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

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ABSTRACT

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

Keywords: Bio-fertiliser; yeast; bacteria; mycotoxins; by-products

1. Introduction

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

The main component of BSG is fibre (30%-50%; w/w), which includes the lignocellulose fraction, protein (19%-30%), hydrolysates of proteins, arabinoxylans and phenolic compounds. For these reasons, BSG represents a nutritionally rich by-product that requires the appropriate procedures for its recovery and re-use. One of the main problems in the re-use of BSG is its high moisture content, which results in its rapid deterioration and logistic difficulties for its storage and transportation. BSG can also be contaminated with mycotoxins, which can arise along the entire production chain, from cultivation of the barley in the field, to its storage and malt production. During all of these phases, contamination by mycotoxigenic fungi represents a high risk, with the consequent release of mycotoxins.

Stabilisation of BSG might be achieved by vermicomposting it, to recycle the nutrients in agriculture and to maintain soil fertility. Indeed, composting and

vermicomposting are two of the best-known processes for biological degradation and stabilisation of organic wastes.

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

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

The importance of earthworm microbial communities is well documented in the vermicomposting of lignocellulosic materials. The decomposition of such raw materials, including BSG, is a particularly difficult process due to the high content of complex

heteropolymers, which confers different characteristics and can inhibit the cellulase enzymes (De Angelis et al., 2011).

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

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

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

2. Materials and methods

2.1 Preparation of earthworm beds

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

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

2.2 Treatment and analysis of earthworm beds

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

use in the subsequent chemical analyses, which were performed in triple and according to the methods reported by Chefetz et al. (1996).

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

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

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

meal and soil standards for calibration. pH was also measured for all of the six samples, in aqueous solution (1:20; v/v) using a glass electrode (XS sensor 250A; Orion, Boston, USA). The C/N ratios were calculated from the TOC and TN.

2.3 FAME of earthworms

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

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

identify the fatty acids, retention times were compared to those obtained for the standard 37-Component Fame Mix (Supelco, Bellefonte, PA, USA).

2.4 Microbial analysis of beds and vermicomposts

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

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

2.5 Mycotoxins determination of beds and vermicomposts

Determination of the BSG mycotoxins was performed using quantitative analysis for the mycotoxins deoxynivalenol, T-2 and HT-2, fumonisins, aflatoxin and ochratoxin A. This was carried out using a ‘rapid one-step assay’ system (Charm Lateral Flow R.O.S.A., Foss A/S, Hillerod, Denmark), as based on ELISA, and following the

212 protocol provided by the manufacturer. The limit of detection of each mycotoxin with
213 this method was 100 ppb for deoxynivalenol, 10 ppb for T-2 and HT-2, 250 ppb for
214 fumonisins, 2 ppb for aflatoxins, and 2 ppb for ochratoxins. Three replicates were
215 performed for each sample. Similar analyses were carried out for the mycotoxins from
216 the beds and the vermicomposts, for ochratoxin A, fumonisins and T-2 and HT-2 using
217 ELISA in 48-well plates (Bio-Shield fumonisin 0.15–-6 ppm; Bio-Shield ochratoxin
218 2.5-40 ppb; Bio-Shield T-2/ HT-2 10-500 ppb; Prognosis-Biotech, Or-Sell, Modena,
219 Italy,). This method used samples that were homogenised, weighed and then extracted
220 with methanol: water (70:30; v/v). After filtration, the samples were ready for the
221 ELISA test at 450 nm absorbance, and the data were analysed using the spreadsheet
222 provided with the test.
223
224 ***2.6 Enzymatic activities of beds and vermicomposts***
225 The enzymatic activities for dehydrogenase, urease and β-glucosidase were determined
226 according to Alef and Nannipieri (1995). Dehydrogenase activity was measured in 10 g
227 of each sample, by estimation of rate of reduction of triphenyltetrazolium chloride to
228 triphenylformazan, after incubation at 37 °C for 24 h, and is expressed as μg
229 triphenyltetrazolium formed g^{-1} h^{-1}. The urease activity was determined as ammonia
230 released from 5 g samples treated with urea and incubated for 2 h at 37 °C. The urease
231 activity is given as μg NH_4–N released g^{-1} h^{-1}. The β-glucosidase activity is given by
232 the p-nitrophenol released from 1 g sample after incubation for 1 h at 37 °C with p-
233 nitrophenylglucoside, and is expressed as μg p-nitrophenol g^{-1} h^{-1}. Each lyophilised
234 sample for analysis of the dehydrogenase, urease and β-glucosidase activities was
235 rehydrated for 3 h before analysis. Enzymatic activities were determined in triplicate
236 samples, and all products were read in LVis plates using a microplate reader

(SpectroStar Nano; BMG Labtech, Ortenberg, Germany), at 480 nm for dehydrogenase, 690 nm for urease, and 400 nm for β-glucosidase.

2.7 Data analysis

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

3. Results and discussion

3.1 Vermicompost BSG is a stabilised fertiliser that is rich in Nitrogen

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

In general, earthworms avoid substrates with pH <4.5, as prolonged exposure to

such pHs can be lethal for them (Edwards and Bohlen, 1996; Dominguez, 2004). Since earthworms have a natural tendency to shift the pH towards values closer to neutrality, the present trial was also used to test their survival capacity and their ability to modify pH values.

The TOC content of these substrates before and after the vermicomposting process decreased significantly for BSG and BSG/CM substrates (Fig. 1; 3.3%, 4.8% respectively), but showed no change for CM. This was paralleled by the before and after organic matter, which also decreased significantly for the BSG and BSG/CM substrates (from 65.0±0.2% to 59.3±0.1% and from 51.6±0.4% to 43.4±0.2%, respectively), again with no difference seen for CM (that remained unaltered to 38.4±0.8%). Some studies have reported that relatively large amounts of TOC are lost in the form of CO_2 (20%-45%) due to the feeding of earthworms on the organic matter and due to microbial degradation (Elvira et al., 1998; Kaushik and Garg, 2003). These modifications promote C loss through microbial respiration, in the form of CO_2, and through mineralisation of organic matter. As partial confirmation of this hypothesis, different dynamics were seen for the CM experimental unit, where the earthworms lived under suitable environmental and balanced biochemical conditions.

Conversely, there were significant increases in TN for the BSG and BSG/CM substrates (from 3.64±0.03% to 5.10±0.02% and from 3.18±0.03% to 3.38±0.01%, respectively), with a significant decrease in CM (from 2.90±0.08% to 2.54±0.07%; Fig. 2). The TN of the final substrates (after the removal of the earthworm from the beds) might be the result of greater withdraw of nitrogen by the earthworms for reproductive purposes. Indeed, earthworms cultured in the presence of the spent grain here (i.e., BSG, BSG/CM) will almost certainly have undergone stress caused by the low pH of these substrates, and especially in the early stages of this vermicomposting. Thus, the

287 larger earthworm populations in CM might have used more nitrogen to produce
288 substances required for individual growth and reproduction, thus lowering the N content
289 in the CM substrate. Using wheat straw as bed, Cortez et al. (1989) demonstrated that
290 earthworms could assimilate the 9.4% of the total N ingested. Furthermore, the
291 observed decreases in the levels of N could be related to the leaching of N for addition
292 of constant water to keep the bed at 80-85% of moisture. Finally, small decreases of
293 Nitrogen could be related to nitrification and denitrification phenomena leading to N_2
294 and N_2O volatilisation (Plaza et al., 2008; Nasir et al., 2014; Nigussie et al., 2016).
295 The TN increases in BSG may be related to the higher rates of earthworms'
296 death and decomposition, before vermicompost was collected, due to the harsh
297 environmental condition in this substrate. Indeed, it has been observed that the N
298 content of the compost depends on the extent of the decomposition (Crawford, 1983;
299 Gaur and Singh, 1995). Also, the action of N-fixing bacteria could be hypothesized
300 (Plaza et al., 2008). As demonstrated by Hand et al. (1988), *E. fetida* in cow dung slurry
301 increases the nitrate-N content. Also, the organic C decreases might be involved in this
302 dynamic, as they can cause N increases that are linked to mucus nitrogenous excretory
303 substances, growth stimulatory hormones, and enzymes from the gut of earthworms
304 (Viel et al., 1987; Tripathi and Bhardway, 2004;). However, to better evaluate the N
305 dynamics during the vermicomposting of BSG, the different chemical forms of N in the
306 organic matrices would need to be evaluated before and after vermicomposting.
307 The C/N ratio represents one of the most widely expressed indices for the
308 maturity of organic matter, as this reflects the mineralisation and stabilisation level
309 (Suthar, 2008). While the C/N ratio for CM before and after vermicomposting
310 processes here were balanced (from 7.68 to 8.75; indicating an equilibrated substrate),
311 the lower values for BSG and BSG/CM before and after vermicomposting (from 10.34

312 to 6.76 and from 9.40 to 7.45, respectively) indicate an advanced degree of organic
313 matter stabilisation throughout this vermicomposting (as shown by Zhang et al., 2015).
314 It has often been reported that the C/N ratio decreases sharply during
315 vermicomposting (Kale, 1998; Gupta and Garg, 2008; Suthar, 2008). This reduction is
316 mainly due to an absolute decrease of Carbon by mineralization and respiration
317 processes (CO_3^- and CO_2) (Nakasaki et al., 1992; Dominguez et al., 1997; Nayak et al.,
318 2013), while Nitrogen varies much less because it is biologically reused. The amount of
319 Nitrogen not used by microorganisms remains into vermicompost and it is therefore
320 available. Also the production of mucus and nitrogenous excreta by earthworms will
321 enhance the levels of N, reducing the C/N ratio at the same time (Senapati et al., 1980).
322 The TEC provides a measurement of the total C in total humic substances like
323 (HSl), and this also significantly increased in the BSG and CM substrates during the
324 vermicomposting (from 21.0±1.2% to 25.2±0.5% and from 17.2±0.8% to 20.6±0.7%,
325 respectively) while no differences were seen for BSG/CM (from 19.1±1.2% to
326 21.0±1.5%; Fig. 3). HSl expressed as proportions of the TEC varied considerably before
327 and after vermicomposting. HSl increased from 11.2±2.3% to 31.3±1.2% for BSG,
328 10.2±1.8% to 18.6±0.7% for CM, and 10.5±0.9% to 23.6±1.0% for BSG/CM (Fig. 4).
329 The results of the two-way ANOVA for the effects of substrate (BSG, CM, BSG/CM,
330 before and after vermicomposting) on the HSl substances showed significant
331 differences for both factors and for their interaction (Table 1). In particular, the Tukey
332 *post-hoc* comparison test showed that HSl contents after the transformation of the
333 substrates were significantly higher in BSG and BSG/CM than in CM. The large TEC
334 contents in all of the substrates after vermicomposting would indicate the achievement
335 of a high degree of maturity and stability of the organic matter(Padmavathiamma et al.,
336 2008; Ngo et al., 2011).

337 Along with the TEC increase after the vermicomposting, the total levels of C in
338 HSl also increased. In particular, the high levels of C in HSl for BSG are almost
339 certainly linked to the large amount of organic C in the unprocessed spent grain (Fig. 1).
340 Both the high TEC and HSl in all of the substrates after vermicomposting would
341 indicate the extended synthesis of organic components recalcitrant to microbial
342 degradation (Plaza et al., 2008).
343
344 ***3.2 Fatty-acids profile provides a biomarker of the health status of E. fetida in BSG***
345 To determine whether BSG is a good substrate for the growth and reproduction of these
346 earthworms, we analysed the fatty-acid profile of *E. fetida* (Table 2). The fatty acids in
347 the whole body or gut of earthworms can be used as a biomarker and as an index of
348 responses to environmental stress (Crockett et al., 2001). In addition, the elevated fat
349 content of *E. fetida* (7.8%) makes this species a good alternative source of protein and
350 fatty acids for animal feed (Gunya et al., 2016). Under the tested conditions here, the
351 pattern of the fatty acids was characterised by the absence of evaluable data for fatty
352 acids with carbon chains shorter than C11 and longer than C20. Only saturated C12,
353 C14, C17 and C20 fatty acids (with prevalence for C17) and unsaturated C18:1, C18:2,
354 C18:3 fatty acids were detected from these earthworms. A comparison of the different
355 growth and reproduction substrates shows that CM had a low content of saturated and
356 unsaturated fatty acids with long and short chains. Linoleic and palmitic oleic acids
357 were the most abundant in *E. fetida* in BSG. Small amounts of linolenic and stearic
358 acids and high contents of myristic, margaric, linolenic, omega 6 and omega 3,
359 arachidonic fatty acids were also found, in agreement with Almeida et al. (2017). The
360 fatty-acid content of the earthworms from the BSG/CM substrate reflects the
361 contribution of BSG. The composition of fatty acids in the earthworm body depends on

both species (Paoletti et al., 2003) and diet (Sampedro et al., 2006). Different studies have reported that *E. fetida* contains large amounts of omega 3 fatty acids (Fadaee, 2012; Gunya et al., 2016). The highest levels seen for the 17:0 fatty acid, followed by 18:2-cis, is in partial agreement with the fatty-acid data of Antisari et al. (2015). Indeed, they identified fatty acids with C chains of >15, but no further information is currently available on fatty acids in earthworms.

3.3 Vermicompost from BSG respects the safety law parameters

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

387 health standards related to animal by-products that are not intended for human
388 consumption, where manure is defined as follows: “excrements and/or urine from
389 animals of breeding, other than farmed fish, with or without litter”. According to Italian
390 legislation (Legislative Decree N° 75/2010, annex 2, point 11 and following),
391 vermicompost refers to worm and insect ejections. In Italy, the microbial quality of
392 vermicompost is regulated by Legislative Decree N° 75/2010. In particular, *Salmonella*
393 spp. should be absent in 25 g of sample, while *E. coli* must not exceed 5×10^3 CFU/g in
394 vermicompost. This decree classifies vermicompost as a soil improver, and it also
395 establishes the parameters for the nitrogen and organic carbon content. If vermicompost
396 is intended to be used in organic farming, the annexes to the legislative decree provide
397 for additional parameters.
398 There is also a difference between vermicompost from manure and
399 vermicompost obtained from organic waste: only the first can be placed on the market,
400 while the second can only be used for self-consumption. From the regulatory point of
401 view, when the humid matrix is used, this does not result in an earthworm
402 vermicompost; this provides instead a mixed composted soil conditioner that has a
403 commercial value one-fifth to one-tenth of earthworm vermicompost produced from
404 manure. Furthermore, the norms established by EC Regulation N° 1069/2009 must be
405 respected. Thus, from the regulatory point of view, the final product that results from
406 the processing of BSG by earthworms should be more accurately defined as a mixed
407 composted soil conditioner. The vermicompost producer must also be registered in the
408 register of fertiliser manufacturers by submitting an application to the Ministry of
409 Agriculture.
410
411 ***3.4 Mycotoxins are degraded during vermicomposting***

412 Preliminary characterisation has shown that BSGs from local breweries are
413 contaminated by ochratoxin A, fumonisins and T-2 and HT-2, while aflatoxins and
414 deoxynivalenol have not been detected (A. Bianco, personal communication). On the
415 basis of this information, ochratoxin A, fumonisins and T-2 plus HT-2 mycotoxins were
416 determined for the BSG and cow manure used for the beds, and for these
417 vermicomposts (Table 3). For the cow manure before and after this vermicomposting,
418 none of these mycotoxins studied here were above the detection thresholds.

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

425 Also, the 338 ppb of T-2 plus HT-2 in the BSG here is above the limit suggested
426 by EC Recommendation 2013/165/EU, although, again, this was significantly reduced
427 to 16 ppb after the BSG alone vermicomposting. The same trend was seen for the
428 BSG/CM substrate, where the initial contamination of 80 ppb T-2 plus HT-2 was
429 reduced to 21 ppb after the BSG/CM vermicomposting. The recommendation
430 (2013/165/EU) indicates T-2 and HT-2 levels <200 ppb (i.e., μg kg^{-1}) in unprocessed
431 cereals, such as maize and barley (including beer barley), and 100 ppb and 50 ppb for
432 cereal products for wheat or other grain-milling products for direct human consumption.

433 Fumonisines were not detected in any of the beds or vermicomposts studied
434 here. However, the analysis carried out on the lyophilised earthworms showed that *E.*
435 *fetida* can bioaccumulate this mycotoxin. In particular, the earthworm growth and
436 reproduction in BSG, cow manure and BSG/CM showed contamination of fumonisins

437 of 0.40 ppm, 0.31 ppm and 1.04 ppm. respectively. The low levels of ochratoxin A and
438 T-2 and HT-2 in the earthworms here were not sufficient to explain the strong reduction
439 in these mycotoxins during the vermicomposting. Thus, it can be hypothesised that
440 partial detoxification of ochratoxin A and T-2 and HT-2 was carried out by *E. fetida* and
441 its associated microbiota.

442 *Eisenia fetida* is considered a representative species of earthworms, with a wide
443 literature available on its ecology and its use in ecotoxicological experiments (OECD
444 207; ISO No.11268-1:2012; ISO No. 1268-2:2012; ISO 11268-3:2014). Generally, the
445 substances analysed in acute toxicity tests with earthworms have mainly been chemical
446 and pharmaceutical soil contaminants; only recently have studies been conducted on the
447 effects of mycotoxins on earthworms. Yang et al. (2015) evaluated the multiple toxic
448 endpoints of naturally occurring mycotoxins in the nematode *Caenorhabditis elegans*
449 model (aflatoxin B1, deoxynivalenol, fumonisin B1, T-2, zearalenone). Delgado (2014)
450 evaluated the potential ecotoxicological risk of fumonisin B1 on terrestrial
451 invertebrates, with their study conducted under controlled laboratory conditions by
452 exposing *E. fetida* to fumonisin B1 in an artificial soil. Szabó-Fodor et al. (2017)
453 studied the possible serious risks of aflatoxin B1 on the earthworm *E. fetida*. The results
454 of these studies confirmed that the tests based on EC Regulation N° 1907/2006 with *E.*
455 *fetida* are applicable and useful in research on toxicities of mycotoxins. This has
456 provided information on possible acute and sub-acute toxic effects, and the effects of
457 mycotoxins on soil invertebrates.

458

459 ***3.5 Enzymatic activities reveal a strict link between microbiota and quality of BSG***
460 ***vermicompost***

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

β-Glucosidase activity did not vary significantly before and after vermicomposting in any of these samples. Before vermicomposting, the β-glucosidase activity of the BSG/CM substrate was 1.7-fold those for BSG and CM. Also, for the BSG/CM substrate after the 5 months of vermicomposting, the β-glucosidase activity was 2.79-fold and 2.01-fold those of the BSG and CM vermicomposts, respectively. β-glucosidase is an extracellular enzyme that is involved in the C cycle (Alvarenga et al., 2008; Bastida et al., 2012) and can be used as an indicator for microbial ability to degrade organic matter (Pankhurst et al.,1997). β-glucosidase degrades glucosides to glucose during cellulose degradation (Esen, 1993). It is believed that β-glucosidase is mainly produced by the fungi in soils (Hayano and Tubaki, 1985). The high β-glucosidase activities seen here might be caused by the greater abundance of fungi,

according to a study conducted by Lazcano et al. (2008), where they reported significant correlation between presence of ergosterol in their substrates and β-glucosidase activity, as also seen in previous studies (Aira et al., 2006).

Several studies have shown that urease activity is influenced by the type of substrate used for vermicomposting (Pramanik et al., 2007; Castillo et al.,2013; Yadav et al., 2015). In particular, the urease activity in the present study was probably favoured by the substrates that were particularly rich in nitrogen (Pramanik et al., 2007), as for those obtained from BSG and BSG/CM. In these samples, the urease activity increased 17.46-fold and 2.37-fold compared to the beds before the 5 months of vermicomposting. The main nitrogenous compounds in BSG are proteins, and high levels of urea might have been generated through their degradation by the microbiota. Thus, increased urea would have led to corresponding increases in the urease activities. On the contrary, the urease activities did not change in the manure after vermicomposting. According to Castaldi et al. (2008), it can be postulated that the major nitrogen compound in cow manure was already stored as ammonium, such that the urease activity during vermicomposting remained constantly high. Urease is an extracellular enzyme that is involved in the N cycle, and it can catalyse the hydrolysis of urea-type substrates to CO_2 and NH_3 (Alvarenga et al., 2008). Urease activity has been used as an environmental stress indicator, in particular in substrates with different levels of nitrogen (Pankhurst et al.,1997; Pascual et al., 2002; Bhattacharyya et al., 2008).

In general, the increased enzyme activities during the vermicomposting of BSG and BSG/CM in particular probably related to increased microbial populations (Fig. 5). The higher enzyme activities in the vermicomposts with respect to the raw materials used for the beds might be due to stimulation of microbial activities during the bioconversion period (Zhang et al., 2000; Pramanik et al., 2007; Yadav et al., 2015).

4. Conclusions

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

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The authors declare that they have no conflict of interest.

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731

732 **Figure captions**

733

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

737

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

741

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

745

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

750

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

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

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

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

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

Fatty acids	CM	BSG	BSG/CM
(C4:0)	< 0.3	< 0.3	< 0.3
(C6:0)	< 0.3	< 0.3	< 0.3
(C8:0)	< 0.3	< 0.3	< 0.3
(C10:0)	< 0.3	< 0.3	< 0.3
(C11:0)	< 0.3	< 0.3	< 0.3
(C12:0)	14.8±0.3	1.5±0.2	9.2±0.3
(C13:0)	< 0.3	1.6±0.2	< 0.3
(C14:0)	4.2±0.2	4.8±0.2	4.5±0.2
(C14:1)	< 0.3	< 0.3	< 0.3
(C15:0)	< 0.3	0.5±0.2	< 0.3
(C15:1)	< 0.3	< 0.3	< 0.3
(C16:0)	< 0.3	1.6±0.2	< 0.3
(C16:1)	< 0.3	1.2±0.2	< 0.3
(C17:0)	18.3±0.3	29.1±0.3	15.4±0.3
(C17:1)	< 0.3	< 0.3	< 0.3
(C18:0)	17.5±0.2	< 0.3	16.3±0.3
(C18:1–trans)	< 0.3	< 0.3	< 0.3
(C18:1–cis)	< 0.3	3.8±0.2	< 0.3
(C18:2– trans)	< 0.3	< 0.3	< 0.3

(C18:2– cis)	< 0.3	27.7±0.3	14.7±0.3
(C18:3) (omega–6)	32.4±0.2	13.2±0.3	19.4±0.3
(C18:3) (omega–3)	< 0.3	7.8±0.3	10.8±0.2
(C20:0)	12.8±0.3	7.2±0.3	9.7±0.3
(C20:1)	< 0.3	< 0.3	< 0.3
(C20:2)	< 0.3	< 0.3	< 0.3
(C20:3) (omega–3)	< 0.3	< 0.3	< 0.3
(C20:3) (omega–6)	< 0.3	< 0.3	< 0.3
(C20:4) (omega–6)	< 0.3	< 0.3	< 0.3
(C20:5) (omega 3)	< 0.3	< 0.3	< 0.3
(C21:0)	< 0.3	< 0.3	< 0.3
(C22:0)	< 0.3	< 0.3	< 0.3
(C22:1) (omega–9)	< 0.3	< 0.3	< 0.3
(C22:2)	< 0.3	< 0.3	< 0.3
(C22:6n3) (omega–3)	< 0.3	< 0.3	< 0.3
(C23:0)	< 0.3	< 0.3	< 0.3
(C24:0)	< 0.3	< 0.3	< 0.3
(C24:1)	< 0.3	< 0.3	< 0.3

Data are mean ± standard deviation of three independent replicates

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

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

Data are means ±standard deviation

ppb, μg/kg; ppm, mg/kg

LOQ, limit of quantification; ochratoxin A, 1.5 μg kg^{-1}; fuminisins, 0.1 mg kg^{-1}; T–2+HT–2, 10 μg kg^{-1}

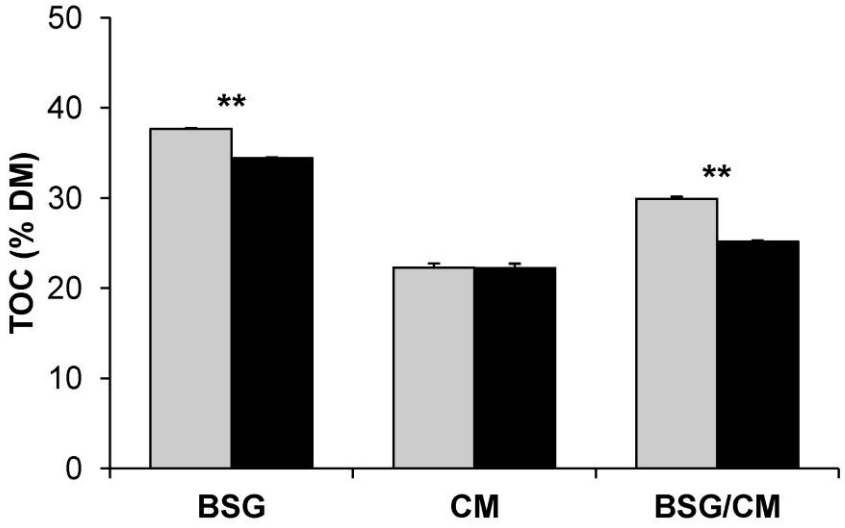


Figure 1

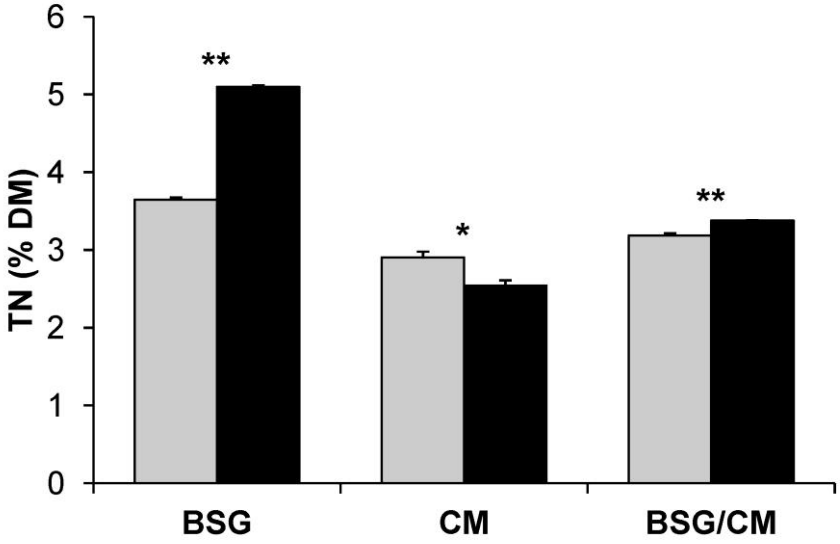


Figure 2

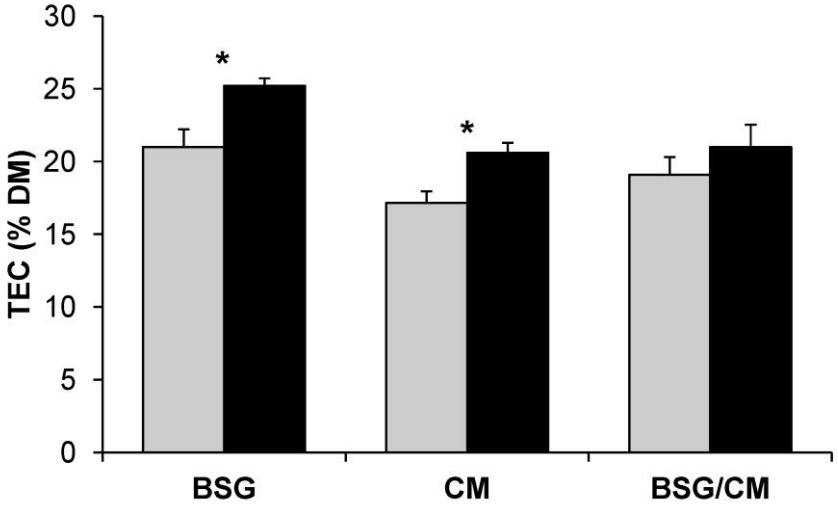


Figure 3

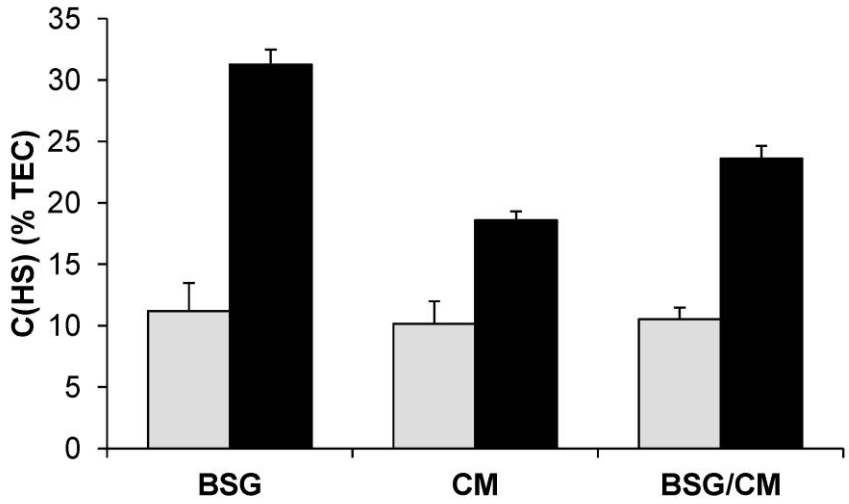
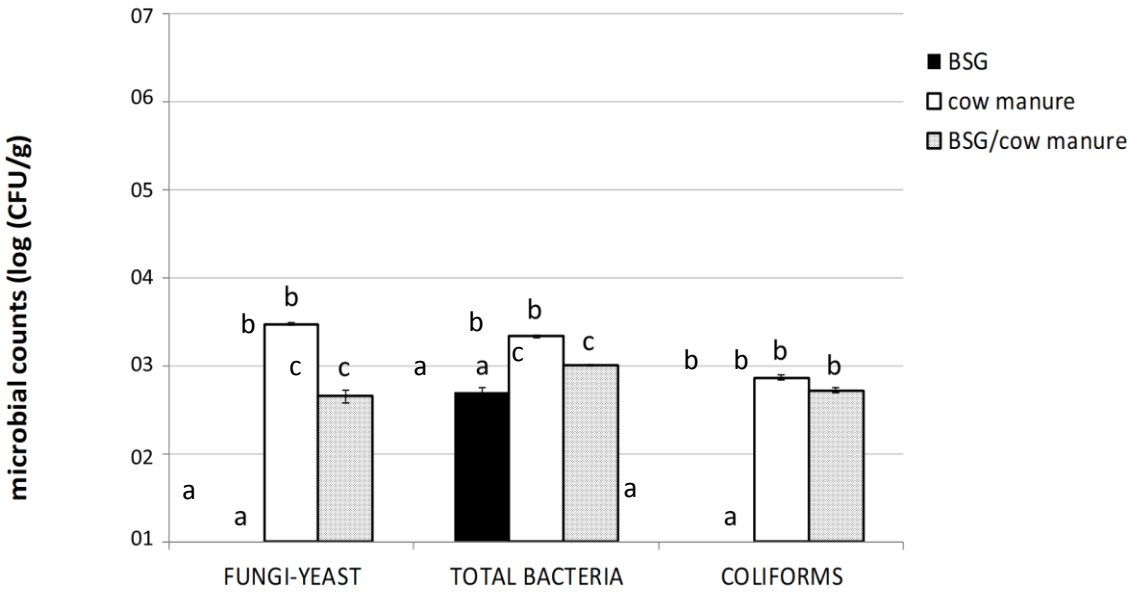


Figure 4

(A)


microbial counts (log (CFU/g)
07
06
05
04
03
02
01
BSG
cow manure
BSG/cow manure
FUNGI-YEAST
TOTAL BACTERIA
COLIFORMS

(B)

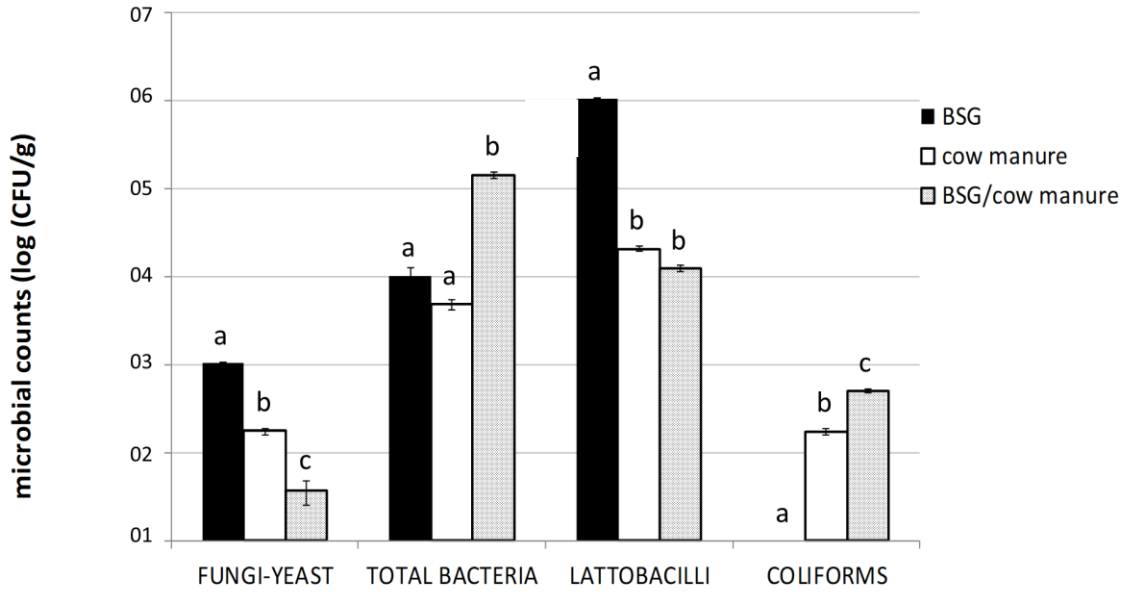
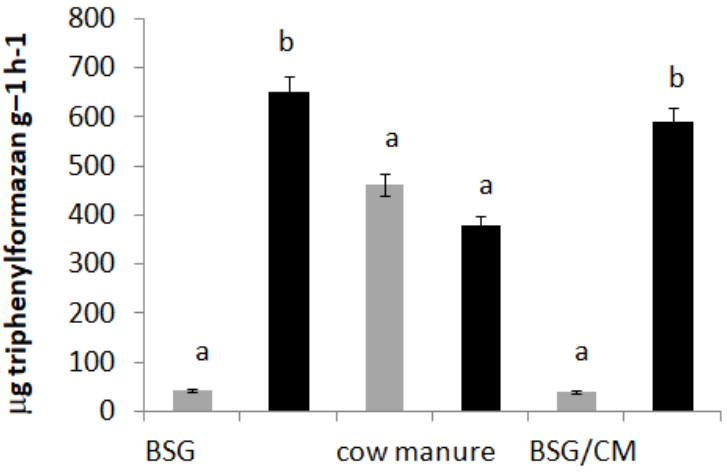
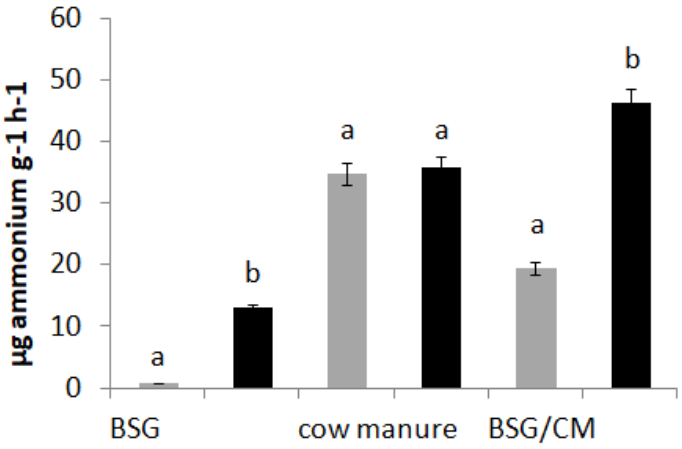

microbial counts (log (CFU/g)
07
06
05
04
03
02
01
BSG
cow manure
BSG/cow manure
FUNGI-YEAST
TOTAL BACTERIA
LATTOBACILLI
COLIFORMS

Figure 5

(A)


µg triphenylformazan g−1 h-1
800
700
600
500
400
300
200
100
0
a
b
a
a
a
b
BSG
cow manure
BSG/CM

(B)


µg ammonium g-1 h-1
60
50
40
30
20
10
0
a
b
a
a
a
b
BSG
cow manure
BSG/CM

(C)

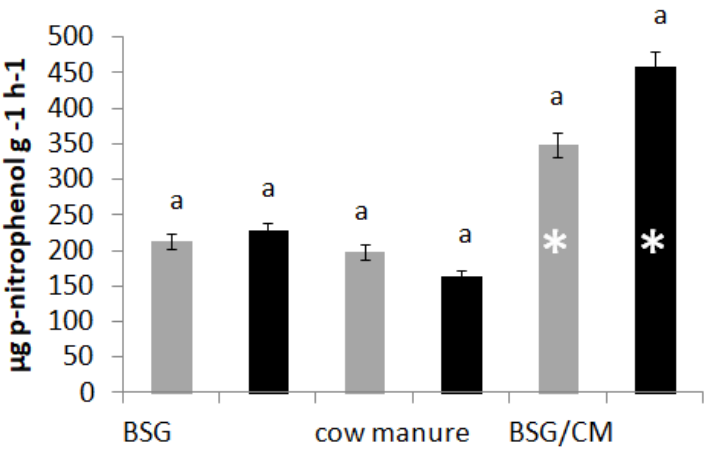

µg p-nitrophenol g -1 h-1
500
450
400
350
300
250
200
150
100
50
0
a
a
a
a
a
a
*
*
BSG
cow manure
BSG/CM

Figure 6