

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

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

3

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23

24 **Highlights**

25

26 • low-input pre-treatment of brewers' spent grain is eco-sustainable

27 • low-input pre-treatment of brewers' spent grain shows accelerated decomposition

28 • Vermicompost from pre-treated brewers' spent grain has good indicators of

29 maturity

30 • Vermicompost from pre-treated brewers' spent grain respects the legal

31 requirements

32 **Abstract**

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

53

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

## 55 **1. Introduction**

56

57 Sustainable agriculture promotes soil health and productivity by favoring the use of  
58 organic fertilizers, and by reducing the dependence on non-renewable resources, such  
59 as petroleum-based fertilizers (Wang et al., 2020). Suitable substrates to produce  
60 organic fertilizers include lemon prunings and cereal straw and bran, and urban,  
61 horticultural, olive, palm, grape, cotton, and brewery wastes (Assandri et al., 2021;  
62 Budroni et al., 2020; Bianco et al., 2020; Saba et al., 2019; Mtui, 2009).

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

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

76 In composting, indigenous microorganisms transform organic matter through  
77 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'

101 spent grains, crop residues, medicinal herbal residues, and leaf litter (Singh et al.,  
102 2021; Saba et al., 2019; Sharma and Garg, 2019). Contrary to animal wastes, crop and  
103 plant wastes are better suited to sub-mesophilic treatments as they are less subjected  
104 to contamination by human and animal pathogens.

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

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

116           Pre-treatments with physical methods have also been proposed to reduce the  
117 spontaneous microbial load of wastes (Rahmati et al., 2020). In particular, drying of  
118 BSG at 45 °C for 48 hours has been shown to make it suitable for direct  
119 vermicomposting (Saba et al., 2019). Also, inoculation of BSG with specific microbial  
120 taxa has been proposed, which can include aerobic and anaerobic cellulolytic bacteria  
121 of the genera *Cellulomonas*, *Pseudomonas*, and *Streptomyces* (Wei et al., 2019).  
122 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- $\alpha$ -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

146 to make BSG palatable for earthworms. This process was carried out without expensive  
147 thermal treatments or chemical and microbiological additives to fully exploit the  
148 microflora naturally associated with raw BSG. Finally, the mature vermicompost from  
149 pre-treated BSG was evaluated through microbiological and chemical analysis, as well  
150 as germination tests, to assess its suitability as a soil conditioner and fertilizer.

151

## 152 **2. Materials and methods**

153

### 154 **2.1. Sampling and pre-treatment of brewers' spent grain**

155 Brewers' spent grain was collected from four microbreweries (MB1, MB2, MB3, MB4)  
156 in Sardinia (Italy), as 15 kg from each microbrewery. These BSG samples were filtered,  
157 divided into sterile plastic bags, and kept at 4 °C until further processing. From these  
158 BSG samples, 500 g aliquots were placed into sterile plastic containers (23 × 15 × 3 cm)  
159 at 22°C in a thermostatic room, with the humidity kept constant at ~70% by periodic  
160 turning and addition of sterile distilled water (every 3 days). Each BSG sample was pre-  
161 treated in triplicate containers. During this period, temperature, pH, and humidity  
162 were monitored daily by averaging three measurements taken at three different points  
163 (at the center, and at the two ends of the containers) at 2 cm from the top layer of  
164 BSG. Samples were taken in triplicate at 17-day intervals, as T0 (6 h after  
165 microbrewery production), T1 (17 days), T2 (34 days), and T3 (51 days). These samples  
166 were subjected to chemical, biochemical, and microbiological characterization. After  
167 51 days of pre-treatment, determined by the stabilization of pH around the neutrality  
168 (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

$$194 \quad \text{NFC (\%DM)} = 100 - (\text{NDF} + \text{CP} + \text{ash} + \text{EE}) \quad (1).$$

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-NH<sub>4</sub><sup>+</sup> and N-NO<sub>3</sub><sup>-</sup> 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

213 **2.4. Culture-dependent microbiological analysis of brewers' spent grain and**  
214 **vermicompost**

215 The main microbiological groups of fresh BSG samples (within 6 h of collection) and the  
216 vermicompost were determined to assess the compliance with legal requirements  
217 (Italian Legislative Decree 75/2010). In particular, the occurrence of pathogenic and  
218 nonpathogenic fungi, yeast, mesophilic bacteria, lactic bacteria, enterococci,  
219 enterobacteria, Actinomycetes, *Pseudomonas* spp., and aerobic and anaerobic spore-  
220 forming bacteria was evaluated according to Grantina-Levina et al. (2013).  
221 Enumeration of *Escherichia coli* was according to ISO 16649-2: 2001. Incidence of  
222 *Salmonella* spp. was determined as described by ISO 6579: 2002.

223

224 **2.5. Culture-independent microbiological analysis of brewers' spent grain and**  
225 **vermicompost**

226 Total DNA from BSG sampled during the pre-treatment, and from the mature  
227 vermicompost, was extracted according to Ahmed *et al.* (2009). DNA concentration,  
228 integrity, and purity were assessed by spectrophotometric analysis (LvisPLATE  
229 SpectroSTAR Nano; BMG Labtech, Ortenberg, Germany) and using 1.5% agarose gel  
230 electrophoresis. The 16S rRNA (for bacteria) and internal transcribed spacer (ITS) rRNA  
231 (for fungi) genes were amplified from 25 ng DNA in a final volume of 50  $\mu$ L, using 0.5  
232  $\mu$ M of each primer (Table S1), 2.5 mM MgCl<sub>2</sub>, 0.5 mM dNTPs, and 1 U Taq DNA  
233 polymerase (Eurx). The specificity of the PCR reactions was confirmed by  
234 electrophoresis on 1.5% agarose gels. The amplified DNA fragments were analyzed by  
235 denaturing gradient gel electrophoresis (DGGE; DCode Universal Mutation Detection

236 System; BioRad, Carlsbad, CA, USA). DGGE was carried out by loading 200 ng PCR  
237 products directly onto polyacrylamide gels under different denaturing conditions, as  
238 polyacrylamide 40% to 60% denaturing gradient for bacteria, and 30% to 60%  
239 denaturing gradient for fungi (where 100% is considered as 7 M urea, 40% deionized  
240 formamide). The PCR products were separated by electrophoresis in TAE buffer, as  
241 reported by Correddu et al. (2019). The gels were stained in TE buffer (pH 8) containing  
242 SYBR safe (Life Technology, Hercules, CA, USA), with the images acquired (ChemiDoc  
243 XRS system; Bio-Rad, Carlsbad, CA, US). The most intense PCR amplicons were excised  
244 from the DGGE gels and 1 µL of solution was used as the DNA template for  
245 amplification of the 357f and 519r regions for bacteria, and the ITS1f and ITS2 regions  
246 for fungi (Table S1). The specificity of the PCR products was assessed by  
247 electrophoresis on 1.5% agarose gels stained with 0.001% SyberSafe (Thermo Fisher  
248 Scientific, USA). The PCR products were sequenced at an external facility (Macrogen,  
249 Amsterdam, The Netherlands), and the sequences obtained were identified by BLAST  
250 analysis against the NCBI nucleotide sequence database, and using the Classifier and  
251 Sequence Match programs of Ribosomal Database Project II  
252 (<http://www.rdp.cme.msu.edu>).

253

## 254 **2.6. Germination index of vermicompost**

255 Germination tests were carried out using cress (*Lepidium sativum*, L.) seeds, according  
256 to the method described by Zucconi et al. (1981). After 3 days of incubation, the  
257 proportions (%) of germinated seeds and the root lengths were recorded. The

258 germination index (GI) was calculated using the mean value from three biological  
259 replicates, as given by Equation (2):

260

$$261 \quad GI (\%) = [(Gt\%) / (Gc\%)] \times [Lt/Lc] \times 100 \quad (2),$$

262

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

266 Phytotoxicity tests were carried out with the vermicompost extracts diluted at  
267 75%, 50%, and 30% in sterile distilled water.

268

## 269 **2.7. Data analysis**

270 The DGGE images were acquired and analyzed using the Infoquest FP V 4.5 software  
271 (Bio-Rad Laboratories, Hercules, CA, USA). The binary matrix (presence/absence of  
272 DGGE bands) constructed using the *Band matching* function of the PAST software  
273 (version 4.01, <https://folk.uio.no/ohammer/past/>) was converted into a distance  
274 matrix (Bray-Curtis method). The intensity of every single band (i.e., peak density) was  
275 used to derive quantitative values for subsequent analysis. The number of OTUs  
276 (bands), the Shannon–Wiener index, and the Simpson (or Dominance) index were  
277 calculated using the PAST software. To evaluate the effects of the sampling period (T0,  
278 T1, T2, T3) and the variation in the biodiversity indices, analysis of variance (ANOVA)  
279 followed by Tukey tests ( $p < 0.05$ ) were carried out using the SPSS software, v19 (SPSS  
280 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

304 converged to values near neutrality at T3 ( $7.32 \pm 0.24$ ). The C/N ratio decreased from  
305 T0 to T2, and then stabilized at T3 ( $14.25 \pm 0.50$ ) across all of the samples. The changes  
306 in the C/N ratio were related to significant variations in the nitrogen contents of the  
307 samples, as the total carbon content of BSG showed no significant variations during  
308 pre-treatment. In particular, the total nitrogen content increased significantly from T0  
309 to T2, and then decreased to T3. Instead, there was an increase in ammonium nitrogen  
310 content of the BSG throughout the pre-treatment period. Modification in these  
311 chemical parameters was accompanied by changes in the temperature of the BSG,  
312 which increased from T0 to T2 and then stabilized at 22°C at T3.

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

319

### 320 **3.2. Evolution of brewers' spent grain microbiota during the pre-treatment**

321         From a microbiological perspective, all of the BSG samples at T0 were within  
322 the quality parameters, as there were no *Enterococci* spp, *Enterobacteria* spp,  
323 *Pseudomonas aeruginosa*, Coliforms, *E. coli*, *Salmonella* spp., and pathogenic fungi  
324 detected (Table S2). The BSG from brewery MB1 showed overall lower contamination,  
325 as fungi and yeast were not detected. According to the origins of the BSG, Lactobacilli

326 were strongly represented, as they made up from 20% to 58% of the total bacterial  
327 community.

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

339         Ordination analysis revealed that the BSG samples across the different times  
340 were more similar than those within the same time points, thus suggesting a lack of  
341 correlation among the composition of the bacterial and fungal communities and the  
342 times of sampling. This was also confirmed by the lack of significance of the  
343 PERMANOVA (experimental factor = time of sampling) (bacteria,  $p = 0.712$ ; fungi,  $p =$   
344  $0.318$ ). Similarly, the composition of the bacterial community was not associated with  
345 the origins (i.e., breweries) of the samples, as they were scattered randomly  
346 throughout the ordination space.

347         To identify the main microbial species involved in the pre-treatment process,  
348 63 bands for the bacterial and fungal communities were extracted, with successful

349 sequencing and identification of 37 DNA fragments from bacteria (190-200 bases) and  
350 26 DNA fragments from fungi (200-300 bases). The relative abundance of the main  
351 bacterial and fungal taxa identified through the pre-treatment process is reported in  
352 Figure 2.

353

### 354 **3.3. Chemical characterization and microbiota of vermicompost**

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

359 The vermicompost obtained was not contaminated by mycotoxins. Ochratoxin  
360 A, fumonisins, deoxynivalenol, T-2, HT-2, and total aflatoxin were identified at  
361 concentrations lower than those recommended by the European Union for food  
362 products and animal feed (Bianco et al., 2018, 2019). Similarly, *Enterococcus* spp.,  
363 Enterobacteria, Coliforms, *Salmonella* spp., and pathogenic fungi were not detected in  
364 the vermicomposts. *Escherichia coli* was only found in a single sample at 60 CFU/g,  
365 which was well below the legal limit of 5,000 CFU/g (L.D. 75/2010). To further  
366 characterize the microbial communities of the vermicompost, 45 DNA fragments from  
367 bacteria (190-200 bases) and 36 DNA fragments from fungi (200-300 bases) were  
368 extracted from the DGGE gels, sequenced, and classified (Figure 3).

369 Culture-independent analyses showed that four of the 27 bacterial taxa  
370 identified were common between the BSG and mature vermicompost: *Weissella*  
371 *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<sub>2</sub>. 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

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

492         Similar to what was observed for Saccharomycetales, the pre-treatment of BSG  
493 resulted in the enrichment of Sordariales (*Botryotrichum domesticum* and *Humicola*  
494 *homopilata*) at T2 and T3. Sordariales are involved in the decomposition of decaying  
495 plant organic matter because of their high enzymatic activity (Marín-Guirao et al.  
496 2019). Sordariales were found positively correlated to increased maize yields.  
497 Particularly, *Botryotrichum* was associated with increases in the fertility of soils with  
498 high organic matter (Marín-Guirao et al. 2019).

499         Conversely, the abundance of different fungal species highly represented in raw  
500 BSG, such as *Achetomium strumarium*, *Trichoderma reesei*, and *Verticillium dahliae*,  
501 showed a reduction during the pre-treatment. *Verticillium dahliae* is a soil-borne  
502 pathogen with a wide host range, comprising over 300 woody and herbaceous plant  
503 species. On the contrary, both *Achetomium strumarium* and *Trichoderma reesei* have  
504 great biotechnological potential. The application of *Achetomium strumarium* was  
505 suggested for remediation processes of wastewater polluted by textile industries, as it  
506 can degrade synthetic azole dyes (Bankole et al., 2017 ). The use of *Trichoderma* spp.  
507 as a biofertilizer or a bioremediation and biocontrol agent is well known (Bhandari et  
508 al., 2021). In particular, the use of *Trichoderma reesei* in agriculture has increased  
509 exponentially in recent years, as it is considered an efficient biotechnological factory

510 for the production of second-generation biofuel from lignocellulosic biomass  
511 (Hinterdobler et al., 2021). Zhang et al. (2021) studied the crucial role of *T. reesei* in  
512 humic substance formation and production of lignocellulosic crop residues. *T. reesei*  
513 promoted the humification process of corn straw and can contribute to the  
514 accumulation and occurrence of stable soil organic matter (Zhang et al., 2021).  
515 *Trichoderma*, and *Fusarium* genera encompass the main aerobic fungi identified as  
516 lignin degraders (Sindhu et al., 2016). Thus, the identification of these fungal genera  
517 suggests active degradation of the most stable polymers during the first stages of BSG  
518 pre-treatment.

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

524 *Thermolithobacter ferrireducens*, identified at higher population during the first  
525 stages of the pre-treatment, has the highest iron reduction rate per cell and is thus  
526 considered of great interest for the functioning of soil and bioremediation.

527 *Pseudomonas stutzeri* can fix nitrogen, increase metal bioavailability, and degrade  
528 compounds such as hydrocarbons and alkanes (Lalucat et al., 2006). It is known that

529 many lactic acid bacteria (LAB) can grow in beer. Thus, *Weissella cibaria* and  
530 *Pediococcus acidilactici* could derive directly from the breweries where the BSG  
531 samples were produced. LAB have complex nutritional requirements and usually  
532 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*

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

564           The fungal community identified in the mature vermicompost was dominated  
565 by Ascomycota, with only one species of Basidiomycota, *Puccinia symphoricarpi*. This  
566 agrees with Huang et al. (2014), who identified Ascomycota and Basidiomycota as the  
567 most common fungal phyla in the vermicompost from fresh fruit and vegetable  
568 wastes. Similarly, Anastasi et al. (2004) reported that the occurrence of Ascomycetes in  
569 vermicompost from plant and animal wastes was twice that in the compost obtained  
570 from the same wastes. The main fungal species identified were *Pichia fermentans*,  
571 *Beauveria bassiana*, *Puccinia symphoricarpi*, *Lachancea thermotolerans*, and  
572 *Verticillium dahliae*, each accounting for >5% of the total fungal community. *Pichia*  
573 *fermentans* and *Verticillium dahliae* were already identified in pre-treated BSG.  
574 Similarly, it is conceivable that the occurrence of *Pichia cecembensis* (4%) and  
575 *Trichoderma reesei* (1%) in mature vermicompost are related to their presence in BSG.  
576 Other fungal species were not identified in BSG. *Beauveria bassiana* is well-known as  
577 an entomopathogenic and plant-growth-promoting fungus (Mantzoukas et al., 2021).  
578 *Lachancea thermotolerans* is also a plant-growth-promoting yeast as it promoted

579 seedling development of *Nicotiana benthamiana* (Fernandez-San Millan et al., 2020).  
580 *Puccinia symphoricarpi* is a phytopathogens that seriously threatens wheat production  
581 by causing leaf, stem, and stripe rust diseases. *Yarrowia lipolytica* (accounting for 4%  
582 of the fungal community in the vermicompost) can grow on different food wastes as it  
583 can degrade hydrocarbons, oil, diesel, and lignocellulosic raw materials (Madzak,  
584 2021). *Y. lipolytica* is also able to grow in media with extremely high (up to 10.0) and  
585 low (pH 3.0) pH values (Madzak, 2021). Together with other phylamentous fungi,  
586 *Aspergillus oryzae* (4% of the fungal community in the vermicompost) is a promising  
587 source of protein and carbohydrate for the food and feed industries, as it can grow on  
588 food wastes (Serba et al., 2020). Interestingly, BSG treated directly with *A. oryzae*  
589 spores was proposed as protein enriched animal feeds (Bekatorou et al., 2005). Species  
590 of the lichen genera *Umbilicaria* (4%) were able to grow in extremely harsh  
591 environments, particularly in regions of higher latitudes or altitudes. The pre-  
592 treatment of BSG did not result in the development of mycotoxigenic fungal species, as  
593 all of the mycotoxins analyzed were below the detection limits.

594         At the phylum level, the bacteria identified in mature vermicompost from the  
595 pre-treated BSG belonged to Actinobacteria, Proteobacteria, and Firmicutes, which is  
596 in agreement with the findings of Srivastava et al. (2021). Actinobacteria and  
597 Firmicutes were also identified among the dominant phyla in the vermicompost from  
598 medicinal herbal residues (ADL, 8%/14% soluble solids), and together with  
599 Proteobacteria, Bacteroidetes, and Chloroflexi, these accounted for 83% to 93% of the  
600 bacterial community (Chen et al., 2018). Members of these phyla are generally  
601 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-NH <sub>4</sub> <sup>+</sup> nitrogen	g/kg	0.08 ±0.04	1.22 ±0.77	5.40 ±3.87	9.00 ±3.41
N-NO <sub>3</sub> <sup>-</sup> 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.

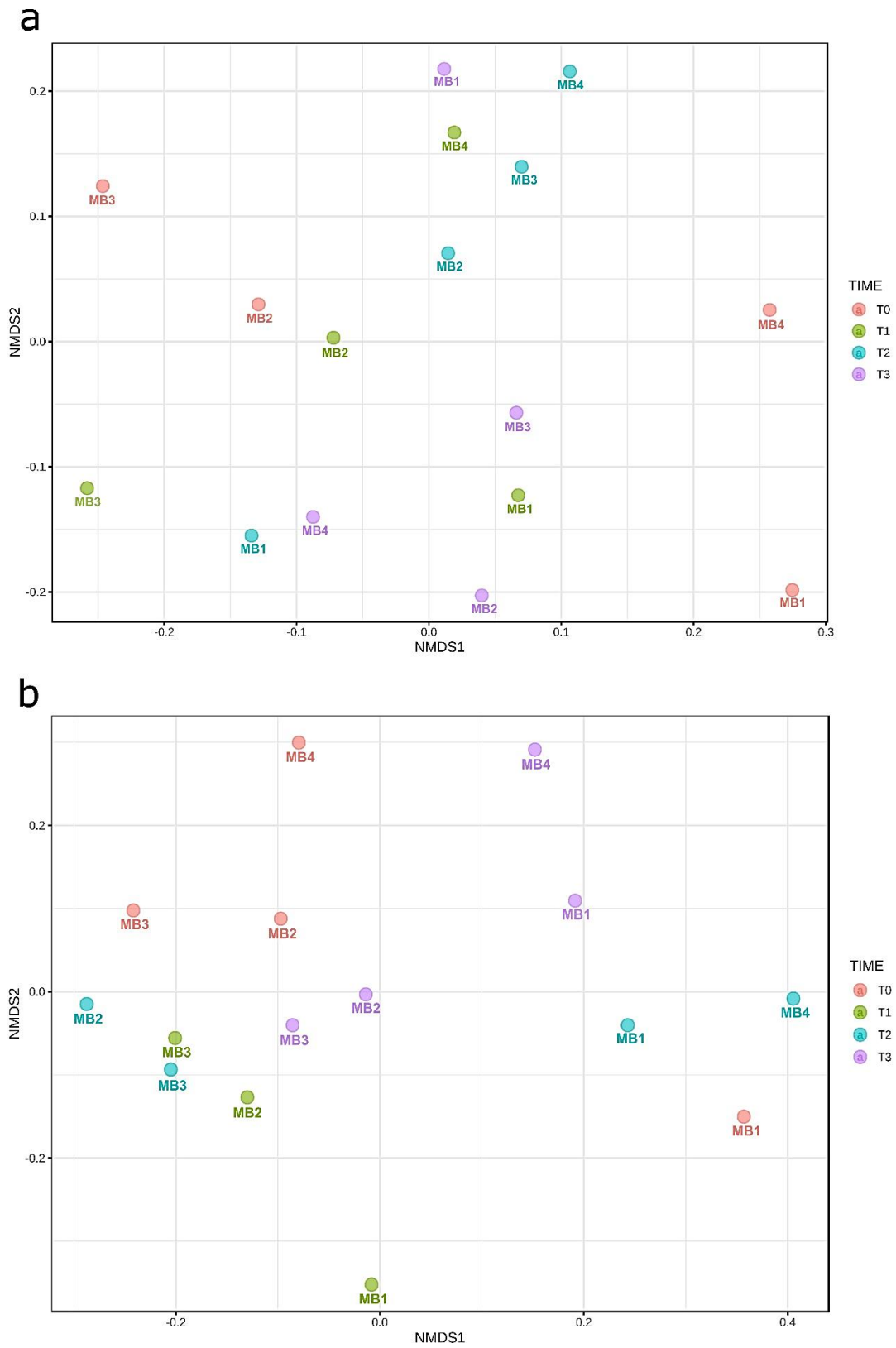
Sampling time	Bacterial community indices			Fungal community indices		
	Chao1	Shannon	Simpson	Chao1	Shannon	Simpson
T0	20.50 ±3.12 <sup>a</sup>	2.53 ±0.24 <sup>a</sup>	0.89 ±0.02 <sup>a</sup>	18.00 ±9.83 <sup>a</sup>	2.38 ±0.61 <sup>a</sup>	0.86 ±0.07 <sup>a</sup>
T1	28.50 ±4.80 <sup>ab</sup>	2.93 ±0.33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

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

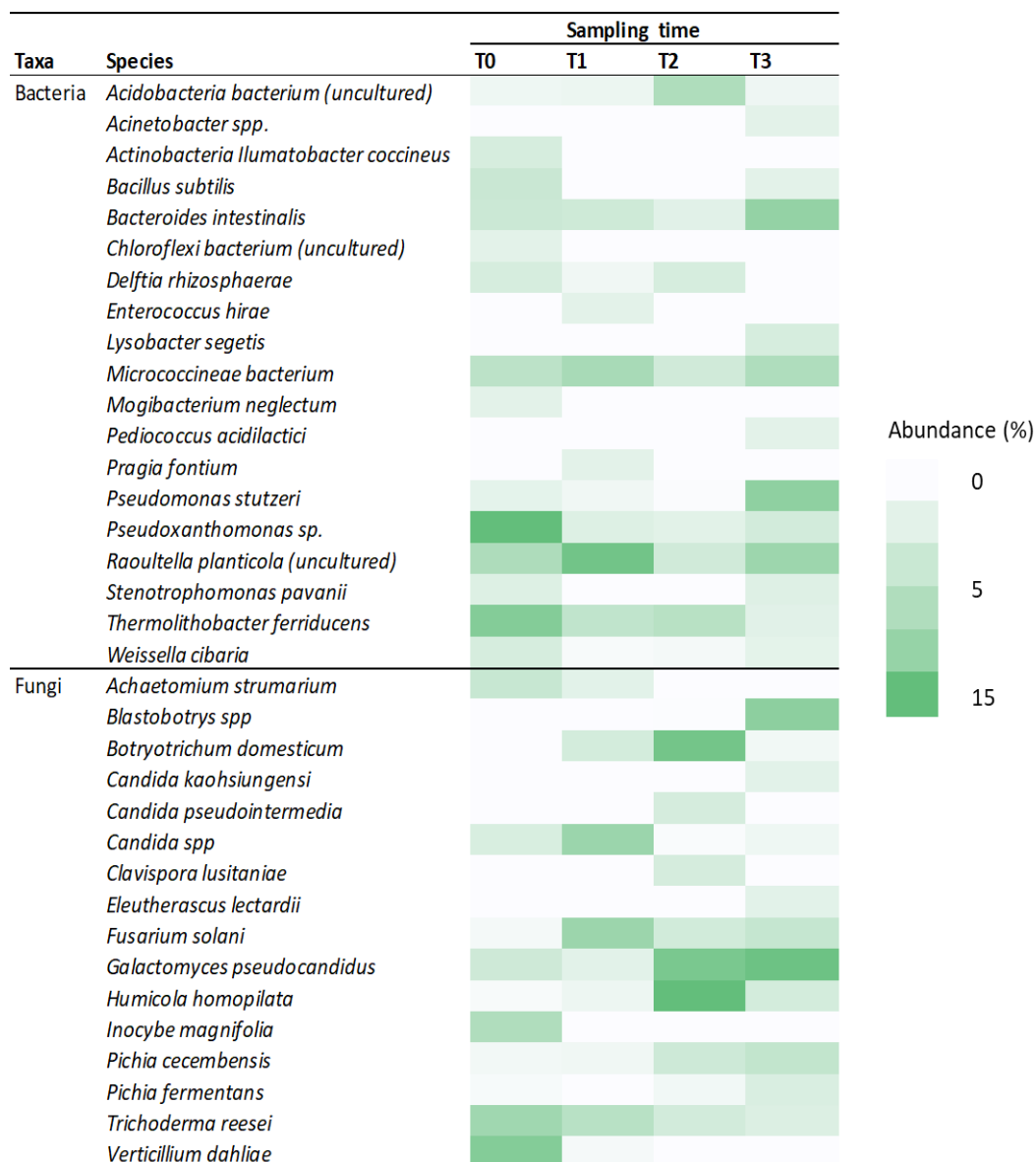
856 **Table 3.** Chemical, biochemical, microbiological and quality parameters of the vermicompost  
 857 from the pre-treated BSG.

Parameter/ Index	Parameter	Units	Mean $\pm$ SD
Chemical	Moisture	%	45.31 $\pm$ 0.41
	pH	-	7.08 $\pm$ 0.21
	Carbon/nitrogen	-	11.00 $\pm$ 1.15
	Organic carbon	g/kg	38.63 $\pm$ 0.56
	Total nitrogen	g/kg	32.8 $\pm$ 0.30
	N-NH <sub>4</sub> <sup>+</sup> nitrogen	g/kg	0.02 $\pm$ 0.01
	N-NO <sub>3</sub> <sup>-</sup> nitrogen	g/kg	0.56 $\pm$ 0.53
	Dehydrogenase	$\mu$ g/TPF	117.62 $\pm$ 53.38
	Dissolved organic carbon	mg/g	1.09 $\pm$ 0.37
Microbial counts	Total bacteria	CFU/g	11.9 $\times 10^6 \pm 95.4$
	Lactobacilli		2.14 $\times 10^6 \pm 17.1$
	Actinomyces		6.32 $\times 10^6 \pm 50.6$
	Aerobic sporigens		162 $\times 10^6 \pm 130$
	Anaerobic sporigens		100 $\times 10^6 \pm 801$
	<i>Pseudomonas</i> spp.		12800 $\pm 5.13$
	Total fungi		44200 $\pm 3.98$
	Total yeast		34400 $\pm 3.09$
Alpha diversity	Chao1 (bacteria)	-	44.25 $\pm 3.10$
	Shannon (bacteria)	-	3.44 $\pm 0.10$
	Simpson (bacteria)	-	0.96 $\pm 0.00$
	Chao1 (fungi)	-	37.50 $\pm 5.20$
	Shannon (fungi)	-	3.20 $\pm 0.09$
	Simpson (fungi)	-	0.95 $\pm 0.00$
Germination	75%*	%	87.63 $\pm 5.68$
	50%*	%	66.93 $\pm 1.79$
	30%*	%	24.53 $\pm 7.08$

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).



## SUPPLEMENTARY MATERIAL

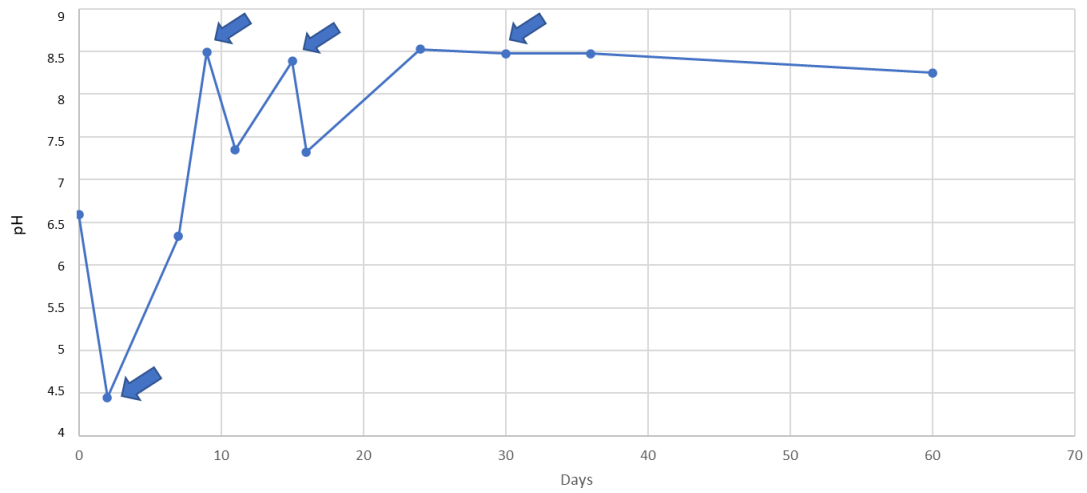
**Supplementary Table S1.** PCR primers used in this study.

Organism	Primer	Sequence (5'→3')	PCR product (bp)	Reference
Bacteria	GC-357f	CGCCCGCCGCGCCCCGCGCCCGCCGCCGCGCC CCCGCCCCCTACGGGAGGCAGCAG	191	Lane, 1991
	519r	ATTACCGCGGCKGCTGG		
	357f	CCTACGGGAGGCAGCAG		
Fungi	ITS1f	TCCGTAGGTGAACCTGCGG	700/900	Gardes and Bruns, 1993
	ITS4	TCCTCCGCTTATTGATATGC		
	ITS1f-GC	CGCCCGCCGCGCGCGGGCGGGGCGGGGG	200/300	Valášková and Baldrian, 2009
		CACGGGGGGCTTGTCAATTAGAGGAAGTAA		
	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  $\pm$  standard deviation, from three independent samples.

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