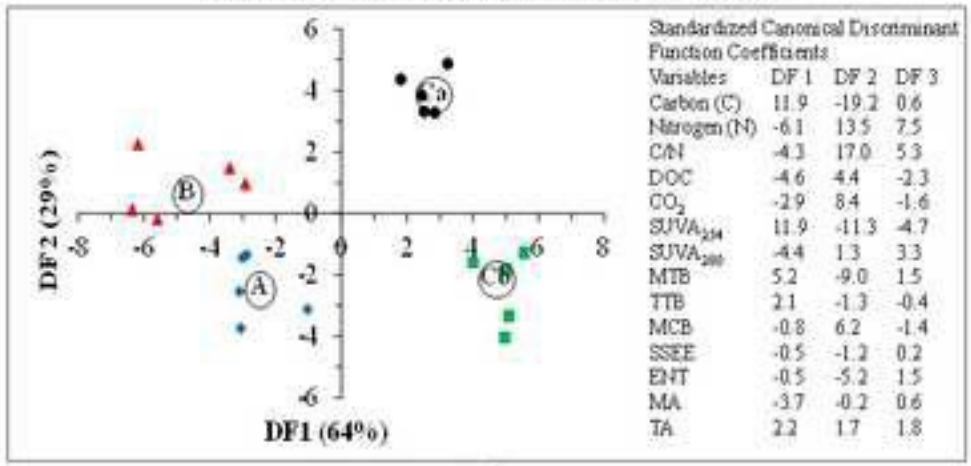
Piles	Deproteinized whey	Bioactivators
A	YES	NO
B	YES	YES
Ca	NO	NO
Cb	NO	YES

Microbiological and Chemical parameters analyzed



Standardized Canonical Discriminant Function Coefficients

Variables	DF 1	DF 2	DF 3
Carbon (C)	11.9	-19.2	0.6
Nitrogen (N)	-6.1	13.5	7.5
C/N	-4.3	17.0	5.3
DOC	-4.6	4.4	-2.3
CO_2	-2.9	8.4	-1.6
$SUVA_{254}$	11.9	-11.3	-4.7
$SUVA_{280}$	-4.4	1.3	3.3
MTB	5.2	-9.0	1.5
TTB	2.1	-1.3	-0.4
MCB	-0.8	6.2	-1.4
SSEE	-0.5	-1.2	0.2
ENT	-0.5	-5.2	1.5
MA	-3.7	-0.2	0.6
TA	2.2	1.7	1.8

Nitrogen supplied by deproteinized whey is the most important factor in affecting the composting process

Highlights

- Composting is an environment-friendly method to reuse winery and dairy by-products
- Deproteinized whey improves composting process
- Bioactivators and deproteinized whey speed up the composting process
- Bioactivators and deproteinized whey provide compost of great stability

Cellulolytic bacteria joined with deproteinized whey decrease carbon to nitrogen ratio and improve stability of compost from wine production chain by-products

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ABSTRACT

Composting residues from wine and dairy chains would contribute to increase the environmental sustainability of the production. The aim of this study was to evaluate the effects of deproteinized whey combined with bioactivators on the composting process. *Bacillus velezensis* and *Kocuria rhizophila*, bacteria with cellulolytic activity, were isolated from raw materials and inoculated in the organic mass to be composted. Piles moistened with deproteinized whey showed the highest reduction of total and dissolved organic carbon due to the stimulation of bacterial activity by nitrogen compounds held within deproteinized whey. Such findings were also confirmed by the speed up of the microbial carbon mineralization. Bioactivators and deproteinized whey speeded up the composting process and returned compost characterized by high stability and quality. The addition of available N is crucial to improve the composting process and can act even better if combined with cellulolytic bacteria.

Keywords: grape marc, pruning residue, bioactivators, *Bacillus velezensis*, *Kocuria rhizophila*

1. Introduction

The wine and dairy supply chains constitute two leading sectors of the agri-food system in Italy. The wine industry covers 8.2% of agricultural production in Italy, which puts onto the market 17.7% of world wine, followed by France (15.9%) and Spain (13.0%) (OIV, 2019). Thus, these three countries alone account for more than 84.8% of total European Union production (DG AGRI, Brussels, 2019).. Such chains produce high quantity of processing wastes, mainly represented by grape marc and pruning residues, with regard to the wine production chain, and deproteinized whey, with regard to the dairy supply chain. The disposal of these wastes, although may be given away for free to farmers for land application, has both economic and environmental negative relapses (Cortés et al., 2020).

The most common use of grape marc and pruning residues is their distribution on agricultural land to increase soil organic matter (Novara et al., 2020). However, having grape marc and pruning residues high C/N ratio (between 25-40) and high content of tannins (Paradelo et al., 2013), they lead to N deficiency and stress for soil microbial community. On the other hand, they hold inorganic nutrients, such as Mg^{2+} and K^{+}, that can be released into the soil following their mineralization (Viel et al., 2017). Furthermore, the burial of pruning residues could also lead to an increase of fungal and bacterial pathogens propagules, so rising the incidence of plant diseases (Sharma et al., 1997). Deproteinized whey is the liquid fraction resulting from the processing of ricotta. Ricotta is a cheese produced by re-cooking residual whey from cheese processing. The whey is re-cooked at high temperatures (80-90 °C) for approximately 20-30 minutes, thus promoting protein flocculation (Settanni et al., 2020). Microbiological analysis conducted on different samples showed the absence of microorganisms due the high temperature processing (Settanni et al., 2020). Deproteinized whey is highly pollutant (Rocha-Mendoza et al., 2020) and, therefore, generally destined for waste disposal (Hausjell et al., 2019). In some typical Sicilian

cheeses (PDO Pecorino Siciliano), deproteinized whey is used for cooking after the moulding process (Gaglio et al., 2021). Furthermore, based on current legislation, the deproteinized whey is considered a special waste due to its high organic content (Italian Legislative Decree n. 152/06). Considering the chemical features of grape marc, pruning residues and deproteinized whey, it is reasonable to combine all these by-products to produce compost to be used in organic farming (Laudicina et al., 2011) according to the Council Regulation (EC) (n. 834/2007 and n. 889/2008). Indeed, the high C/N ratio of grape marc and pruning residues, that may slow down the composting process (Palaniveloo et al., 2020), can be decreased by the N organic compounds within deproteinized whey (Daniel et al., 1999).

Obtaining compost from the combination of grape marc and deproteinized whey could contribute to increasing the environmental sustainability of wine and dairy production chains, also reducing the disposal costs of the respective by-products.

Composting is an intense biological process consisting of a rapid succession of specialized microbial populations secreting various enzymes which drives the organic wastes transformation into humus-rich complex mixtures (Zang et al., 2017). Microorganisms selected and inoculated into the materials to be composted allow for more effective management of waste materials. In fact, they show superior degradative capacities compared to indigenous microorganisms naturally present in the raw materials (Wan et al., 2020). For this reason, to speed up the composting process microbial inoculation is recommended (Ma et al., 2019). Naturally occurring microorganisms in the composting system would be the best candidates for the compost inoculations, in order to accelerate the process. Inoculants generally consist of microbial strains that possess versatile enzymatic capabilities (Jurado et al., 2015). Several species of bacteria (*Bacillus* spp.) and filamentous fungi (*Aspergillus* spp. and *Trichoderma* spp.) are able to facilitate the composting process (Wan et al., 2020). Wei et al. (2007) underline the usefulness of inoculating a blend of

microorganisms into the masses to be composted (*Bacillus casei*, *Lactobacillus buchneri*, *Candida rugopelluculosa*, and *Trichoderma* spp.). In addition, actonimycetes (*Mycobacterium* sp., *Micromonospora* sp. and *Saccharomonospora* sp.) with high degradative activity towards lignocellulose have been successfully used for composting the straw from several cereals (Wei et al., 2019). However, whether the inoculation composting system has an ideal performance is sometimes uncertain because of the competition between the exogenous inoculants and native indigenous microbes, inoculation timing and the quantity and type of microbial inocula (Zhao et al., 2017).

In Italy, recent studies have shown that compost made from industrial wastes can be an ideal source to isolate cellulolytic bacteria (Amore et al., 2013). Moreover, studies dealing with thermotolerant and thermophilic microorganisms during composting phases have highlighted their importance in improving the process (Di Piazza et al., 2020). Generally, these studies try to develop commercial products with selected microorganisms to be used in both domestic and industrial composting, in order to improve the composting process and decrease its costs.

Similarly, the aim of our work was to produce high-quality compost by combining the residues from the cultivation of the grapevine and the by-products of wine chain with deproteinized whey, but contemporarily inoculating the organic mass to be composted with strains of bioactivators at high cellulolytic activity, isolated from the raw materials, in order to speed up the composting process.

2. Material and methods

2.1. Composting site

The composting process was carried out at Cantine Europa (Petrosino, Trapani, Italy; 37°43′1″ N; 12°31′51″E). This site, located at 50 m a.s.l., shows a semiarid Mediterranean climate. The hottest

months are July and August, with an average month temperature of 26 °C, while the coldest are January and February with an average temperature of 10-12 °C. The average annual rainfall is 450 mm, and the wettest months are from November to February. In the year of the study (2018), the average annual temperature was 18°C, with the highest temperatures reached in summer (37 °C); in winter, temperature did not fall below 3 °C.

2.2. Isolation of bioactivators

Presumptive bioactivators were obtained from the raw materials (grape marc, green herbaceous crop residues, pruning residues) used for the composting process. The culture media used for the isolation of presumptive cellulose-degrading bacteria was BC medium, prepared according to the protocol described by Viel et al. (2017). Bacterial colonies were purified by streaking in the same medium. The purified colonies were preserved at -18 °C for further identification and screening for cellulase production.

2.3. Selection of bioactivators

For screening of cellulolytic activity, bacterial isolates were individually transferred in CMC agar plates for 48 h of incubation at 30°C (Yin et al., 2010). After growth of bacteria, the CMC agar plates were flooded with 1% Congo Red and allowed to stand for 15 min at room temperature. The Petri dishes were subsequently treated with a 1 M solution of NaCl in order to highlight the presence of halos. The presence of clear halos at the edges of growing colonies indicated the ability of isolates to hydrolyse cellulose (Irfan et al., 2012). Bacteria producing the largest halo of cellulose hydrolysis were selected as the primary criterion of selection and the growth dynamics was evaluated at different pH and temperatures. The quantitative expression of cellulolytic activity was expressed as the radius of the halo (mm) formed in a Petri dish around the colony was measured.

2.4. Phenotypic and genotypic characterization of bioactivators

Presumptive bioactivators were phenotypically characterized by cell morphology (cocci and rods), Gram reaction (KOH method) and catalase activity (determined by transferring fresh colonies from a Petri dish to a glass slide and adding H_2O_2 5 %, v/v). Molecular identification of cellulolytic bacteria was carried out by the method as described by Weisburg et al. (1991) using the primers rD1 (5′-AAGGAGGTGATCCAGCC-3′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′). The PCR mixture (30 µL total volume) included 62.5 ng of target DNA, 1 × Taq DNA polymerase buffer with 2 mM $MgCl_2$ (ThermoFisher Scientific, Monza, Italy), 0.25 mM of each dNTP, 0.2 µM of each primer and 1.5 U of Taq DNA polymerase (ThermoFisher Scientific, Monza, Italy). PCR conditions were as follows: initial denaturing step at 95°C for 3 min; 30 cycles (1 min at 94°C, 45 s at 54°C, 2 min at 72°C); and an additional final chain elongation step at 72°C for 7 min. The amplicons corresponding approximately to 1400 bp were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Sequences were manually corrected and assembled using Chromas 2.6.2. (Technelysium Pty Ltd, Australia). The PCR products were visualized by UV transillumination on a 2% (w/v) agarose gel (Safe Imager™ Transilluminator, Invitrogen, Italy), stained with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA). The GeneRuler 100 bp Plus DNA Ladder (M-Medical S.r.l., Milan, Italy) was used as a molecular weight marker. The resulting DNA was sequenced using the same primers employed for the PCR amplifications. The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at NCBI web site and those of the sole type strains within the database EzTaxon, located at the EzTaxon web site. All isolates were processed by RAPD analysis with three primers (M13, AB111, and AB106) used singly by means of Thermal cycler (Swift™ MaxPro, Esco Technologies, Inc., USA). The amplified products

were separated by electrophoresis, visualized, and acquired by KODAK Gel Logic 100 System (Kodak, Rochester, USA). The analysis of the RAPD patterns was performed with the Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium).

2.5. Inoculum development

The cultures of the selected strains were streaked in CMC-agar and subsequently inoculated for 24 h at 30°C in a culture broth prepared as described by Irfan et al. (2012). The medium was inoculated with 1 mL of selected bacterial isolates and incubated for fermentation in a shaker (IKA-Werke HS-501 Digital S1, Staufen, Germany) at 35˚C for 24 h, with agitation speed of 140 rpm. At the end of the fermentation period, the broth was centrifuged at 12000 rpm for 10 min at 4˚C and the pellet was used as an inoculum of compost piles.

2.6. Composting plan and sampling

On June 2018, twelve static piles (3 piles per treatment), each with a volume of 3 m^3, hereafter referred to as A, B, C_a and C_b, were set up mixing grape marc coming from crushing of Grillo grapes with green herbaceous crop residues. Each pile consisted of three layers of grape marc mixed with green herbaceous crop residues interspersed with two layers of pruning residues from the past winter pruning. Each pile was therefore composed of: (i) 50% (v/v) of grape marc (C/N 34, pH 4.2), (ii) 30% (v/v) of green herbaceous crop residues, (iii) 20% (v/v) of pruning residues. The B and C_b piles were inoculated with a mixture of the selected bioactivators. A solution (18 L) containing the bioactivators with a ratio of 1:1 of the two selected bacteria species were added to reach a concentration of 6-7 Log CFU g^{-1} composting material. Furthermore, in the A and B piles, the moisture content was maintained by spraying the deproteinized whey (pH 6.2; electrical conductivity 17 dS m^{-1}; total N 1.15 g L^{-1}), while in the C_a and C_b piles the moisture content was

maintained adding equivalent volumes of tap water. Indeed, weekly 125 litres of water or deproteinized whey were added for moisture content maintenance at about 50%. The deproteinized whey added to composting mass was without native microflora. Finally, four treatments were set up as follows: A, pile not inoculated and wetted with deproteinized whey; B, pile inoculated with bioactivators and wetted with deproteinized whey; Ca, pile not inoculated and wetted with water; Cb, pile inoculated and wetted with water.

The piles were covered with Flortex® 55/300 green polypropylene nonwoven sheet (Edilfloor, Sandrigo, Italy) with draining and hydrophobic properties. The piles were turned after 33, 76 and 95 days since their set up to maintain the temperature below 70-75°C, supply oxygen to microorganisms, homogenize the composting mass and guarantee the redistribution of microorganisms.

For each pile, eight sub-samples of equal volume were taken after identifying three equidistant sections along the basal perimeter. In correspondence of each section, two samples were taken at two different heights (one sample at 1/3 and one sample at 2/3 of the pile height). Moreover, two samples were taken in depth towards the pile heart. On-site, the eights samples were mixed and transported to the laboratory in sterile disposable bags at 4 °C.

2.7. Measurement of temperature and moisture content of piles

The temperature of the piles was monitored using Escort iMini data loggers (Cryopak, USA) located in the heart of each pile. The temperature and moisture content values throughout the composting process were determined hourly during the composting process. Data were elaborated using a ConsolePlus software (ver. 1.16.59, Saak Dertadian, USA).

2.8. Microbiological analysis

The evaluation of the dynamics of microbial populations during the composting process was carried out following the procedure described by Viel et al. (2017). The monitoring of pathogenic microorganisms (*Salmonella* spp., *Shigella* spp. and *Enterobacteriaceae*) was performed according to the official methodology reported in ANPA (2003).

2.9. Chemical characterization of compost

Total carbon, nitrogen, and hydrogen were determined, during the composting process, on samples collected at days 7, 15, 45, 75, 105, by using a Perkin-Elmer 2400 CHNS/O elemental analyser. Dissolved organic matter (DOM) was extracted at the same days by shaking 10 g of compost with 100 mL of distilled water for 2 hours. Then, the suspension was filtered by using Whatman 42 filter paper. The specific ultraviolet absorbance at 254 and 280 nm (SUVA254 and SUVA280, respectively) of DOM were obtained by an UV-Vis spectrophotometer. The SUVA corresponded to the UV absorbance measured at 254 and 280 nm (UVmini-1240, Shimadzu, Japan) and normalized by dividing by concentration of DOC solution (Jouraiphy et al., 2008). The amount of carbon held by DOM (dissolved organic C, DOC) was determined by the hot digestion–oxidation (sulphuric acid-dichromate mixture) method.

To monitor the emission of CO_2 as a measure of the microbial respiration, at days 1, 7, 15, 30, 45, 75 and 105 of composting process, twenty grams of each compost sample were weighed in 200 mL glass jars and sealed with rubber stoppers holding silicon septa. The CO_2 accumulated in the headspace of the glass jars, after three days of incubation at 25 °C, was determined by a gas chromatograph (Thermo ScientificTM TRACE GC, Milano, Italy), equipped with a thermal conductivity detector, and a Poropak Q column (helium was the carrier). The C mineralization rate, calculated dividing by 3 the CO_2 accumulated in the headspace and expressed as mg CO_2–C kg^{-1} dry soil day^{-1}, was fitted to the following double exponential decay function (Robertson et al., 1999): Mineralized $C = C_L\, e^{-k_1 t}$

+ $C_S\ e^{-k_2 t}$, where C_L is the labile C at time zero (i.e. the intercept value), k_1 is the decay rate constant of C_L, C_S is the stable C at time zero (i.e. the intercept value), k_2 is the decay rate constant of C_S, and t is the incubation day.

2.10. Statistical analysis

Compost samples were analysed in duplicate. Reported data are means ± standard deviations of three true replicates (n=3). All data were subjected to two-way ANOVA repeated measures. How the moisture was maintained (by only water or even deproteinized whey) and the presence or not of bioactivators, as well as their interaction, were the tested factors.

Discriminant analysis (DA) was performed to differentiate treatments and to identify the major sources of difference between groups. DA effectively projects data into the space of linear combinations of the variables that account for the greatest proportion of between-groups variance relative to within-groups variance. DA was carried out on standardized data simultaneously entering all independent variables. The procedure generated a discriminant function (DF) based on linear combinations of the predictor variables providing the best discrimination between groups. The four treatments were used to divide the dataset into pre-defined groups. The magnitudes of the Standardized Canonical Discriminant Function Coefficients (SCDFCs) were used to indicate how strongly the discriminating variables affect the score. Statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc. 1996).

3. **Results and Discussion**

3.1. Moisture content and temperature of piles

The moisture content of the compost piles (50% on average) remained constant during the first two months of the composting process with no differences among treatments (Fig. 1a). Then, moisture

content decreased by 5% and remained constant until the end of the process. During the composting, three thermic phases were individuated: mesophilic during the first 20 days, thermophilic from 20 to 45 days, cooling phases after 45 days (Fig. 1b). At the beginning, the temperature linearly increased from 30°C to 78°C probably due to the microbial decomposition of easily degradable organic substances (Wei et al., 2014). From day 33 to 45, the temperature linearly decreased from 78°C to 50°C, on average, probably due to mixing which stopped the thermophilic phase; thereafter, up to day 90, the temperature remained fairly constant at 50°C, while slowly decreased towards the end of composting. As for moisture content, there was no treatment effect on compost temperature.

3.2. Isolation, characterization and selection of bioactivators

Eighty-eight bacterial pure cultures were isolated and purified from BC medium plates, specifically 57 isolates were obtained from grape marc, while 31 isolates from herbaceous crop residues. Sixty-three isolates were able to grow at 60 °C (thermophilic), while 25 isolates were classified as mesophilic (30 °C). All isolates were Gram+ and catalase positive. Observations carried out under an optical microscope allowed the isolates to be subdivided into 22 cocci and 66 rod-shaped. Only 8 isolates showed an evident cellulolytic activity, with the presence along the margin of the colony of a halo indicating the activity of the hydrolysis of cellulose (Hendricks et al., 1995) The halo radius values of the 8 strains of bacteria were found to be in the range of 7.3 - 8.8 mm. The cellulolytic bacteria were represented by two species: *Bacillus velezensis* and *Kocuria rhizophila* (Table 1). Specifically, the CMP3, CMP9, CMP12, CMP52, CMP72 isolates of *B. velezensis* had the same polymorphic profile with the exception of the CMP9 strain, which differed from the other isolates also for the source of isolation. While the isolates CMP36 and CMP92 of *K. rhizophila* were different strains. *Bacillus velezensis* is known for its keratinolytic, proteolytic and cellulolytic

activities (Ye et al., 2018). In addition, some strains are known as plant growth promoters and producers of metabolites with antifungal activity (Torres et al., 2020). *Kocuria rhizophila* is a bacterium resistant to up to 10% NaCl and in some crops leads to a significant increase in growth, biomass production, seed germination and photosynthetic capacity (Li et al., 2020). A mixture consisting of one strain of each species (*B. velenzensis* CMP52 and *K. rhizophila* CMP36) that showed the highest cellulolytic activity, was inoculated into B and C_b piles.

3.3. Microbiological properties of compost during the composting process

3.3.1. Total Bacterial count

Counts of total bacterial during the mesophilic and thermophilic composting phases (Fig. 2a, b) were always higher, about 1-2 logarithmic cycles, in piles wetted with deproteinized whey (A and B) compared to those with water (C_a and C_b) and progressively decreased as the composting proceeded. The bacterial counts were similar to those reported by Viel et al. (2017).

3.3.2. Cellulolytic bacteria

Counts of mesophilic cellulolytic bacteria (Fig. 2c) did not show a univocal pattern. In piles moistened with deproteinized whey, they were one logarithmic cycle higher than piles moistened with water at day 7 and 30 of the composting process. From day 45 till the end of the composting process, mesophilic cellulolytic bacteria did not show differences among the treatments.

Counts of thermophilic cellulolytic bacteria (Fig. 2d) compared to mesophilic ones, were lower than about 1 logarithmic unit on average. Piles moistened with deproteinized whey showed the highest counts of thermophilic cellulolytic bacteria from day 7 to day 30. After day 45, they decreased, and no differences occurred among treatments. Such results indicated that deproteinized whey was the most important factor in affecting total and cellulolytic bacteria at the first stages of composting,

probably because of the N compounds it holds which allowed a higher concurrent bacterial C immobilization, i.e, exponential growth, with a consequent faster composting process (Bohacz, 2018, Harindintwali et al., 2020).

3.3.3. Actinobacteria

Mesophilic actinobacteria slowly decreased during the composting process and did not show differences among treatments (Fig. 3a). On the other hand, thermophilic actinobacteria increased from day 0 to day 15, then decreased up to day 75 and finally remained constant up to the end of the composting process (Fig. 3b). Notably, piles moistened with deproteinized whey showed the highest counts of thermophilic actinobacteria from day 7 to day 45. The presence of actinobacteria is extremely important during composting process as they take part in numerous processes ranging from the decomposition of organic substances (cellulose and lignin) to potential biocontrol (Abdulla, 2007).

3.3.4. Filamentous and cellulolityc fungi

Mesophilic and thermophilic filamentous fungi are well-known as important agents of cellulose and lignin degradation (Hatakka and Hammel, 2011). During the composting process, both fungi types gradually decreased and did not show significant differences among treatments (Fig. 3c, d). Counts of both mesophilic and thermophilic cellulolytic fungi (Fig. 3e, f), after 30 days of composting, were higher than filamentous fungi. Such differences were the highest towards the end of the composting process when counts were, on average, 2-3 logarithmic cycles higher for mesophilic (Fig. 3e) and thermophilic (Fig. 3f) cellulolytic fungi than filamentous ones. The presence of mesophilic and thermophilic cellulolytic fungi is fundamental for the humification process, which characterizes the quality of the compost (López et al., 2006). The synergistic action of the

consortium by bacterial and fungal species is crucial for the quality of compost expressed in terms of humification (van Heerden et al., 2002).

3.3.5. Pathogenic bacteria

In the early stages of composting, counts of *E. coli*, *Salmonella* spp. and *Shigella* spp. were greater than normative levels. Self-sterilization induced by high temperatures during the thermophilic phase of the composting process (> 60 °C) led to the disappearance of *Salmonella and E. coli* and the values recorded dropped below to the normative levels (Fig. 2e) (Pinter et al., 2019). Counts of Enterobacteriaceae decreased after the end of the thermophilic phase for all piles. Pathogenic bacteria were not affected by any experimental factors and their counts were similar to those reported by Bustamante et al. (2008) and Hassen et al. (2001).

3.4. Chemical properties of compost during the composting process

3.4.1. pH

The pH of compost ranged from 6.8 to 7.8 (data not shown), being higher in compost wetted with deproteinized whey. Wang et al. (2015) observed during the first 5-10 days of composting different materials (dairy cattle manure, chicken manure, tomato stem waste, green waste, cabbage waste, kitchen waste, and municipal solid waste), firstly an increasing and then a decreasing trend of compost reaction from 8.6 to 8.0. They ascribed such trend primarily to the alkalinization by evolved ammonia, then to the production of low molecular organic acids and nitrification. The final pH of compost is widely used to evaluate the quality of compost because it influences both soil pH and the bioavailability of nutrients to plants. The optimal pH of a given composting mixture of residues is 6.9–8.3 (Prasad and Chualain, 2003). All compost piles attained the standard for pH, indicating that they were suitable to be applied to soil.

3.4.2. Carbon and nitrogen, and C/N ratio

Organic carbon (C), as the fundamental carbon and energy source for microorganisms, undergoes several complex biological transformations and its content is a reliable index to reflect the maturity and quality of the compost. The amount of C at the beginning of the composting process was on average 52% (w/w, Fig. 4a). During the composting, C content declined by 15% in A, 16% in B, 9% in C_a and 11% in C_b, i.e., piles added with bioactivators and moistened even with deproteinized whey showed the highest C decrease. However, the four treatments did not significantly differ among them. This result may suggest that both bioactivators and deproteinized whey may trigger off the biodegradation of organic matter, probably due to the higher microbial biomass and the supply of nitrogen that stimulate bacteria activity.

At the beginning of composting, deproteinized whey increased nitrogen, on average, by 0.2 g kg^{-1} (data not shown). Then, during the composting, total nitrogen % content increased in all the treatments, likely as a consequence of the C content decrease, although a concomitant N_2 fixation cannot be excluded. At the end of the composting process, nitrogen was by 0.3 % higher in soils inoculated with the bioactivators than in those not inoculated. The initial C/N ratio of the composting biomass was 28 (Fig. 4c). During composting, the C/N ratio continuously declined thus reaching values, at day 105, of 14.3, 11.8, 17.7 and 15.3 in compost A, B, C_a and C_b, respectively. The C/N ratio is the most significant parameter to define the performance of the composting process; indeed, it has been extensively used as criterion to evaluate the compost maturity, stability, and safety (Cui et al., 2017; Wang et al., 2015). If there is shortage of nitrogen availability, the decomposition rate of the materials will proceed more slowly as a consequence of the slowdown of microbial activity. By contrast, with an excess of nitrogenous substances, a volatilization of N as ammonia occurs. Generally, a C/N ratio equal to 12 is the threshold to be reached for compost

maturity (Wang et al., 2017). Only compost B, i.e. compost inoculated with bioactivators and moistened with deproteinized whey, showed at day 105 a C/N slightly less than 12 suggesting a satisfactory compost maturation (Fig. 4c). Such behaviour may be due to the highest abundance of mesophilic and thermophilic bacteria. It is also noteworthy that the largest decreases of the C/N ratio occurred in compost inoculated with bioactivators, probably as a result of the highest decomposition rate of total organic matter as a consequence of the highest abundance of total bacteria, actinobacteria and thermophilic cellulolytic bacteria. The C/N decrease during composting may be also ascribed to the enhanced assimilation of organic materials even triggered by the bioactivators (Paradelo et al., 2013).

3.4.3. Evolution of DOC and DOM during the composting process

At day 7, DOC was higher in compost piles moistened with deproteinized whey compared to those with only water (Fig. 4d). During the first 45 days, DOC declined rapidly in all compost piles but more sharply in those moistened with deproteinized whey. Such behaviour may be ascribed to a greater immobilization and/or mineralization of available and easily biodegradable organic substrates such as amino acids, peptides, carbohydrates, and organic acids by the proliferating bacteria, even increased by the N compounds held within the deproteinized whey (Hsu and Lo, 1999). During the whole composting period, DOC decrease was significantly higher in compost B, thus suggesting that the addition of deproteinized whey and bioactivators promote the biodegradation of easily available organic compounds. DOM is a vital type of organic matter to promote the cycling of carbon and microbial activity during the composting process (Wei et al., 2014). Moreover, DOM may hold a heterogeneous mixture of both humic substances and enzymes, thus working as energy source and catalysts microorganisms to improve the compost humification (Laudicina et al., 2013; He et al., 2014). During composting, $SUVA_{254}$ and $SUVA_{280}$ of DOM

increased (Fig. 4e and 4f). The highest substantial increases of $SUVA_{254}$ in composts B and C_b indicated the increase of aliphatic compounds and unsaturation degree of humic substances, likely as a result of the rapid transformation of non-aromatic compounds (He et al., 2014) due to the inoculation with the bioactivators.

$SUVA_{280}$, an index for detecting aromatic compounds in DOM, markedly increased in compost B and C_b throughout composting process. These results revealed that the water-soluble aromatic compounds accumulated, and the stability of DOM increased (Zhao et al., 2018) following the inoculation with bioactivators.

3.4.4. CO_2 emission rates

The long-term release of CO_2 from compost under optimal conditions can be used through mathematical models to monitor the functional pools of organic matter, commonly referred to as active or labile and passive or stable fractions of organic matter. Emission rates of CO_2 decreased during the composting process following a two-orders exponential decay pattern. The CO_2 emission rate during the first 15 days was highest in composts A and B, followed by compost C_b, thus suggesting that bioactivators and deproteinized whey stimulated microbial activity, as previously reported for observed DOC patterns. After day 30, CO_2 emission rate continued to decrease in all treatments but more slowly in compost B and C_b, suggesting that the amount of biologically available C ran out before in compost inoculated with bioactivators (Fig. 4b).

The labile C pool (C_L) was the highest in compost A and B, with no differences between them, followed by C_b and finally by C_a, and this trend may be explained in terms of the whey addition. Notably, from the decay rate constants of both labile and stable C pools (k_1 and k_2, respectively), we may infer that, in the presence of whey, bioactivators increased the C mineralized from both pools

while, without whey, bioactivators decreased the C mineralized from both pools (Fig. 4b), likely because since the first stages of composting they met N deficiency.

3.5 Discriminant analysis

Discriminant analysis generated three discriminant functions (DFs) and found 14 variables, which discriminated among treatments (Fig. 5). Carbon content and $SUVA_{254}$ had the highest values of SCDFCs on DF1 which explained 64% of between-group mean differences and separated piles moistened with deproteinized whey (A and B) from those moistened with water (Ca and Cb). Also, N content and MTB, however, had high SCDFCs on DF1.

Carbon and N contents, C/N ratio and $SUVA_{254}$ had SCDFCs higher than 10 on DF2 which explained 29% of variance and separated treated piles, regardless of experimental factors (whey and bioactivators), from not treated one (only water). Notably, based on DF2, treatment A was more similar to C_b than B, thus suggesting that whey and bioactivators may have a significant synergistic effect. DF3 explained less than 10% of variance. Overall results from DA suggested that deproteinized whey is the most important factor in discriminating the piles and hence affecting the composting process by lowering the C/N ratio and increasing the humification degree (high SUVA254) of compost. Mesophilic total and cellulolytic bacteria also play an important role in discriminating the treatments.

4. Conclusions

The compost piles were obtained with an innovative protocol based on the reuse of deproteinized whey and on the inoculation of the composting mass with bioactivators selected from the raw material subjected to the composting process. Deproteinized whey was the most important factor in speeding up the composting process due to the addition of organic nitrogen. Piles moistened with

deproteinized whey and inoculated with bioactivators showed the lowest C/N ratio and the highest $SUVA_{254}$ and $SUVA_{280}$ indexes, thus suggesting a compost of great stability. Further studies will be aimed at evaluating the antagonistic activity of bioactivators and their influence on the composition of the microbiota in the different composts. The composting of these waste products can play a role within a vision of circular bioeconomy, as it allows to find an alternative way of disposal to the current processes, which are expensive and polluting.

Acknowledgements

This work was supported by the Ministry of the Economic Development, General Management for Business Incentives [Research project "Integrated approach to product development innovations in the leading sectors of the Sicilian agri-food sector", F/050267/03/X32 - COR 109494 CUP: B78I17000260008. Also, the winery CANTINE EUROPA Società Cooperativa Agricola SS 115 Km 42.400 − 91020 PETROSINO (Trapani, Italy) and, in particular, the president Dr. Nicolò Vinci and Mr. Franco Zerilli, are acknowledged for the equipment and support during the preparation of the compost piles.

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Figure captions

Fig . 1. Moisture content (%) and temperature (°C) measured at the center of the compost piles during the composting process. Results are mean±standard deviation of three measurements.

Fig. 2. Counts (Log CFU g^{-1}) of (a), mesophilic total bacteria (MTB); (b), thermophilic total bacteria (TTB); (c), mesophilic cellulolytic bacteria (MCB); (d), thermophilic cellulolytic bacteria (TCB); (e), *Salmonella* spp., *Shigella* spp., *Escherichia coli* and *Enterococcus faecalis* (SSEE); (f), *Enterobacteriaceae* (ENT) during the composting process. Results are mean±standard deviation of three measurements.

Fig. 3. Counts (Log CFU g^{-1}) of (a), mesophilic actinobacteria (MA); (b), thermophilic actinobacteria (TA); (c), mesophilic filamentous fungi (MF); (d), thermophilic filamentous fungi (TF); (e), mesophilic cellulolytic filamentous fungi (MCF); (f), thermophilic cellulolytic filamentous fungi (TCF) during the composting process. Results are mean±standard deviation of three measurements.

Fig. 4. Patterns of chemical parameters determined during the composting process: (a) total carbon; (b) carbon mineralization; (c) total carbon to nitrogen ratio (C/N ratio); (d) dissolved organic carbon (DOC); (e) specific ultraviolet absorbance at 254 nm ($SUVA_{254}$); (f) specific ultraviolet absorbance at 280 nm ($SUVA_{254}$). Results are mean±standard deviation of three measurements.

Fig. 5. Discriminant plot of the four treatments based on the chemical and microbiological parameters determined on composting mass during the composting process.

Figure1

Click here to access/download;Figure;Fig. 1.jpg

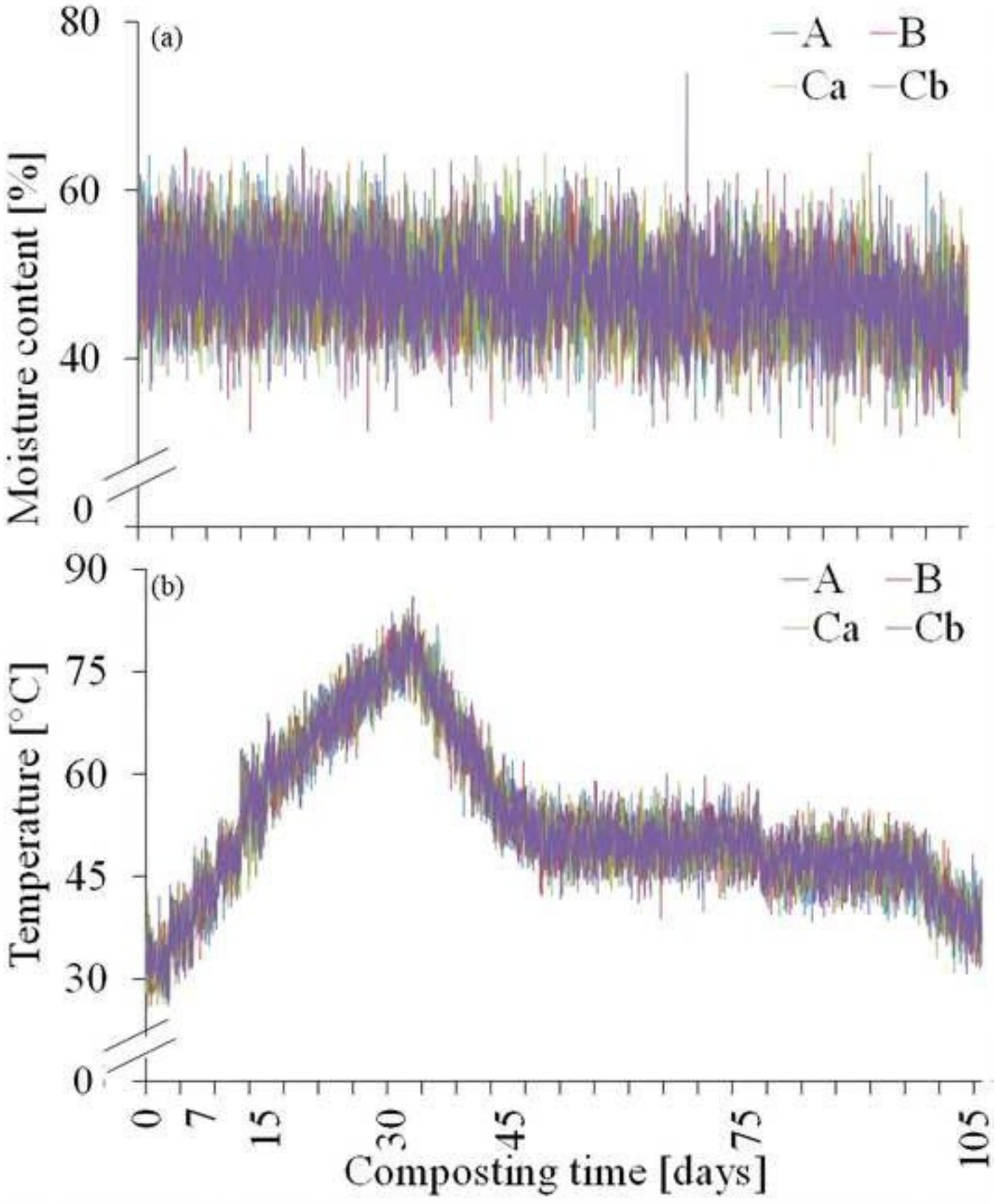

Figure2

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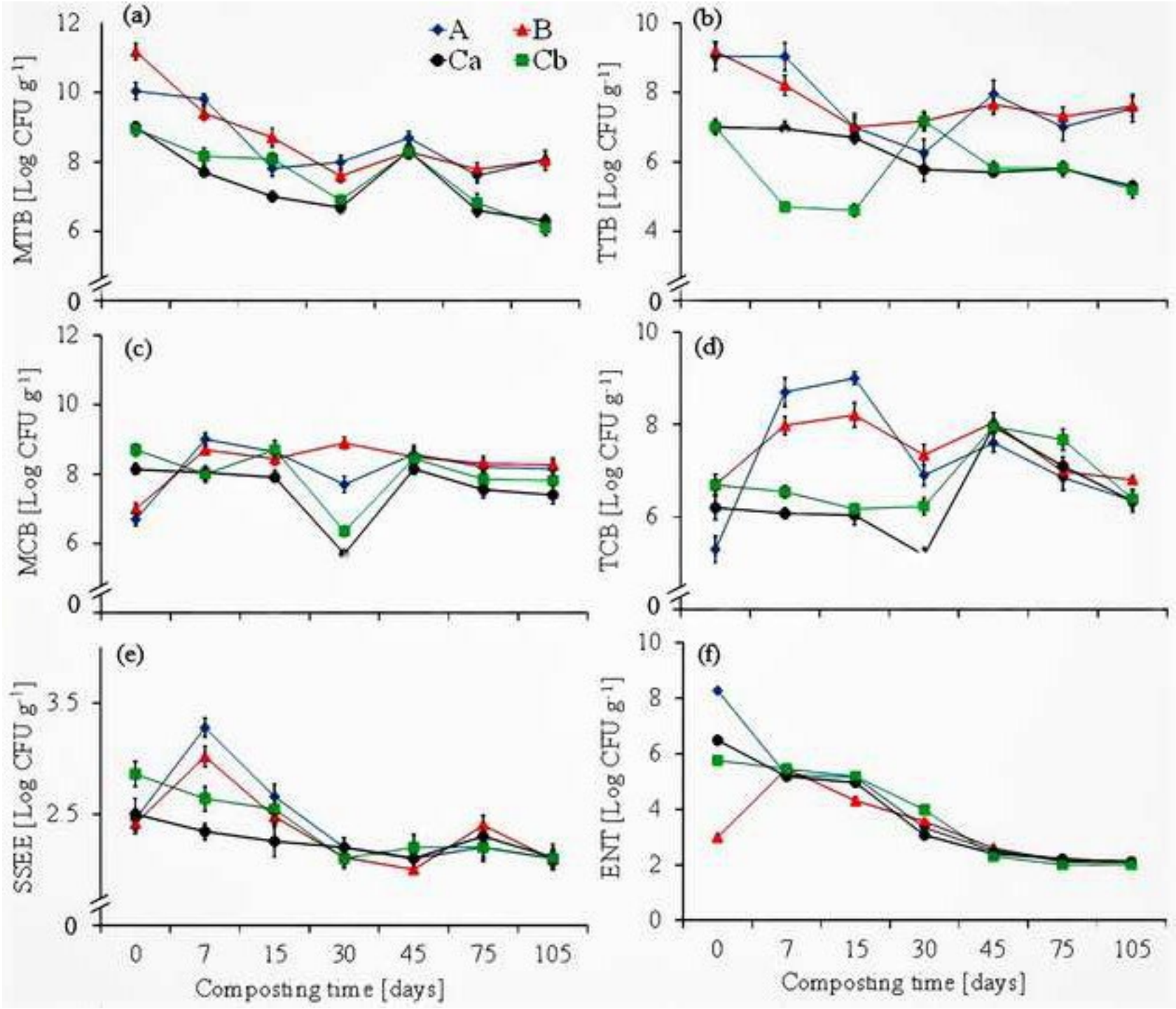

Figure3

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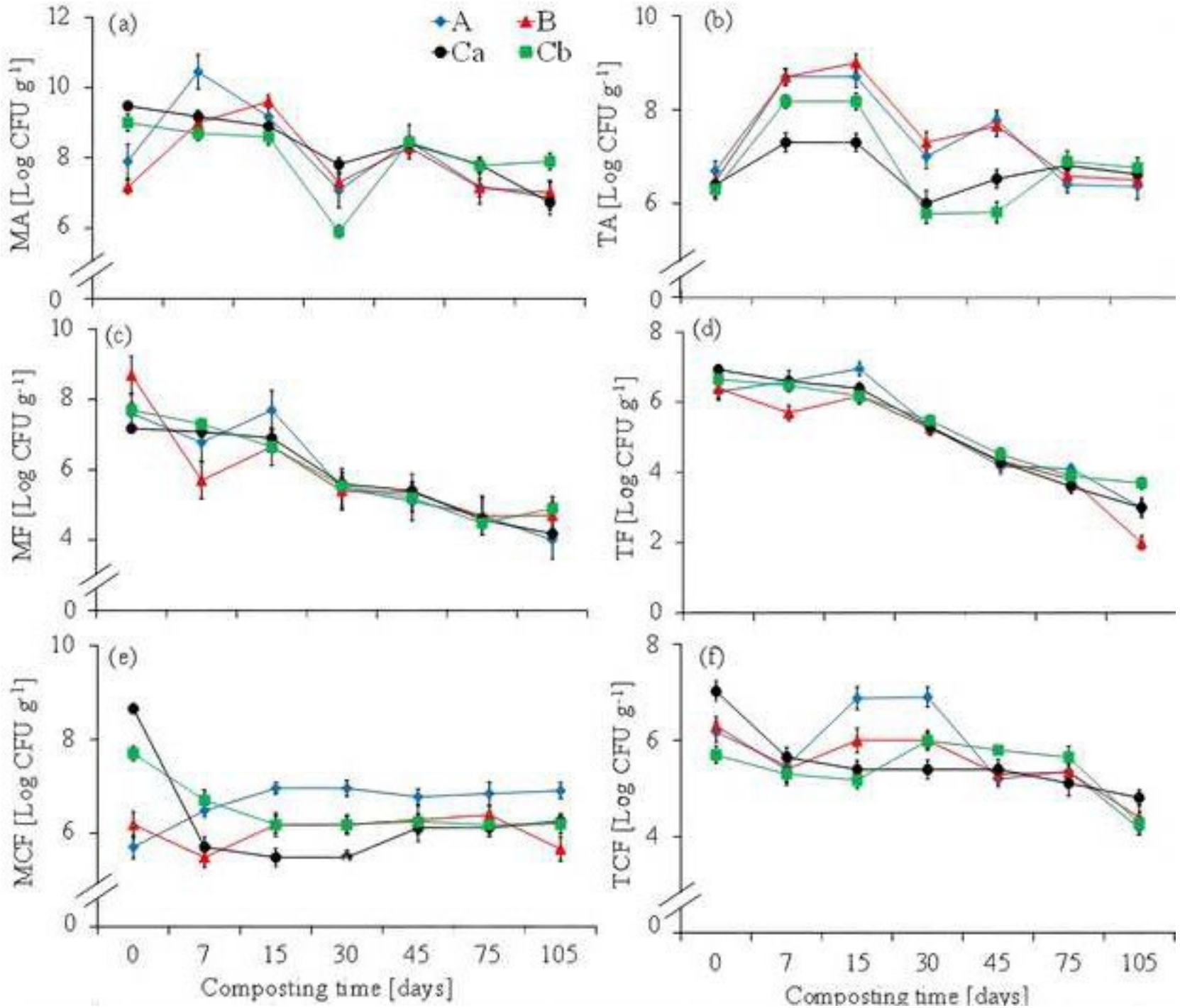

Figure4

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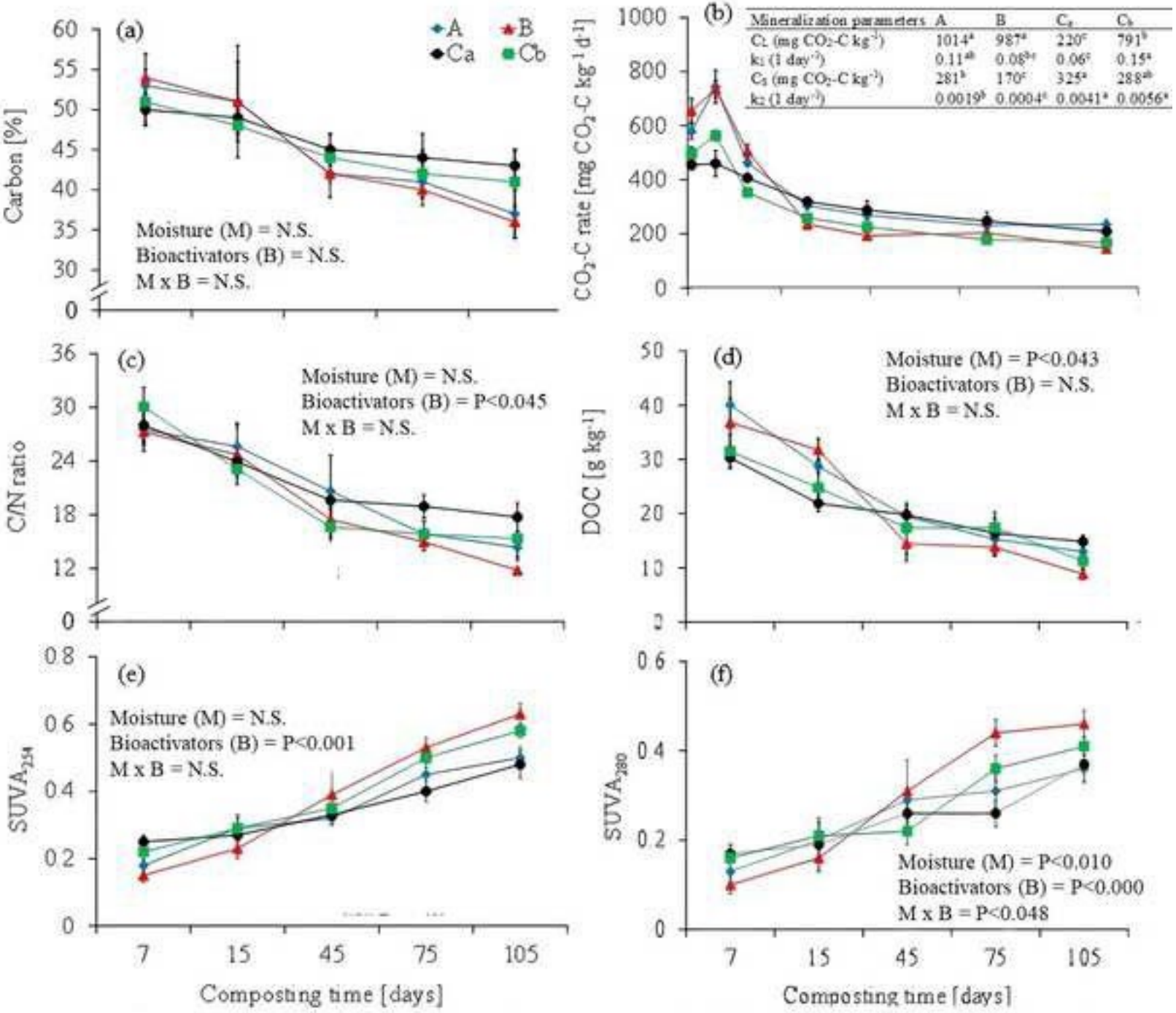

Figure5

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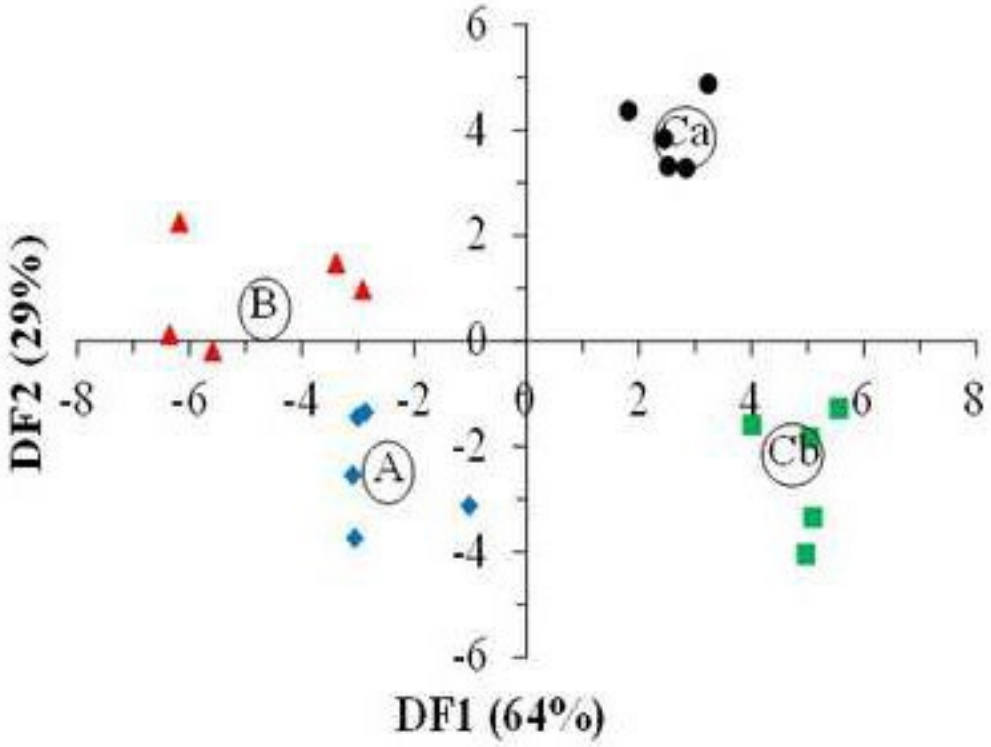

Standardized Canonical Discriminant Function Coefficients

Variables	DF 1	DF 2	DF 3
Carbon (C)	11.9	-19.2	0.6
Nitrogen (N)	-6.1	13.5	7.5
C/N	-4.3	17.0	5.3
DOC	-4.6	4.4	-2.3
CO_2	-2.9	8.4	-1.6
$SUVA_{254}$	11.9	-11.3	-4.7
$SUVA_{280}$	-4.4	1.3	3.3
MTB	5.2	-9.0	1.5
TTB	2.1	-1.3	-0.4
MCB	-0.8	6.2	-1.4
SSEE	-0.5	-1.2	0.2
ENT	-0.5	-5.2	1.5
MA	-3.7	-0.2	0.6
TA	2.2	1.7	1.8

Table 1. Molecular identification by PCR amplified products of 16S rDNA of compost raw material bacteria isolates.

Isolation source	**Code Isolate**	**Species**	**% similarity (accession no. of closest relative) by**		**Sequence length (bp)**	**Accession no.**
			BLAST	**EzTaxon**		
Grape marc	CMP3	*Bacillus velezensis*	100 (MT375545.1)	99.93 (CR-502)	1426	MZ129212
Grape marc	CMP 9	*Bacillus velezensis*	100 (MT375545.1)	99.93 (CR-502)	1426	MZ129213
Grape marc	CMP12	*Bacillus velezensis*	100 (MT375545.1)	99.93 (CR-502)	1426	MZ129214
Grape marc	CMP36	*Kocuria rhizophila*	100 (MK465367.1)	99.79 (TA68)	1396	MZ128817
Herbaceous crop residues	CMP 43	*Bacillus velezensis*	100 (MK641661.1)	99.85 (CR-502)	1417	MZ129215
Grape marc	CMP52	*Bacillus velezensis*	100 (MK780002.1)	99.93 (CR-502)	1417	MZ129216
Grape marc	CMP72	*Bacillus velezensis*	100 (MT375545.1)	99.93 (CR-502)	1417	MZ129217
Grape marc	CMP92	*Kocuria rhizophila*	100 (MK465367.1)	99.93 (TA68)	1406	MZ128818

CRediT author statement

Cellulolytic bacteria joined with deproteinized whey decrease carbon to nitrogen ratio and improve stability of compost from wine production chain by-products

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