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Research article

Influence of earthworms on the microbial properties and extracellular enzyme activities during vermicomposting of raw and distilled grape marc

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ABSTRACT

The treatment of winery wastes by using appropriate management technologies is of utmost need in order to reduce to a minimum their disposal and avoid negative environmental impacts. This is of particular interest for grape marc, the main solid by-product of the winery industry. However, comparative studies on a pilot-scale dealing with the impact of earthworms on marc derived from both red and white grape varieties during vermicomposting are still scarce. The present study sought to evaluate the changes in the biochemical and microbiological properties of red and white raw marc in the presence and the absence of the earthworm species *Eisenia andrei*. The distilled marc obtained through distillation of the red grape marc was also considered under this scenario. Samples were taken after 14, 28, 42, and 63 days of vermicomposting. On day 14 earthworms led to a pronounced increase in most of the enzymatic activities, but only in those vermireactors fed with raw marc from the red grape variety. Alfa- and beta-glucosidase as well as chitinase and leucine-aminopeptidase activities were between 3 to 5-times higher relative to the control, while alkaline phosphomonoesterase was even up to 14-fold higher with earthworm presence. From day 28 onwards the magnitude of earthworms' effect on the studied enzymes was also dependent on the type of grape marc. Reduced values of basal respiration, ranging between 200 and 350 mg CO_2 kg OM h^{-1} and indicative of stabilized materials were found in the resulting vermicomposts. Moreover, the content of macro- and micronutrients in the end products matched with those considered to have the quality criteria of a good vermicompost. Altogether, these findings reinforce the effectiveness of vermicomposting for the biological stabilization of grape marc with the dual purpose of fertilizer production and environmental protection.

1. Introduction

The winemaking industry has been gaining noticeable attention for centuries from an economic, social and cultural perspective (Spigno et al., 2019; Pinter et al., 2019). The International Organization of Vine and Wine estimated that about 260 million hl were produced globally in 2020 (http://www.oiv.int). The European Union (EU) comprises 44% of world's wine-growing areas, with Spain, France and Italy as the three Member States accounting for 76% of EU areas under vines. The increasing activity that it awakens comes hand in hand with the necessity of searching for profitable and sustainable options aiming at the management and valorization of the generated solid and liquid winery by-products (Gómez-Brandón et al., 2019a; Cortés et al., 2020; Ilyas et al., 2021; Portilla-Rivera et al., 2021). Putting the accent on this matter it is of particular interest for grape marc given that approximately 18–25% of the grape mass results into this by-product during wine production (Chowdhary et al., 2021). Approximately 1 kg of grape marc comes from the production of 5 L of wine, accounting for a worldwide production of 10–13 Mtons per year. In this regard, vermicomposting has been found as an effective option to dispose of and treat large quantities of raw marc derived either from white (Domínguez et al., 2014; García-Sánchez et al., 2017; Almeida-Santana et al., 2020) or red grape varieties (Gómez-Brandón et al., 2019b, 2020a) on a real-scale application.

A way to economically valorize grape marc is through distillation that permits the recovery of ethanol for its use in the elaboration of

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alcoholic beverages (Ratna et al., 2021). The feasibility of the vermicomposting process has also been demonstrated for processing distillery residues derived from the wine sector (Nogales et al., 2005; Hanc et al., 2019; Gómez-Brandón et al., 2020b,c; Nogales et al., 2020). Treating both raw and distilled grape marc through vermicomposting led to rapid shifts in their microbial biomass and activity (Gómez-Brandón et al., 2011; Castková and Hanc, 2019), and in the composition of the microbial communities within the first two weeks of vermicomposting (Gómez-Brandón et al., 2019b, 2020c). These compositional changes were further accompanied by an increase in the abundance of putative genes related to metabolic processes potentially beneficial for plant growth and development (Kolbe et al., 2019; Gómez-Brandón et al., 2020b,c). In particular, these authors reported higher abundances of genes involved in plant hormone synthesis, and in the biosynthesis of antibiotics in the grape marc-derived vermicompost when compared to the starting material.

The abovementioned studies were primarily focused on only one type of marc, either raw or distilled, and did not include a control without earthworms. White and red winemaking processes involve a different fermentation process (Domínguez et al., 2017) and, raw marc's properties undergo strong selective pressures through distillation due to high ethanol concentrations, low pH, reduced oxygen levels and temperature fluctuations (Nogales et al., 2005). These distinct procedures likely result in compositional differences between raw marc derived from white and red grape varieties, and with regard to distilled marc as reported by Gómez-Brandón et al. (2020b,c) in terms of bacterial richness and diversity. It is known that the starting material largely influences earthworms' activity with consequences on the dynamics of the vermicomposting process and on the potential usefulness of the vermicompost as an organic amendment (Domínguez et al., 2019).

Following this rationale, the present study sought to evaluate the role of the earthworm species *E. andrei* during vermicomposting of raw and distilled grape marc from a biochemical and microbiological perspective. For each type of marc, pilot-scale reactors designed to handle large amounts of substrate were set up and performed at the maximum earthworm density capacity throughout the trial. Control reactors in the absence of earthworms were also included. Specifically, the impact of the earthworm *E. andrei* was assessed on microbial biomass carbon and on basal respiration used as a proxy of microbial activity, as well as on the abundances of bacteria and fungi over a vermicomposting period of 63 days. The shifts in a wide number of extracellular enzymatic activities involved in the main nutrient cycles were evaluated throughout the vermicomposting process. From a functional perspective, the activity of extracellular enzymes has received considerable attention because these enzymes contribute to the processes controlling decomposition and respond promptly to environmental changes (Acosta-Martínez et al., 2018). They are considered as sensitive indicators of biological processes and investigating the effects of earthworms on extracellular enzyme activity during vermicomposting may enhance the understanding of the dynamics of the process.

2. Material and methods

2.1. Grape marc

The white grape marc (*Vitis vinifera* L. cv. Albariño) was kindly supplied by Terras Gauda winery in Pontevedra (Galicia, northwestern Spain). The red grape marc (*Vitis vinifera* L. cv. Mencía) and its distillery effluent were kindly provided by the Abadía da Cova winery located in Lugo (Galicia, northwestern Spain). Both grape varieties Albariño and Mencía represent 95% of the annual white and red grape harvest in northwestern Spain, respectively. The three types of marc were stored at 4 °C until needed, and turned and moistened with water for two days prior to the vermicomposting trial. Their main physico-chemical properties are given in Table 1.

Table 1
Main characterization of the three types of grape marc used in this study. Values are means ± standard error. Nutrient data are on a dry weight (dw) basis.

	Albariño raw marc	Mencía raw marc	Mencía distilled marc
Moisture (%)	71.98 ± 0.64	77.61 ± 0.95	70.71 ± 0.68
OM (%)	90.63 ± 0.82	92.48 ± 0.72	91.59 ± 0.64
pH	4.36 ± 0.04	3.76 ± 0.05	4.30 ± 0.07
EC (mS cm^{-2})	1.34 ± 0.15	0.84 ± 0.02	0.89 ± 0.03
Total C (%)	51 ± 0.14	50 ± 0.15	51 ± 0.14
Total N (%)	1.85 ± 0.02	2.24 ± 0.04	1.91 ± 0.02
Ca (mg kg^{-1} dw)	3206 ± 52	2308 ± 66	3239 ± 69
K (mg kg^{-1} dw)	19,723 ± 372	16,602 ± 1265	18,834 ± 513
P (mg kg^{-1} dw)	2481 ± 29	2675 ± 47	2245 ± 37
Mg (mg kg^{-1} dw)	1045 ± 14	1029 ± 19	838 ± 11
Mn (mg kg^{-1} dw)	15 ± 0.16	30 ± 0.29	27 ± 0.66
Fe (mg kg^{-1} dw)	136 ± 56	93 ± 18	90 ± 8
S (mg kg^{-1} dw)	1188 ± 10	1307 ± 22	1220 ± 26

OM: organic matter.
EC: electrical conductivity.

2.2. Vermicomposting set-up and sampling design

Vermicomposting was carried out in rectangular metal pilot-scale vermireactors (2 m long x 50 cm wide x 1 m high; Fig. 1) housed in a greenhouse belonging to the facilities of the University of Vigo (Galicia, northwestern Spain). The initial set up of the vermireactors was performed as shown in Gómez-Brandón et al. (2019b, 2020a,c). Briefly, each reactor contained a base layer of vermicompost (12 cm height) as a bed for the earthworms prior to adding the respective grape marc. A plastic mesh (5 mm mesh size) was placed on top of the vermicompost bedding to permit earthworm migration. The vermireactors were continuously fed for almost a year with the distinct types of marc. The density of the earthworm population (*Eisenia andrei*) was determined by a random collection of 10 samples, each 6 cm deep (five from above and five from below the plastic mesh) of the material in the vermireactor. Prior to start the 63-day trial, the vermireactors were operating at the maximum earthworms' density capacity, having an average value of 11, 115 ± 2827 individuals m^{-2} that corresponds with a mean biomass of 1361 ± 415 g m^{-2}.

For the purpose of the present study, a new layer of each grape marc (50 kg fresh weight, fw) was placed and no more substrate was added to the vermireactor until the end of the trial, that is for the 63-day period. The species *E. andrei* is characterized by a high rate of consumption,

Fig. 1. Overview of the pilot-scale vermireactors used in the present study. Fresh layer of grape marc on top of the plastic mesh for the performance of the vermicomposting trial in the presence of the earthworm *E. andrei*.

digestion and assimilation of organic matter and the fresh marc layer was completely processed by the earthworms after 63 days. Control reactors with the same abovementioned dimensions were considered for the experimental set-up and consisted of each type of marc without the presence of earthworms. Waste mixtures were turned once a week and the moisture content in each vermibed was maintained around 75% throughout the 63-day trial by periodic sprinkling of adequate quantity of water, if required. To prevent moisture loss, all of the reactors with and without earthworms were covered with a shade cloth throughout the study period.

For the characterization of the biochemical and the microbiological properties, the grape marc layer (6 cm height) of the reactors with and without earthworms was divided into eight equal sections, and one sample (30 g) was taken at random from above the mesh in each section with a cylindrical sampler (7.5 cm diameter x 12 cm height) after 14, 28, 42 and 63 days of vermicomposting. The samples from each section were stored at 4 °C for microbial biomass carbon, basal respiration and enzyme activity measurements; and at −20 °C prior to DNA extraction and real-time PCR.

2.3. Physico-chemical and nutrient analyses

Samples were dried 24 h at 105 °C and combusted 5 h at 550 °C in a muffle furnace (Carbolite, CWF 1000) for the determination of the moisture and organic matter content respectively. Electrical conductivity (EC) and pH were measured in aqueous extracts (1:10 mass/volume) by using a Crison conductivity metre CM35 and a Crison MicropH 2000 pH metre, respectively. Total C and N contents were assessed in oven-dried (60 °C) samples, in a Carlo Erba (EA 1108 CHNS–O) 1500 C/N analyser. The total content of macro- and micronutrients was determined from extracts of dried samples previously subjected to nitric-perchloric digestion by optical emission spectrometry with inductively coupled plasma (ICP-OES) following the USEPA 3050 B method (USEPA, 1996).

2.4. Microbial biomass and microbial activity

Microbial biomass C was determined by the chloroform fumigation–extraction method using a $K_{EC} = 2.64$ (Vance et al., 1987). Microbial activity was assessed as basal respiration by measuring the rate of evolution of CO_2 after 6 h of incubation. The evolved CO_2 was trapped in NaOH and then measured by titration with HCl to a phenolphthalein endpoint after adding excess of $BaCl_2$ (Anderson, 1982).

2.5. DNA extraction and real-time PCR

DNA was extracted from 0.25 g (fw) of grape marc using the MO-BIO PowerSoil® kit (MoBio Laboratories Inc., Carlsbad, California) according to manufacturer's protocols. DNA quality and quantity were evaluated using BioTek's Take3™ Multi-Volume Plate (Sinergy™ 2 Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc.). Quantitative real-time PCR (qPCR) analysis was performed to determine the 16S rRNA gene copy number of bacteria, and the 18S rRNA gene copy number of fungi by using the primer pairs 1055f/1392r and FF390/FR1 respectively, as previously described by Gómez-Brandón et al. (2021).

2.6. Enzymatic activities

A total of twelve hydrolytic enzymatic activities were measured in the sample extracts by applying a heteromolecular exchange process (Cowie et al., 2013; Bardelli et al., 2017): (i) *C-cycle*: α- and β-glucosidases, α- and β-galactosidases, cellobiohydrolase, and xylosidase; (ii) *N-cycle*: chitinase and leucine-aminopeptidase; (iii) *P-cycle*: acid and alkaline phosphomonoesterases, and phosphodiesterase; (iv) *S-cycle*: arylsulphatase. An amount of 0.25 g of sample (fw) was subjected to a bead-beating procedure (Bardelli et al., 2017) in the presence of 3% lysozyme solution in 0.1 M NaCl, pH 6 by using a Retsch 400 beating mill at 30 strokes s^{-1} for 3 min. The supernatant containing the desorbed enzymes was then centrifuged at 20,000 g for 3 min. Afterwards, 20 μL of diluted extracts were pipetted in duplicate on 384-well microplates with 50 μL of appropriate buffer in order to quantify enzyme activities using a Synergy HT microplate reader (BIO- TEK). The activities were expressed as nanomoles of 4-methyl-umbelliferone (MUF) h^{-1} g^{-1} dry sample except for leucine-aminopeptidase, which activity was expressed as nanomoles of 7-amino-4-methyl coumarine (AMC) h^{-1} g^{-1} dry sample.

2.7. Statistical analyses

The impact of earthworms on microbiological properties and enzyme activity measurements during the 63-day trial was analysed by repeated measures analysis of variance (ANOVAR). Single reactors were considered as subjects. Earthworm treatment (presence and absence) and the type of marc (Albariño and Mencía raw marc, and Mencía distilled marc) were fixed as between-subject factors, and the sampling time (14, 28, 42 and 63 days) was fixed as a within-subject factor. The choice of the ANOVAR test was based on the fact that the same subjects (i.e., the reactors in the current study) were measured on the same outcome variable under the different time points. The normality and the variance homogeneity of the dataset were tested prior to ANOVAR. The sphericity violation was corrected (if necessary) with the Geisser-Greenhouse procedure. A two-way analysis of variance (ANOVA) was chosen to evaluate the changes in the nutrient content in the presence and the absence of earthworms because in this case we only focused on a single time point (that is, after 63 days). Whenever it was necessary, data were transformed to meet the normality assumptions, followed by pairwise comparison tests (Tukey HSD test) when differences were significant.

3. Results and discussion

3.1. Microbial biomass and activity during vermicomposting of grape marc

Previous studies have emphasized the importance of the starting material for driving the composition and activity of microbial communities during vermicomposting (Lores et al., 2006; Yakushev et al., 2011; García-Sánchez et al., 2017). In fact, the impact of *E. andrei* on microbial biomass carbon (Cmic) and basal respiration used as proxy of microbial activity varied with the type of marc (Fig. 2), and this effect was time-dependent (Cmic: ANOVAR $F_{6,126} = 7.9$, $p = 0.00008$; basal respiration: ANOVAR $F_{6,126} = 13.2$, $p < 0.00001$). For instance, no significant differences were found in Cmic with earthworm presence in the reactors fed with Albariño raw marc during the 63-day trial (Fig. 2A). However, lower Cmic levels were recorded in the presence than in the absence of earthworms at the latter time points in those reactors treated with raw and distilled marc from Mencía's grape variety (Fig. 2C, E).

Despite the lack of differences in Cmic, lower respiration values relative to the control were observed for Albariño raw marc after 14 days of vermicomposting (Fig. 2B). A decrease in basal respiration was recorded for all three types of marc after 28, 42 and 63 days as a result of earthworm activity (Fig. 2B, D, F). This reduction was more pronounced for the raw marcs, being 2–3 times lower compared to the control, than for the distilled marc (1.5 times lower). The decreasing trend on microbial activity with earthworm presence has already been reported in previous pilot-scale vermicomposting trials with grape marc (Gómez-Brandón et al., 2019b, 2020c; Kolbe et al., 2019; Nogales et al., 2020), or other plant-derived materials (leguminous shrubs: Domínguez et al., 2019; Rosado et al., 2022). In the short-term, epigeic earthworms are known to modify microbial populations via the fragmentation and the ingestion of fresh organic matter during the transit through the earthworm gut. This leads to an increased surface area available for

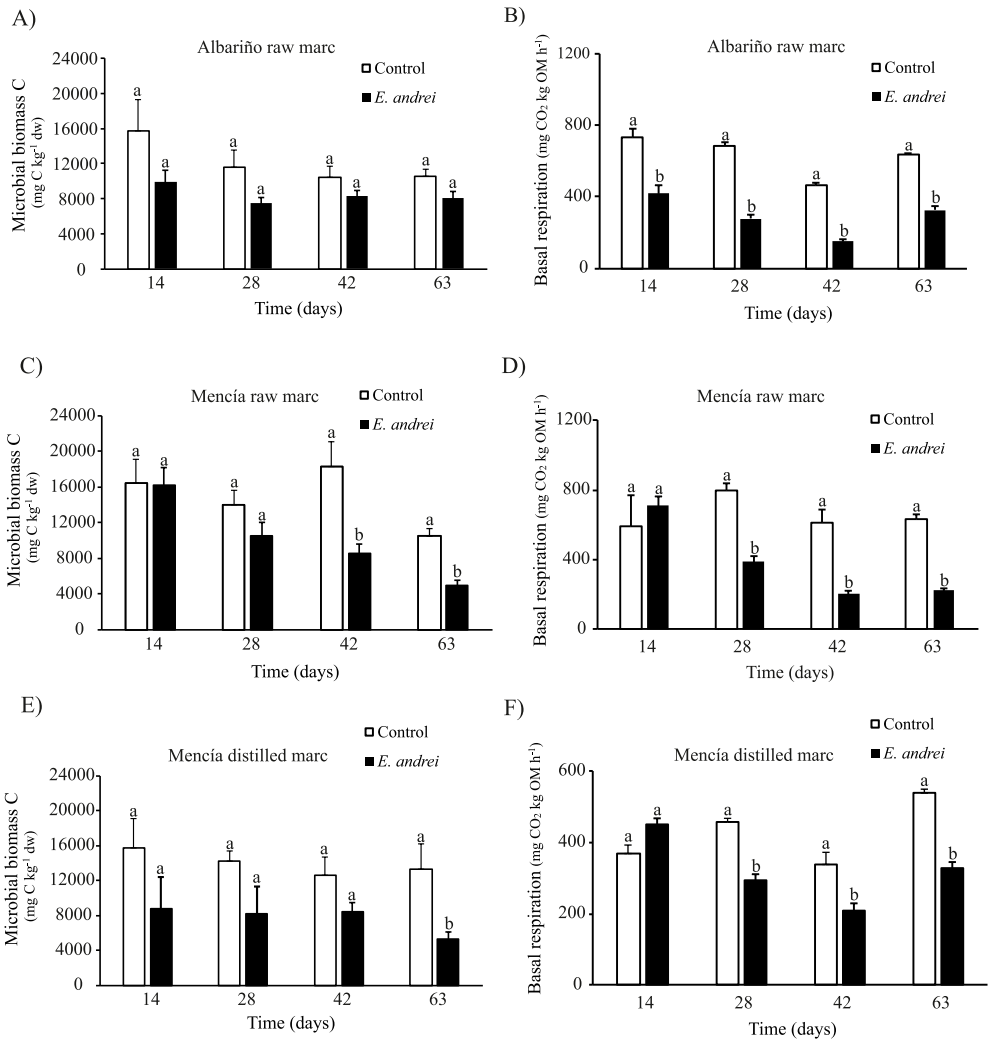

Fig. 2. Microbial biomass carbon and microbial activity assessed as basal respiration in the presence and the absence of earthworms during vermicomposting of raw grape marc from the cultivar Albariño (A, B), and raw and distilled marc from the cultivar Mencía (C–F). Values are means ± standard error. For each time point different letters indicate significant differences in the presence of *E. andrei* relative to the control without earthworms (Tukey HSD test).

microbial colonization and an accelerated rate of organic matter decomposition during vermicomposting (Domínguez et al., 2010). Nonetheless, Gómez-Brandón et al. (2021) did not observe differences in microbial respiration relative to the control without earthworms, irrespective of the type of marc and the age of vermireactor's layers. These authors used a continuous-feeding vermicomposting system, while here no more fresh grape marc was added to the vermireactors after the start of the trial and over the period of 63 days. Besides this distinction, of note is also that in the current study the reactors operated at the maximum earthworm density capacity. Aira et al. (2008) reported that earthworm density linearly increased CO_2 efflux and pools of labile C and inorganic N in a mesocosm trial with the earthworm species *E. fetida*. This clearly points toward a strong and linear-density dependent response of the C and N mineralization to the earthworms' density.

The respiration values in the final grape marc-derived vermicomposts fell between 200 and 350 mg CO_2 kg OM h^{-1}, similar to those reported in former studies (Gómez-Brandón et al., 2020c). This points to the effectiveness of vermicomposting at biologically stabilizing the raw and distilled grape marc, as shown by the lower and stable values of basal respiration in the presence than in the absence of earthworms from day 28 onwards (Fig. 2). This is in agreement with the findings from Gómez-Brandón et al. (2011) who showed, at a laboratory scale, that the activity of *E. andrei* promoted the stabilization of grape marc in the short-term, as reflected by decreases in the labile carbon pool and microbial biomass and activity compared to the control.

The impact of *E. andrei* on the abundances of bacteria and fungi also varied depending on the type of marc and sampling time (ANOVAR, bacteria: $F_{6,54} = 9.3$, $P = 0.0001$; fungi: $F_{6,54} = 12.1$, $P < 0.00001$). Despite the consistent effects on total microbial activity and in line with the trend in Cmic, the presence of *E. andrei* did not significantly affect neither the bacterial nor the fungal abundances in the reactors fed with Albariño raw marc over the course of vermicomposting (Fig. 3A and B). Cell death and the subsequent release of genetic material by plant residues and microbes are a primary source of extracellular DNA in environmental matrixes (Probst et al., 2021). A fraction of extracellular DNA can persist in the environment due to physical protection against enzymatic denaturation (Nagler et al., 2018), and its turnover rate can range from a few hours to several months and years (Agnelli et al., 2004). As such, it is plausible that some recalcitrant extracellular DNA not belonging to microbes could have been isolated from the grape marc-derived vermicompost samples following DNA extraction and real-time PCR. This could explain why the abundance of bacteria and fungi in the Albariño raw marc was not reduced throughout the 63-day period, even though a decrease in microbial activity was found over time. This is, however, beyond the scope of this study and should be further investigated.

In the case of raw and distilled marc from Mencía's grape variety, the bacterial abundance was circa 2-times higher in the reactors with

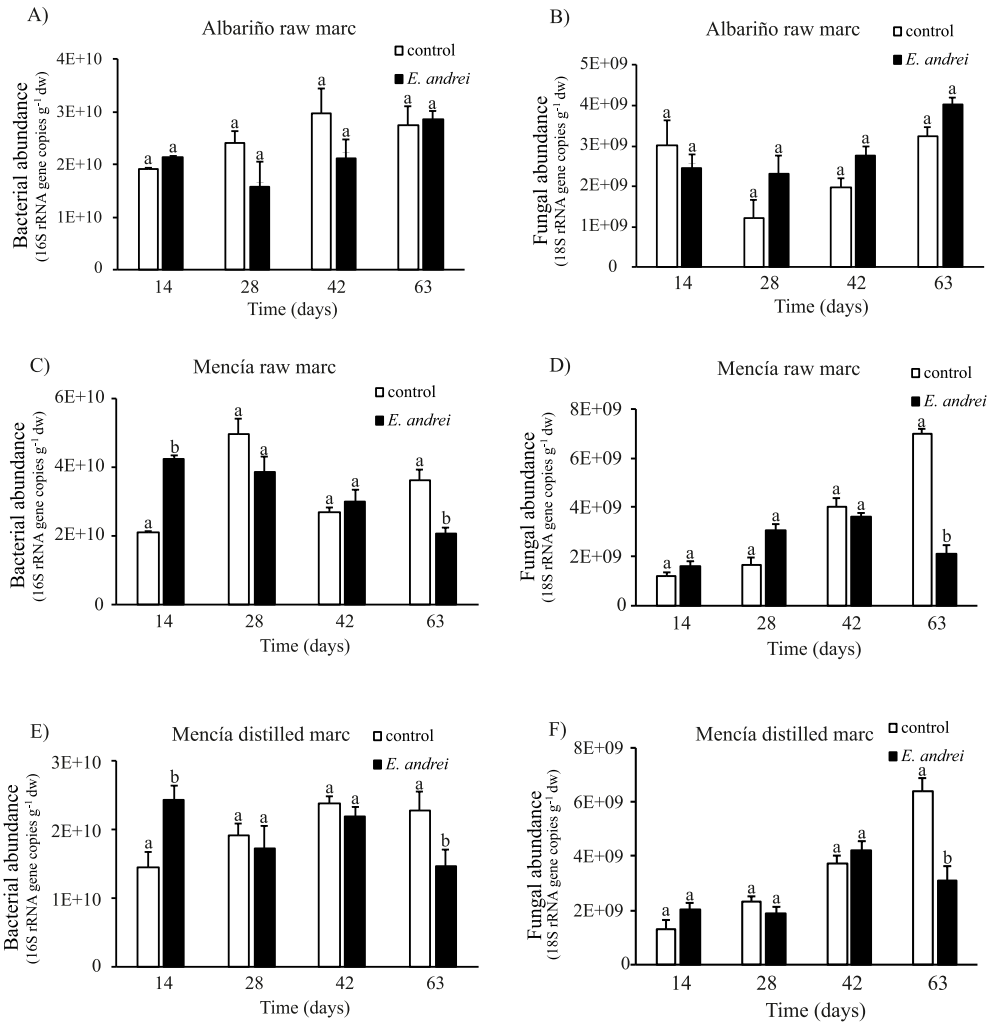

Fig. 3. Bacterial and fungal abundances, assessed by real-time PCR, in the presence and the absence of earthworms during vermicomposting of raw grape marc from the cultivar Albariño (A, B), and raw and distilled marc from the cultivar Mencía (C–F). Values are means ± standard error. For each time point different letters indicate significant differences in the presence of *E. andrei* relative to the control without earthworms (Tukey HSD test).

earthworms compared to the control on day 14, whilst lower abundances of both bacteria and fungi with earthworm presence were recorded on day 63 (Fig. 3C–F). These findings are in line with those from Gómez-Brandón et al. (2019b, 2020c) who reported a rapid increase in bacterial richness and diversity in Mencía distilled and raw marc within the first 14 and 28 days of vermicomposting, respectively. Unlike fungi, bacteria have a more exploitative nutrient use strategy by rapidly using newly produced labile substrates. At the end of the process, it is plausible that epigeic earthworms may have reduced the bacterial and fungal abundances indirectly by the depletion of the resources used by microbes, due to the acceleration of organic matter decomposition as vermicomposting progresses.

3.2. Enzymatic activities during vermicomposting of grape marc

Studies monitoring the succession of enzymatic activities during vermicomposting of grape marc already exist (García-Sánchez et al., 2017; Hrebeckova et al., 2019; Almeida-Santana et al., 2020; Nogales et al., 2020). A main contribution of the present study is to consider a wider range of enzymatic activities in the presence and the absence of earthworms providing a comprehensive picture of the role of *E. andrei* on the dynamics of the process. On day 14, we found that earthworms significantly increased the activity of all C-associated enzymes, except for alfa-galactosidase, in the vermireactors fed with raw marc from Mencía's grape variety (Tables 2, S1). As an example, alfa- and beta-glucosidase activities were circa 3- times higher when compared to the control treatment (Table 2). Likewise, earthworms' presence resulted in short-term increases in the N-, P- and S-related enzyme activities in the Mencía raw marc (Tables 2, S1). After 14 days of vermicomposting, a 5-fold increase was recorded in the chitinase, leucine-aminopeptidase and arylsulphatase activities in comparison with the control (Table 2). In the case of alkaline and acid phosphomonoesterases their activities were around 14- and 4-times higher in the presence than in the absence of earthworms in the Mencía vermireactors at this time point (Table 2). Earthworms can alter nutrient cycling and increase N and P uptake by plants through a combination of biochemical and chemical pathways (Medina-Sauza et al., 2019). Taken together, these findings are consistent with those observations providing evidence of the enhancing effect of the earthworm *E. andrei* on certain enzyme activities involved in the breakdown or mineralization of N and P into inorganic forms that can be used by plants.

The rapid increase in these enzymatic activities was consistent with a higher bacterial abundance in the presence of earthworms on day 14 (Fig. 3C). At this time point similar levels of microbial activity than those in the control were also found with earthworms' presence in the reactors treated with Mencía raw marc. Soil microorganisms are known to secrete extracellular enzymes to break down polymerized soil organic matter into assimilable small molecules in order to fulfill nutrient and

Table 2
Changes in the enzymatic activities in the absence and the presence of E. andrei during vermicomposting of raw and distilled marc. Values are means ± standard error. Units are given as nanomoles of MUF h-1 g-1 dry weight. For each type of marc different letters indicate significant differences ($p < 0.05$; ANOVAR followed by Tukey HSD test) in the presence of E. andrei in comparison with the control without earthworms within each time point.

Time (days)					
Enzymatic activities	Treatment	14	28	42	63
C-cycle alfa-glucosidase					
Albariño raw marc	Control	151 ± 16a	212 ± 21a	107 ± 10a	94 ± 6a
	E. andrei	121 ± 9a	76 ± 3b	44 ± 5b	27 ± 2b
Mencía raw marc	Control	74 ± 1a	132 ± 20a	333 ± 26a	88 ± 15a
	E. andrei	211 ± 14b	139 ± 7a	76 ± 4b	66 ± 6a
Mencía distilled marc	Control	175 ± 18a	246 ± 32a	153 ± 29a	88 ± 4a
	E. andrei	172 ± 15a	155 ± 14b	128 ± 23a	55 ± 5a
beta-glucosidase					
Albariño raw marc	Control	246 ± 36a	423 ± 12a	316 ± 27a	484 ± 44a
	E. andrei	204 ± 18a	169 ± 7b	113 ± 7b	110 ± 10b
Mencía raw marc	Control	141 ± 14a	775 ± 140a	571 ± 90a	349 ± 89a
	E. andrei	369 ± 45b	328 ± 16b	268 ± 21b	279 ± 21a
Mencía distilled marc	Control	522 ± 47a	673 ± 82a	484 ± 91a	334 ± 23a
	E. andrei	431 ± 39a	371 ± 41b	273 ± 10b	209 ± 17a
alfa-galactosidase					
Albariño raw marc	Control	177 ± 27a	164 ± 12a	188 ± 29a	197 ± 21a
	E. andrei	143 ± 19a	195 ± 26a	142 ± 17a	135 ± 19a
Mencía raw marc	Control	1085 ± 60a	1504 ± 312a	448 ± 42a	745 ± 78a
	E. andrei	544 ± 60b	382 ± 77b	781 ± 127b	981 ± 81a
Mencía distilled marc	Control	81 ± 8a	166 ± 23a	161 ± 32a	132 ± 10a
	E. andrei	127 ± 15a	137 ± 20a	101 ± 10a	64 ± 6a
beta-galactosidase					
Albariño raw marc	Control	73 ± 10a	151 ± 13a	95 ± 8a	109 ± 7a
	E. andrei	70 ± 14a	55 ± 4b	35 ± 2b	30 ± 2b
Mencía raw marc	Control	32 ± 3a	253 ± 27a	152 ± 31a	115 ± 38a
	E. andrei	170 ± 28b	128 ± 8b	97 ± 7a	86 ± 8a
Mencía distilled marc	Control	165 ± 9a	216 ± 32a	172 ± 34a	113 ± 15a
	E. andrei	126 ± 12a	103 ± 12b	83 ± 6a	55 ± 4b
Cellobiohydrolase					
Albariño raw marc	Control	9 ± 3a	20 ± 2a	16 ± 2a	22 ± 2a
	E. andrei	6 ± 1a	8 ± 0.7b	5 ± 0.9b	4 ± 0.6b
Mencía raw marc	Control	9 ± 0.9a	65 ± 10a	47 ± 8a	25 ± 8a
	E. andrei	23 ± 2b	22 ± 5b	14 ± 3b	14 ± 1a
Mencía distilled marc	Control	35 ± 5a	46 ± 6a	35 ± 8a	27 ± 3a
	E. andrei	43 ± 6a	30 ± 6a	34 ± 8a	14 ± 2a
Xylosidase					
Albariño raw marc	Control	305 ± 75a	2892 ± 307a	1277 ± 204a	516 ± 66a
	E. andrei	1632 ± 288b	926 ± 37b	331 ± 12b	287 ± 39a
Mencía raw marc	Control	120 ± 13a	414 ± 189a	903 ± 49a	317 ± 45a
	E. andrei	562 ± 146b	1102 ± 308b	799 ± 77a	663 ± 83b
Mencía distilled marc	Control	551 ± 72a	1097 ± 150a	641 ± 172a	267 ± 34a
	E. andrei	436 ± 56a	545 ± 113b	693 ± 142a	696 ± 87a

Time (days)					
Enzymatic activities	Treatment	14	28	42	63
N-cycle Chitinase					
Albariño raw marc	Control	216 ± 59a	677 ± 84a	287 ± 14a	332 ± 43a
	E. andrei	246 ± 15a	147 ± 11b	135 ± 21a	245 ± 40a
Mencía raw marc	Control	80 ± 8a	572 ± 53a	510 ± 49a	601 ± 135a
	E. andrei	493 ± 29b	464 ± 51a	639 ± 77a	620 ± 80a
Mencía distilled marc	Control	342 ± 34a	401 ± 58a	202 ± 41a	162 ± 10a
	E. andrei	212 ± 25a	228 ± 28a	201 ± 28a	182 ± 19a
Leucine-aminopeptidase					
Albariño raw marc	Control	495 ± 96a	2422 ± 164a	691 ± 68a	425 ± 45a
	E. andrei	795 ± 115a	398 ± 20b	346 ± 17a	330 ± 22a
Mencía raw marc	Control	229 ± 17a	1010 ± 344a	1629 ± 58a	404 ± 46a
	E. andrei	1211 ± 200b	708 ± 66a	786 ± 71b	968 ± 74b
Mencía distilled marc	Control	600 ± 87a	617 ± 54a	348 ± 67a	339 ± 40a
	E. andrei	377 ± 45a	469 ± 78a	430 ± 75a	450 ± 25a
S-cycle Arylsulphastase					
Albariño raw marc	Control	1.13 ± 0.06a	6.25 ± 1.01a	7.23 ± 0.62a	7.49 ± 1.04a
	E. andrei	2.10 ± 0.26a	5.54 ± 0.57a	14.16 ± 1.03b	14.99 ± 0.63b
Mencía raw marc	Control	1.44 ± 0.12a	10.39 ± 1.62a	14.36 ± 1.71a	8.14 ± 0.90a
	E. andrei	7.40 ± 0.81b	13.13 ± 1.28a	10.62 ± 1.02a	9.79 ± 1.37a
Mencía distilled marc	Control	2.71 ± 0.46a	4.85 ± 0.12a	4.00 ± 1.05a	3.35 ± 1.22a
	E. andrei	3.23 ± 0.62a	8.30 ± 1.29a	16.23 ± 2.56b	15.82 ± 1.04b
P-cycle Acid phosphomonoesterase					
Albariño raw marc	Control	234 ± 22a	1219 ± 72a	768 ± 58a	521 ± 31a
	E. andrei	242 ± 36a	287 ± 28b	219 ± 14b	354 ± 30a
Mencía raw marc	Control	154 ± 17a	616 ± 95a	993 ± 148a	659 ± 50a

(*continued on next page*)

Table 2 (*continued*)

Time (days)					
Enzymatic activities	Treatment	14	28	42	63
	E. andrei	630 ± 76b	664 ± 102a	387 ± 37b	497 ± 43a
Mencía distilled marc	Control	1345 ± 95a	1499 ± 175a	738 ± 158a	486 ± 40a
	E. andrei	1268 ± 102a	738 ± 73b	476 ± 63b	435 ± 42a
Alkaline phosphomonoesterase					
Albariño raw marc	Control	220 ± 43a	4464 ± 420a	2485 ± 172a	3171 ± 341a
	E. andrei	1242 ± 112b	1522 ± 88b	2542 ± 300a	3095 ± 344a
Mencía raw marc	Control	217 ± 26a	387 ± 70a	1639 ± 271a	4871 ± 528a
	E. andrei	3027 ± 216b	4261 ± 491b	5517 ± 539b	5521 ± 464a
Mencía distilled marc	Control	934 ± 93a	1529 ± 131a	1220 ± 211a	1365 ± 177a
	E. andrei	939 ± 129a	2233 ± 352a	3252 ± 515b	3287 ± 151b
Phosphodiesterase					
Albariño raw marc	Control	35 ± 3a	332 ± 25a	210 ± 10a	205 ± 16a
	E. andrei	153 ± 7b	143 ± 6b	164 ± 11a	184 ± 16a
Mencía raw marc	Control	35 ± 3a	47 ± 11a	265 ± 35a	266 ± 47a
	E. andrei	288 ± 18b	329 ± 22b	324 ± 32a	378 ± 34a
Mencía distilled marc	Control	151 ± 22a	205 ± 17a	137 ± 22a	125 ± 10a
	E. andrei	130 ± 15a	242 ± 33a	246 ± 31a	224 ± 11a

energy demands (Burns et al., 2013). Nonetheless, unlike the red raw marc, no significant differences relative to the control were observed in the respective Mencía distilled marc for any of the studied enzymes after 14 days of vermicomposting (Table 2). The high temperatures characteristic of the distillation process might have distinctly affected the availability of the substrates for the enzymes and led to a different dynamics of the enzyme activities within the first two weeks of vermicomposting.

Later on, between days 28 and 63, the magnitude of earthworms' effect on the studied enzyme activities compared to the control varied depending on both the type of marc and the time of sampling (Table S1). For instance, among the C-associated enzymes, β-glucosidase activity was between 1.5 and 2.5-times lower in the presence than in the absence of earthworms in all three types of marc on days 28 and 42 (Table 2). However, on day 63, such a decrease with earthworm presence was only recorded for Albariño marc, being 4.4-times lower than in the control (Table 2). Cellobiohydrolase activity followed a similar trend than β-glucosidase from day 28 onwards (Table 2), except for the distilled marc for which no differences were recorded due to earthworm activity. Nonetheless, on days 42 and 63, arylsulphatase and alkaline phosphomonoesterase activities were about 4- and 2- times higher relative to the control in the vermireactors fed with Mencía distilled marc (Table 2).

The fraction of organic compounds available during vermicomposting is considered as a major driving force of extracellular enzymes (Benitez et al., 2005). Bearing this in mind, it is likely that the accelerated depletion of resources due to earthworm activity results in reduced enzyme activities towards the end of the process. Supporting this, lower levels of most of the C-associated enzymes were generally found for all three types of marc in the reactors with earthworms on days 42 and 63, when compared to the earlier time points (days 14 and 28; Table 2). This reinforces the use of enzyme activities as indicators of the suitability of vermicomposting for the biological stabilization of raw and distilled marc. Other enzymes like arylsulphatase, alkaline phosphomonoesterase and phosphodiesterase showed higher activities at the latter time points (Table 2). A plausible explanation relies on the increasing concentrations of humic substances that appear as vermicomposting progresses providing chemical support for binding extracellular enzymes, and protecting them against proteases or adverse environmental conditions (Castillo et al., 2013).

3.3. Physico-chemical and nutrient characterization in the grape marc-derived vermicomposts

After 63 days of vermicomposting the moisture content was about 75% for all the three types of marc (Table 3), which is considered an optimum level for the growth and reproduction of epigeic earthworms and for a good performance of the vermicomposting process (Domínguez et al., 2016). The values of C/N ratio (13–24) achieved for the final vermicomposts were within the ranges recommended by Goyal et al. (2005) (<25) for quality compost production. Also, the nutrient concentrations in the various vermicomposts meet the required international standards and guidelines for quality organic fertilizers in the United States, Canada and the European Union (Brinton, 2000). At the end of the trial the earthworm species *E. andrei* did not have a significant

Table 3
Nutrient content of the raw and distilled marc in the presence and the absence of earthworms at the end of the vermicomposting trial, that is on the day 63. Values are means ± standard error. In each row different letters indicate significant differences ($p < 0.05$; ANOVA followed by Tukey HSD test) in the presence of E. andrei relative to the control without earthworms for each type of grape marc.

	Albariño raw marc		Mencía raw marc		Mencía distilled marc	
	Control	*E. andrei*	Control	*E. andrei*	Control	*E. andrei*
Moisture (%)	75 ± 0.6a	76 ± 1a	74 ± 1a	73 ± 0.8a	76 ± 1a	74 ± 1a
OM (%)	94 ± 2.2a	93 ± 0.4a	95 ± 1.4a	94 ± 0.7a	94 ± 2a	92 ± 0.6a
Total C (%)	53 ± 0.1a	50 ± 0.2a	52 ± 0.3a	50 ± 0.1a	53 ± 0.1a	49 ± 0.1a
Total N (%)	2.1 ± 0.02a	2.1 ± 0.05a	2.5 ± 0.05a	2.3 ± 0.07a	2.6 ± 0.06a	2.3 ± 0.06a
C to N ratio	25 ± 0.3a	24 ± 0.8a	21 ± 0.4a	22 ± 0.4a	20 ± 0.5a	21 ± 0.2a
Ca (mg kg^{-1} dw)	4149 ± 49a	4347 ± 108a	3680 ± 49a	3669 ± 55a	3257 ± 52a	4391 ± 196a
K (mg kg^{-1} dw)	20,660 ± 713a	13,489 ± 798b	23,654 ± 568a	11,956 ± 351b	17,721 ± 748a	13,161 ± 415b
P (mg kg^{-1} dw)	2866 ± 78a	1857 ± 87b	3116 ± 61a	1818 ± 96b	2582 ± 56a	1864 ± 38b
Mg (mg kg^{-1} dw)	1277 ± 19a	1149 ± 52a	1135 ± 15a	988 ± 40a	976 ± 31a	1047 ± 49a
Mn (mg kg^{-1} dw)	18 ± 0.2a	20 ± 1a	40 ± 0.4a	37 ± 3a	39 ± 0.6a	43 ± 2a
Fe (mg kg^{-1} dw)	115 ± 4a	157 ± 8a	92 ± 4a	134 ± 33a	98 ± 3a	129 ± 6a
S (mg kg^{-1} dw)	1408 ± 35a	1461 ± 71a	1579 ± 32a	1650 ± 64a	1528 ± 31a	1141 ± 45a

OM: organic matter.

effect on the total content of nutrients regardless the type of marc (Table 3), with the exception of K and P. In agreement with Gómez-Brandón et al. (2021), earthworms promoted a decrease in the total content of K and P in either raw or distilled marc (ANOVA, K content: $F_{2,24} = 16.8$, $P = 0.00003$; P content: $F_{2,24} = 5.8$, $P = 0.009$). The values of these nutrients in the presence of *E. andrei* were around 1.5-times lower than those in the control (Table 3). On the contrary, an increasing trend in the levels of potassium as a result of earthworms' activity was reported by Domínguez and Gómez-Brandón (2013) on a vermicomposting system with sewage sludge and cattle manure. Unlike grape marc, cattle manure or other types of manure have already passed through the vertebrate gut (i.e., cow, pig, horse). However, our study started with a plant material of lignocellulosic nature and the resulting vermicompost can be thought to represent the process of a single gut – that is, the starting material passed only through the earthworm gut.

Taken together, the return of nutrients through recycled material via vermicomposting may serve as a small but efficient puzzlestone for the transition of extensive to sustainable agriculture. Nutrient and carbon cycling is a prerequisite for sustainable economy and particularly concerning agriculture and horticulture. The potential usefulness of vermicompost as a biofertilizer may help to achieve climate-friendly solutions promoting smart agricultural soil management. Appropriately used, grape marc-derived vermicomposts may act as bioinoculums providing a community of microorganisms that contribute to the functioning of the ecosystem and the maintenance of its productivity on the long term.

4. Conclusions

The findings of the present study reinforce the suitability of vermicomposting as an environment-friendly approach for the biological stabilization of raw marc derived from white and red winemaking processes in pilot-scale systems. Likewise, vermicomposting was found to be effective for the treatment and stabilization of distilled marc obtained from the red grape variety. Overall, reduced values of basal respiration, ranging between 200 and 350 mg CO_2 kg OM h^{-1} and indicative of stabilized materials were found in the resulting vermicomposts. Likewise, most of the C-associated enzymes had reduced activities as a result of earthworms' activity towards the end of the trial. Moreover, the C to N ratio and the content of macro- and micronutrients achieved for the end products matched with those considered to have the quality criteria of a good vermicompost. Further agronomic studies to determine the optimal amendment rate to ensure high nutrient release and synchrony for crop uptake, improved yield, and nutritional quality of crops are crucial with regards to the field application of grape marc-derived vermicomposts.

Credit author statement

María Gómez Brandón: Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **Flavio Fornasier**: Methodology, Writing – review & editing. **Nariane de Andrade**: Methodology, Writing – review & editing. **Jorge Domínguez**: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Funding acquisition, Writing -review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

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