vermicompost. Indeed, the vermicompost showed good indicators of maturity and
615 respected the law requirements related to pathogenic microorganisms and
616 mycotoxins, as well as phytotoxicity. Finally, the pre-treatment allowed the growth of
617 BSG naturally-associated fungal and bacterial species that eventually shaped the
618 microbiota of the mature vermicompost, thus increasing its biological quality.
619
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845

846 **Table 1.** Chemical analysis of the BSG samples during the pre-treatment (n=12).

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
$N\text{-}NH_4^+$ nitrogen	g/kg	0.08 ±0.04	1.22 ±0.77	5.40 ±3.87	9.00 ±3.41
$N\text{-}NO_3^-$ 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

847 Data are means ±standard deviation across the four breweries
848 nd, not detected; TPF, Triphenylformazan
849

850 **Table 2**. Alpha-diversity indices of the bacterial and fungal communities during the BSG pre-

851 treatment.

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]

852 Data are means ±standard deviation, across the four breweries

853 Means with different superscript letters are significantly different within columns as

854 determined by ANOVA followed by Tukey HSD test ($p < 0.05$)

855

856 **Table 3**. Chemical, biochemical, microbiological and quality parameters of the vermicompost
857 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
	$N\text{-}NH_4^+$ nitrogen	g/kg	0.02 ±0.01
	$N\text{-}NO_3^-$ 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	11.9×10^6 ±95.4
	Lactobacilli		2.14×10^6 ±17.1
	Actinomyces		6.32×10^6 ±50.6
	Aerobic sporigens		162×10^6 ±130
	Anaerobic sporigens		100×10^6 ±801
	Pesudomonas spp.		12800 ±5.13
	Total fungi		44200 ±3.98
	Total yeast		34400 ±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

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

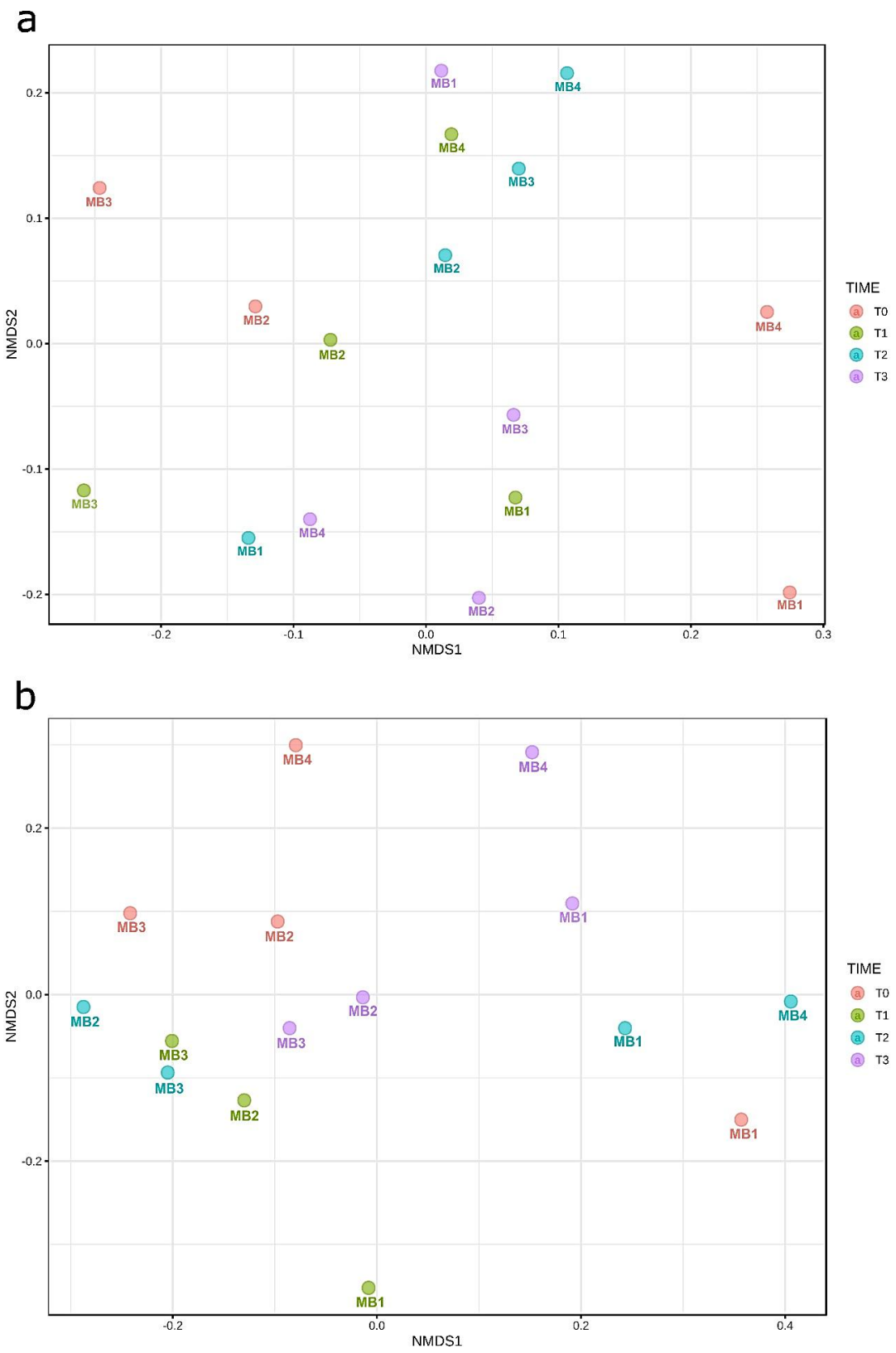


Figure 1. Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distances for the bacterial (**a**) and fungal (**b**) communities.

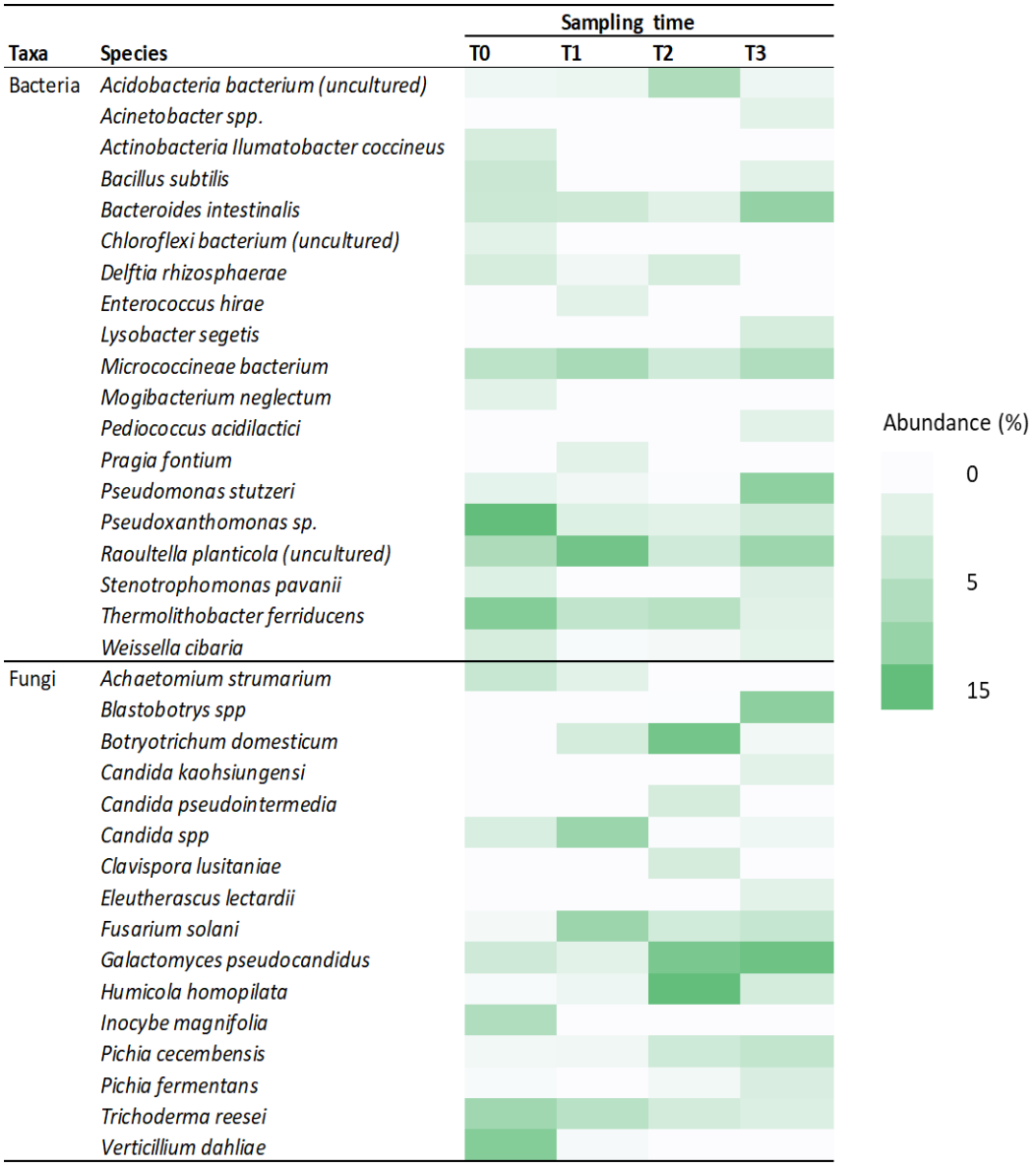


Figure 2. Relative abundance of fungal and bacterial species identified during the BSG pre-treatment. The intensity of DGGE bands (i.e., peak density) was used to derive quantitative values. T0 (6 h after microbrewery production), T1 (17 days), T2 (34 days), and T3 (51 days).

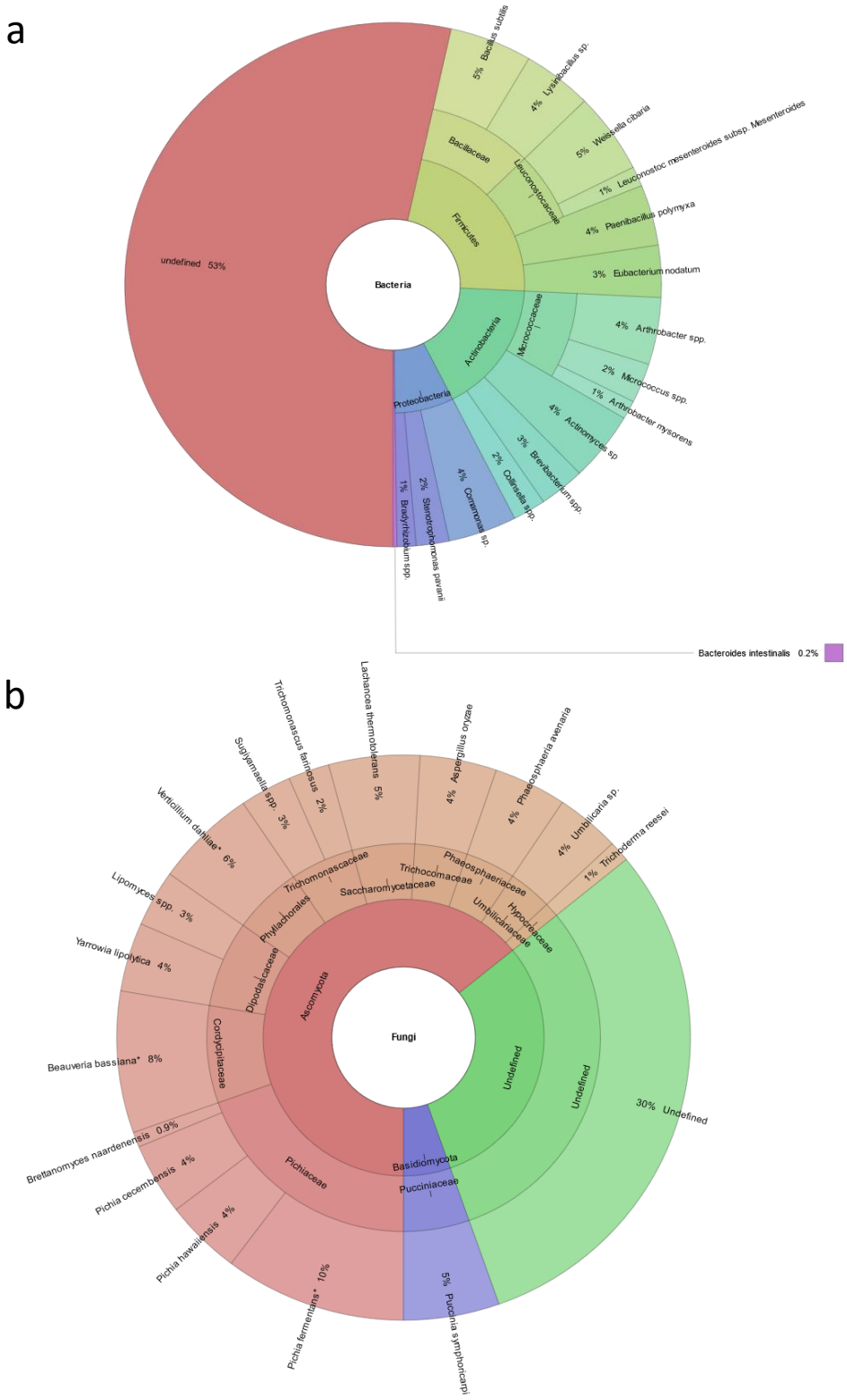


Figure 3. Bacterial (**a**) and fungal (**b**) communities of mature vermicompost obtained from pre-treated BSG. The intensity of DGGE bands (i.e., peak density) was used to derive quantitative values

SUPPLEMENTARY MATERIAL

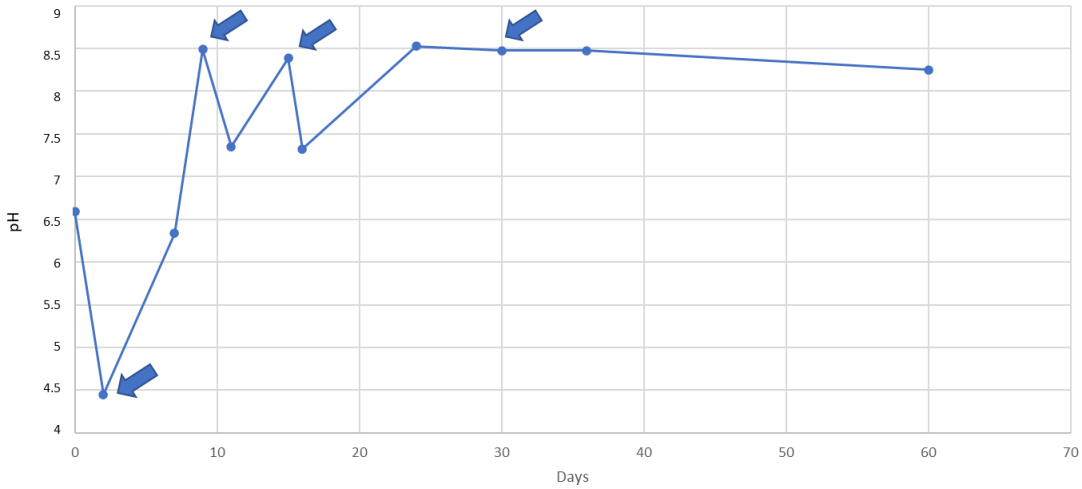
Supplementary Table S1. PCR primers used in this study.

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 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.