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Mealworm frass as a potential biofertilizer and abiotic stress tolerance-inductor in plants

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A B S T R A C T

Frass from the insect *Tenebrio molitor* can be applied as an organic fertilizer due to its nutritional content. In this study insects were fed on different types of food producing excrement with different compositions. This allowed us to compare the potential effect of the excrement on plant growth promotion and to test its ability to increase tolerance to abiotic stresses such as drought, excess water and salinity. The best results were obtained when the insects were fed a diet low in fat and starch content. Moreover, a microbiological study using the excrement produced showed that the presence of microorganisms improved plant growth parameters, in the absence or presence of stress conditions. The analysis of the microbiome through massive parallel sequencing, as well as the analysis of the capacity of several microbial isolates to promote plant growth, showed that several microorganisms present in the excrement are plant growth promoters (PGPs). Our findings indicate that the frass produced by these insects, due to its nutritional content and associated microbiota, can be potentially used as a biofertilizer in organic farming. However, this potential dramatically varies according to the insect's diet. Further analyses should be performed to set the optimal time and amount of application for the different aimed plants.

1. Introduction

The development and application of new organic products with the ability to promote plant growth is a possible way to address the challenges of increasing food production while protecting the environment (Carvalho, 2017; García-Fraile et al., 2015; Menéndez and Garcia-Fraile, 2017; Shankar and Shikha, 2017).

Insects are a group of animals whose excrement can contain large amounts of nutrients in forms that are easily assimilated by plants. Hence the presence of numerous studies suggesting the possible use of insect excrement, such as that from grasshoppers (Fielding et al., 2013), bees (Mishra et al., 2013), ants (Pinkalski et al., 2017) and cabbage moths (*Mamestra brassicae*) (Kagata and Ohgushi, 2012), as organic fertilizers. In addition, other researchers have shown how the intestinal microbiota of different insects, like the diamondback moth (*Plutella xylostella*), can contain microbes that act as plant growth promoting microorganisms (PGPMs). Many of these PGPMs can fix atmospheric nitrogen, produce indole acetic acid (IAA) and salicylic acid (SA), solubilize phosphates, promote zinc absorption, and produce glucanases, chitinases and ACC deaminase. Some isolated strains have been shown to promote the growth of tomato plants, as well as to inhibit the invasion of plant pathogenic fungi such as *Rhizoctonia solani* or *Sclerotinia sclerotiorum* (Indiragandhi et al., 2008). Interestingly, these authors report how changes in an insect's diet can drastically modify the results observed (Fielding et al., 2013; Indiragandhi et al., 2008; Kagata and Ohgushi, 2012; Mishra et al., 2013; Pinkalski et al., 2017).

Yellow mealworm (*Tenebrio molitor*) is an insect whose excrement can provide valuable nutrients to plants when used as an organic fertilizer. It has been reported that the NPK balance is 3.5-1.5-1.5 (Liu et al., 2003). Furthermore, it is known that the application of this fertilizer increases the weight of bean (*Phaseolus vulgaris*) seeds by 18% (Liu et al., 2003) and that aqueous extracts made using excrement increases wheat seed germination (*Triticum aestivum*) by 4% (Li et al., 2013). In all these studies, the authors suggest these differences in productivity are not solely due to the nutritional composition of the excrement and propose that microorganisms present in the feces could play an important role in plant growth promotion. In fact, the importance of microorganisms in plant growth promotion has already been described in detail (García-Fraile et al., 2015; Menéndez and Garcia-Fraile, 2017). Therefore, the aims of this study were (i) to test how different diets influence the properties of *T. molitor* frass, (ii) to analyze the ability of the excrement to promote plant growth (iii) to

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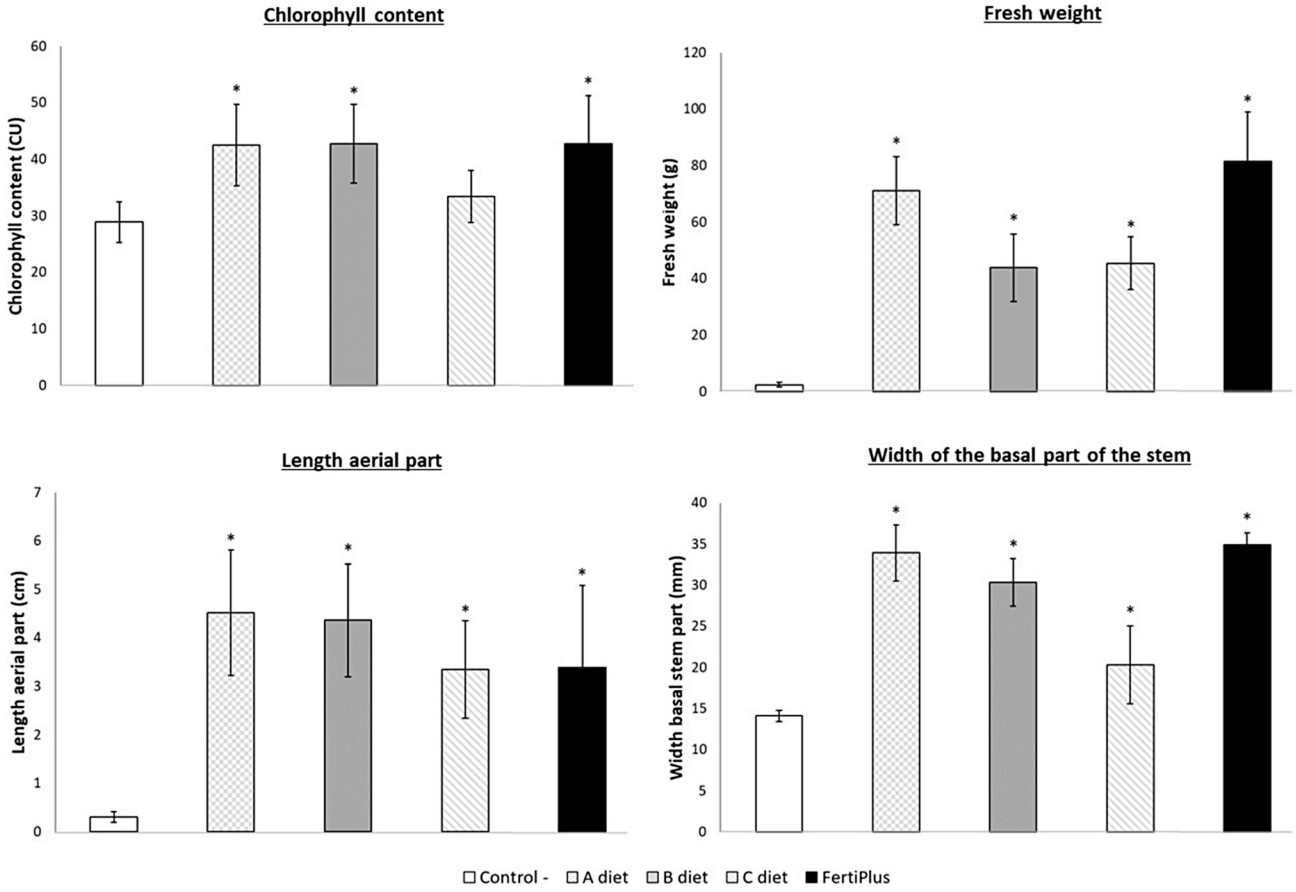

Fig. 1. Plant growth parameters of chard fertilized with *T. molitor* feces obtained from different diets. Asterisks indicate statistically significant differences between groups.

Table 1
Effect of the different *T. molitor* diets on macronutrients and micronutrients feces contents.

	C (g/100 g)	N (g/100 g)	C/N	P (g/100 g)	K (g/100 g)	S (g/100 g)	Ca (g/100 g)	Mg (g/100 g)	Mn (mg/Kg)	Fe (mg/Kg)	Mo (mg/Kg)
Diet A	38.90	2.92	13.32	1.53	1.86	0.18	0.10	0.54	171.89	140.68	0.56
Diet B	38.80	2.67	14.53	1.44	1.97	0.17	0.09	0.52	155.90	127.00	0.53
Diet C	42.44	7.75	5.48	1.02	1.15	0.28	0.11	0.34	83.40	129.03	0.96

determine if this frass induces resistance to abiotic stress in plants and (iv) to analyze its associated microbiota and its plant growth promoting mechanisms.

2. Material and methods

2.1. Insect farming

The insect rearing system was similar to that used by Dhinaut et al. (2018), with the following modifications: the insects were reared and maintained in an insectary at 24 ± 2 °C with 70% relative humidity in permanent darkness, and allowed access to wheat bran and water *ad libitum*. In addition, their diets were supplemented with different components, indicated in percentage of weight. Diet A contained 66% carbohydrates, which were mainly celluloses and hemicelluloses, 6% fat and 28% protein; diet B contained 77% carbohydrates, of which 12% was starch; 6% fat and 28% protein and diet C contained 49% carbohydrates, again mainly celluloses and hemicelluloses, and 12% fat and 39% protein.

Each starting population was comprised of virgin adult beetles of a specific age (10 ± 2 days post-emergence), which were obtained from randomly selected pupae from an outbred stock maintained in the conditions described above. At the end of the insects' life cycle, the excrements were collected through screening.

2.2. Analysis of the biofertilizer potential of T. molitor

Plant seeds were germinated in Petri dishes containing agar-water (1%w/v) at room temperature in the dark. The seeds had previously been surface sterilized in NaClO (5%v/v) for 5 min followed by immersion in 70% ethanol for 5 min. Then the seeds were washed several times in sterile distilled water and twelve seedlings, per treatment, were transferred into individual pots.

To test for plant growth promotion, chard plants (*Beta vulgaris* var. *cicla*) were grown in 3 L pots containing fluvial soil mixed with perlite (3/1). Excrement, obtained from the different diets, was added 2% in volume. An unfertilized soil-perlite mixture was used as the negative control and FertiPlus® (4-3-3) was used as the positive control. After the plants were grown in greenhouse conditions for one month, they were harvested and the fresh and dry weights of the material were recorded. A total of 15 different plants per treatment were analyzed as different biological replicas.

In addition, bean plants (*Phaseolus vulgaris*) were cultivated in 1 L pots, containing sterile vermiculite as the substrate, and were used to

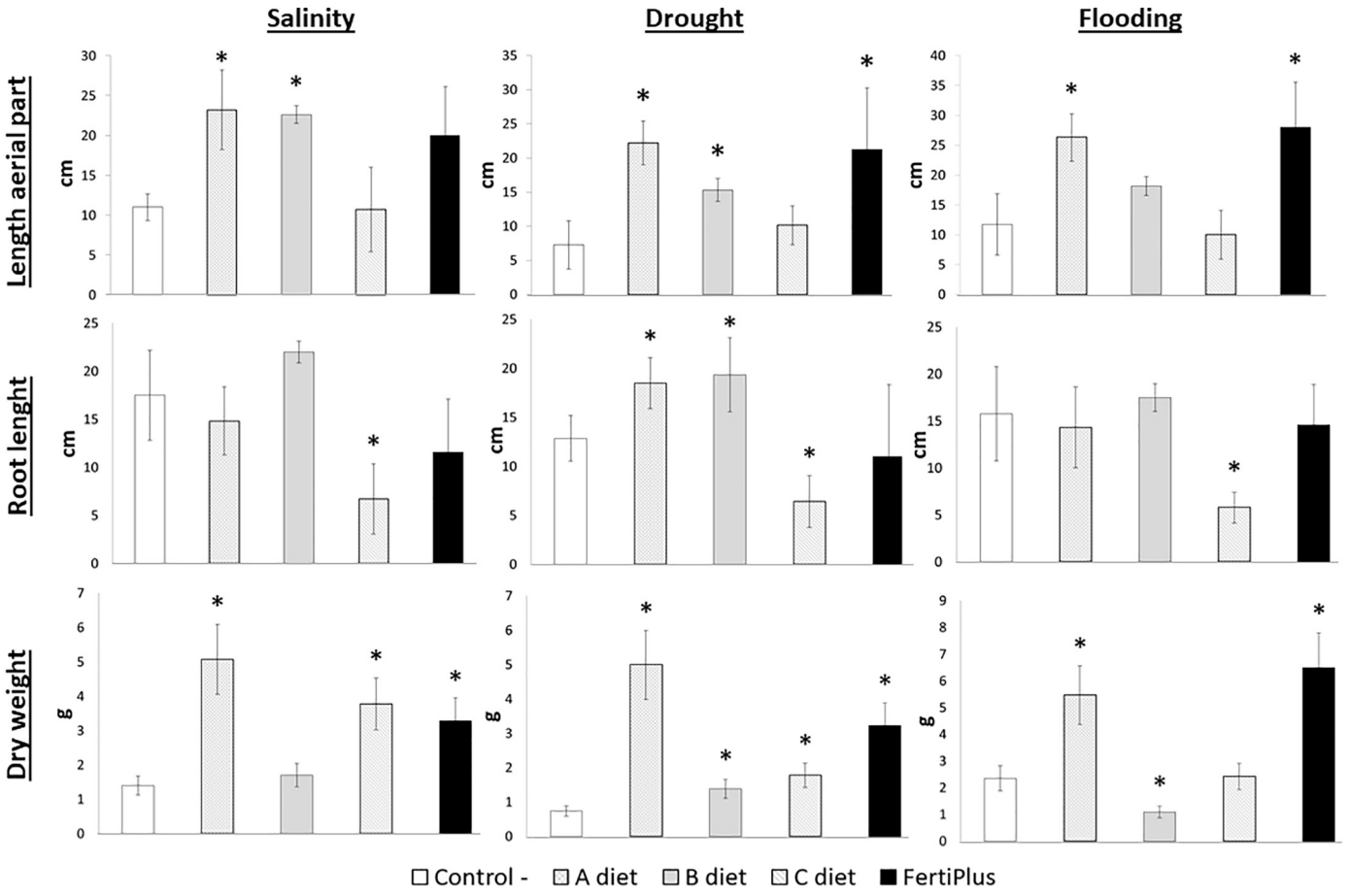

Fig. 2. Plant growth parameters of *P. vulgaris* fertilized with *T. molitor* feces from different diets under different stress conditions. Asterisks indicate statistically significant differences between groups.

determine the potential of the different types of excrement to increase plant tolerance against abiotic stresses such as drought, excess water and salinity. As previously mentioned, the negative control consisted of unfertilized vermiculite and the positive control was fertilized with the FertiPlus®. Due to the nutritive innocuousness of the vermiculite, plants were watered with Fåhraeus medium (Vincent, 1970). The plants used to study salt stress resistance were watered with a NaCl solution (200 mM), and the plants used to test resistance to water-logging were grown in pots in trays containing a continuous supply of water. Drought stress was caused by not watering the plants for 7 days. One month after the beans had been exposed to the different stress conditions, the plant material was collected and data on the length of the aerial part of plant and the roots were recorded in 15 plants per treatment; the dry weights after the plants had been desiccated were also determined. Additionally, the same measurements were also taken from the control plants, which had not been exposed to the stress conditions.

To determine if the microbiome present in the feces was able to promote plant growth and abiotic stress tolerance, the excrement resulting from the best diet was tindalized and added to the plants as a fertilizer in the conditions abovementioned, to compare the results with those from non-sterilized excrements.

2.3. Analysis of the nutritional content of feces

The feces of *T. molitor* fed on different diets were used to determine N, P, K, C, S, Ca, Mn, Fe, Mo and Mg contents. The analysis was performed at the Ionomics Service of CEBAS-CSIC (Spain) using the instrument Elemental Analyst model TruSpec CN628 in the case of N, and the ICP THERMO ICAP 6500DUO for the analysis and detection of the remaining macro- and micronutrients.

2.4. Amplicon sequencing and analyses of microbial communities

The analysis of the microbial community was carried out using total DNA extracted from 200 mg of feces using the NucleoSpin® Soil kit (Macherey Nagel), following the manufacturer's instructions.

PCR amplification of the bacterial 16S rRNA V4 region was achieved using the barcoded primers 515F and 806R (Bates et al., 2011). PCR amplification of the fungal ITS2 region from DNA and cDNA was performed using barcoded gITS7 and ITS4 (Ihrmark et al., 2012). For each of the amplicons, three PCR reactions containing 2.5 µl of 10× buffer for DyNAzyme DNA Polymerase, 0.75 µl polymerase (2 U/µl DyNAZyme II DNA polymerase 1:24 Pfu DNA polymerase), 0.5 µl of PCR Nucleotide Mix (10 mM), 0.75 µl of BSA (20 mg/ml), 1 µl of each primer (0.01 mM), and 1 µl of template DNA were prepared. For PCR amplification, the following cycling conditions were set: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. Amplicons were sequenced on an Illumina MiSeq platform.

The amplicon sequencing data were processed using SEED (Větrovský and Baldrian, 2013) following the procedure described by Žifčáková et al. (2015). Bacterial and fungal sequences were analyzed individually. The chimeric sequences found using UPARSE (Edgar, 2013) were deleted. Sequences shorter than 150 bp or longer than 340 bp were eliminated and singletons were also discarded. Operational taxonomic units (OTUs) were defined as sequences clustered at a 97% similarity (Lundberg et al., 2012). Consensus sequences were built for each cluster with MAFFT (Katoh et al., 2009) and the hits closest to the

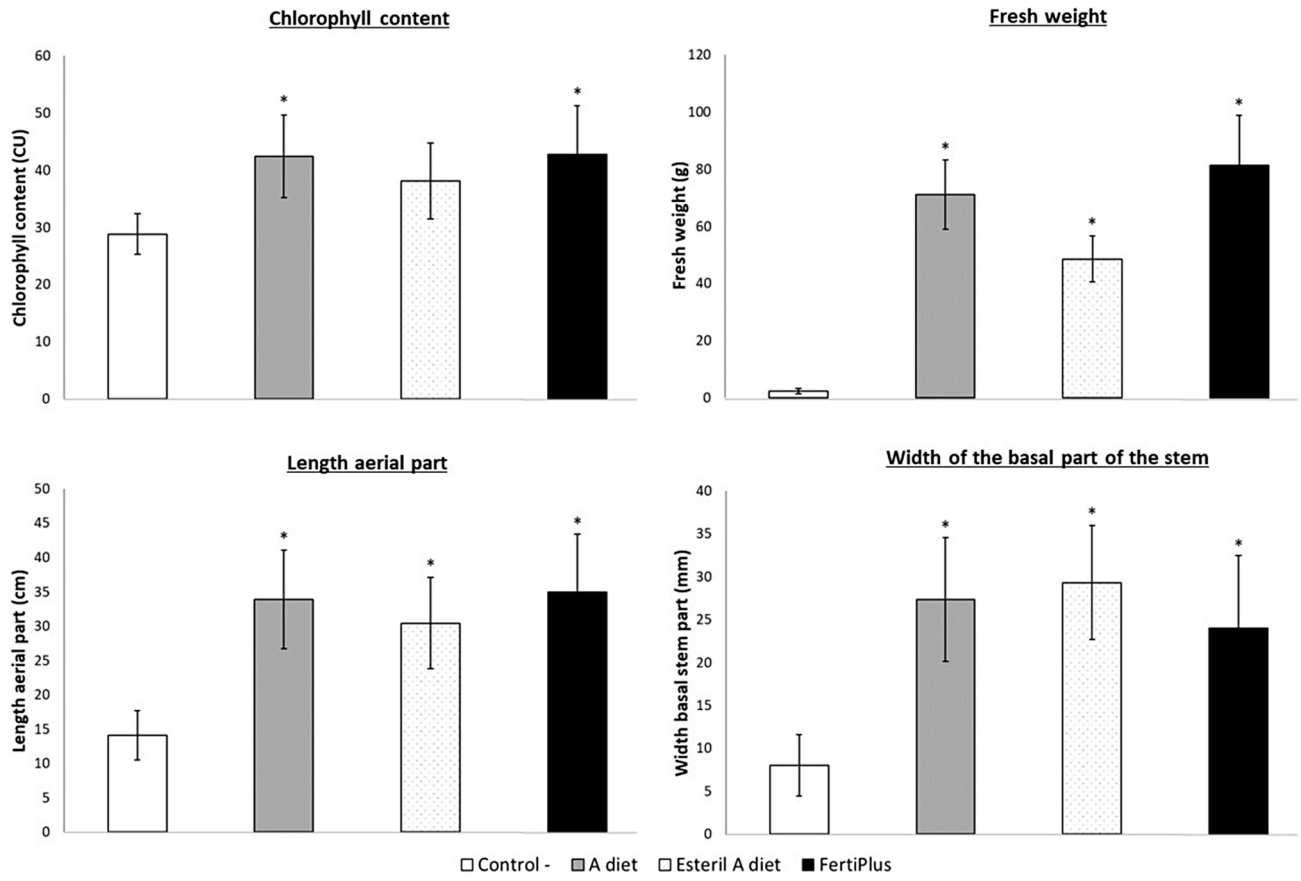

Fig. 3. Comparison of the effect of sterile and non-sterile *T. molitor* feces on plant growth in chard. Asterisks indicate statistically significant differences between groups.

consensus sequences were identified using BLASTn (Chen et al., 2015b). In each case, non-bacterial or non-fungal sequences were removed.

2.5. Isolation and identification of microbial strains

The isolation of bacteria and fungi was achieved using the feces of *T. molitor* fed on diet A. Ten grams of excrement was resuspended in 90 ml of sterile water and another 10 g was ground using a sterile pestle and then diluted in 90 ml of sterile water. Both suspension cultures were kept in an orbital shaker for 1 h at 300 rpm. Serial dilutions were made in sterile water and 100 µl of each dilution were plated onto Petri dishes containing different types of media: Nutritive Agar (NA, Sigma), Tryptose Soy Agar (TSA) and Saboureaud with Cloranphenicol (SAB). Additionally, two other types of media were inoculated with *T. molitor* feces: MFsm and MFm (K_2HPO_4 2,5 g/l, KH_2PO_4 2,5 g/l, $(NH_4)_2HPO_4$ 1 g/l, $MgSO_4{\cdot}7H_2O$ 0,2 g/l, $FeSO_4{\cdot}7H2O$ 0,01 g/l, $MnSO_4{\cdot}7H_2O$ 0,007 g/l, Agar 15 g/l, *T. molitor* feces 10 g/l and water q.s. to 1 l). MFm medium has frozen and grinded feces.

The microbial isolates were identified using genomic DNA extracted from pure cultures as described in Fabryová et al. (2018). The 16S rRNA gene of each bacterial strain was amplified as described by Rivas et al. (2007) and the ITS sequence was amplified in fungi as described by Hubka and Kolarik (2012).

Electrophoresis of PCR products was carried on a 1.5% agarose gel for 2 h at 60 V. The bands corresponding to the 16S rRNA gene and the ITS sequence, identified using a 1Kb ladder (GeneRuler Thermo®), were excised from the gel. The DNA was purified, sequenced by Macrogen S.L. and processed using MEGA7 software (Kumar et al., 2016). The sequences of ~1400 bp (bacterial 16S rRNA) and ~700 bp (fungal ITS) in size were identified by comparison with those available in GenBank, using the BLASTn program (Chen et al., 2015b) and EzTaxon tool (O.S. Kim et al., 2012).

2.6. Analysis of plant growth promoting (PGP) traits

Solubilization of insoluble phosphate was performed by incubating the isolated microbial strains in YED-P Petri dishes (Peix et al., 2001) at 28 °C for one week. The presence of a clear halo around the colonies was considered as a positive result for the solubilization of phosphate.

The detection of potassium solubilization was assessed by inoculating the isolates in Aleksandrov medium as described by Hu et al. (2006). The inoculated plates were incubated at 28 °C for one week, and again, the presence of clear halos around the colonies was interpreted as potassium solubilization.

Auxins production was analyzed by the colorimetric method described by Khalid et al. (2004), and the analysis of the production of aminocyclopropane-1-carboxylic acid (ACC) deaminase was performed in minimum liquid medium (Jiménez-Gómez et al., 2018) with ACC as the carbon source. The experiment was performed by inoculating sterile glass tubes with 5 ml of medium and 10 µl of a cell suspension. After 1 week of incubation at 28 °C, the turbid appearance of the liquid medium indicated microbial growth and the production of ACC deaminase.

Siderophore production was analyzed in M9-CAS-AGAR medium as described by Jiménez-Gómez et al. (2018) for bacteria and O-CAS medium (Pérez-Miranda et al., 2007) for fungi.

Finally, the ability of the bacterial isolates to fix nitrogen was assessed by testing their ability to grow in modified JMM medium, with and without tryptophan (Jiménez-Gómez et al., 2018). Modified JMM, plus NH_4NO_3, was used as the positive control. Two milliliters of medium and 10 µl of cells in suspension were cultivated in vials for one week at 28 °C. A turbid appearance was interpreted as the presence of

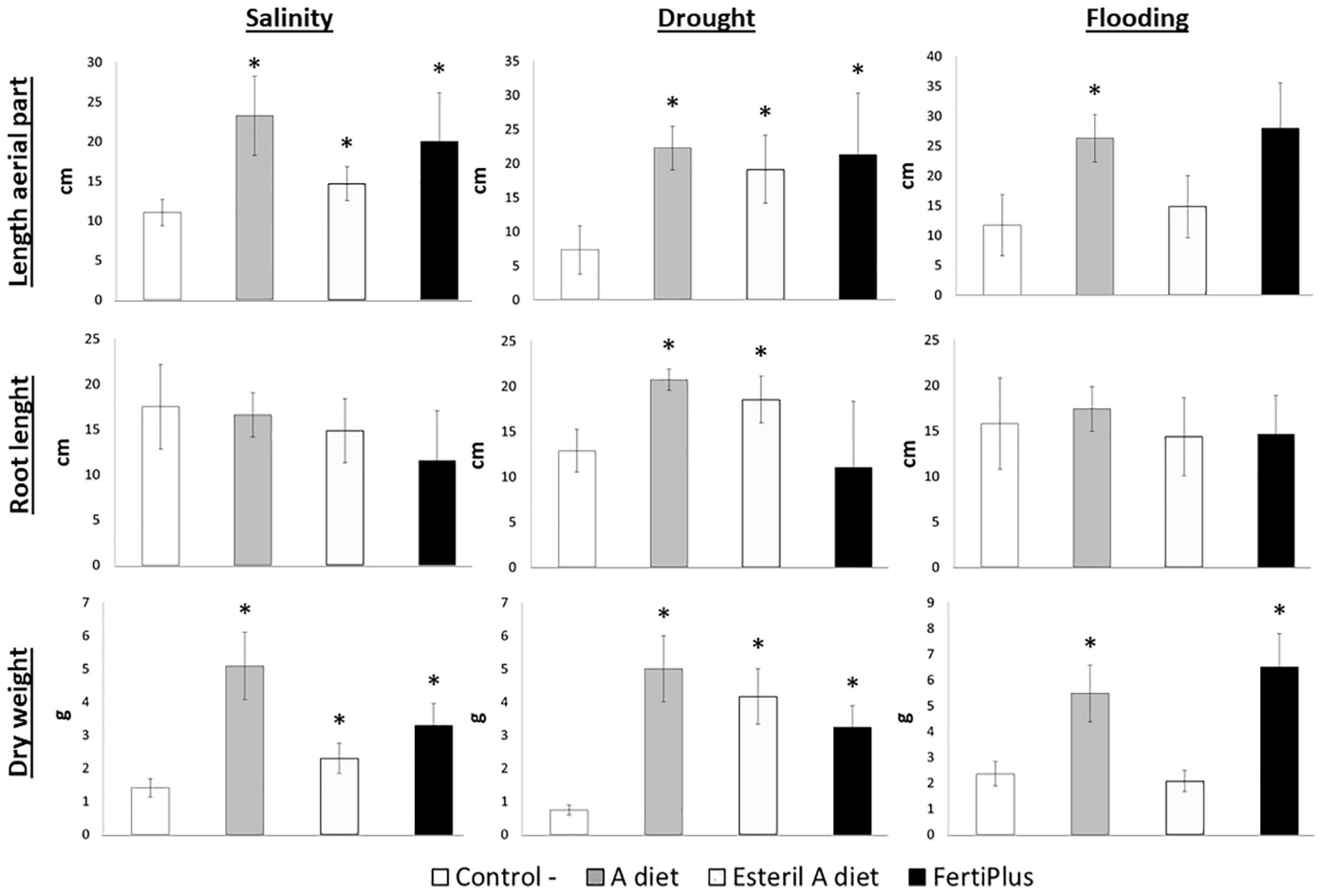

Fig. 4. Comparison of the effect of sterile and non-sterile *T. molitor* feces on plant growth parameters of *P. vulgaris* under stress conditions. Asterisks indicate statistically significant differences between groups.

growth and thus the ability to fix nitrogen.

2.7. Statistical analyses

The statistical analysis of the data was carried out using the Statistix 8.0 software. Analysis of variance (ANOVA) was used for each measured variable to assess the overall significance (*t*-tests) of main effects and interactions between the different fertilizers for each assay against the negative control. Differences with a *P*-value < 0.05 were considered statistically significant and marked with an asterisk. For the elaboration of the different graphs, Microsoft ® Excel 2016 software was used.

3. Results

3.1. Analysis of the biofertilizer potential of T. molitor feces

The results of the greenhouse experiments using chard plants are presented in Fig. 1. It was tested whether the feces obtained from insects fed on different diets were suitable to use as biofertilizers. It was found that the chlorophyll content of chard leaves, the fresh weight, the length of the aerial part and width of the basal part of the stem of these plants increased with the application of all of the fertilizers, as compared to the control plants. Plant biomass was significantly higher after the application of the excrement resulting from diet A, as well as the positive control, than the biomass of plants fertilized with excrement from diets B and C. In addition, the width of the basal part of the stem was found to be significantly greater in plants that had been treated with excrement resulting from C diet. But the increase was even greater in plants that had been treated with diets A and B and the positive control biofertilizer.

3.2. Analysis of the nutritional content of T. molitor feces

Analysis of the nutritional content of feces of *T. molitor* fed on different diets (Table 1) showed that diet C contained the highest N content. In fact, the amount of N was more than double than that of the other diets. It was determined that the C/N ratio of the excrement from this diet was the lowest. It was also determined that the excrement from diet C had more C, S, Ca, and Mo content overall (42.44 g/100 g, 0.28 g/100 g, 0.11 g/100 g and 0.96 mg/Kg, respectively). The highest amount of K content (1.97 g/100 g) was found in feces resulting from diet B. However, the highest value of P (1.53 g/100 g), Mg (0.54 g/100 g), Mn (171,89 mg/kg) and Fe (140,68 mg/kg) contents were detected in diet A.

3.3. Analysis of the potential of T. molitor feces to induce tolerance to abiotic stresses

Under most of the stress conditions, the plant growth parameters appeared to be improved in plants fertilized with the feces from diets A and B, as well as with the positive control involving a commercial biofertilizer (Fig. 2). In contrast, the *T. molitor* feces obtained from diet C do not appear to improve the parameters of plants grown under stress conditions, as compared to the negative control. Regarding the length of aerial part of the plant, the lowest growth of the plants under all

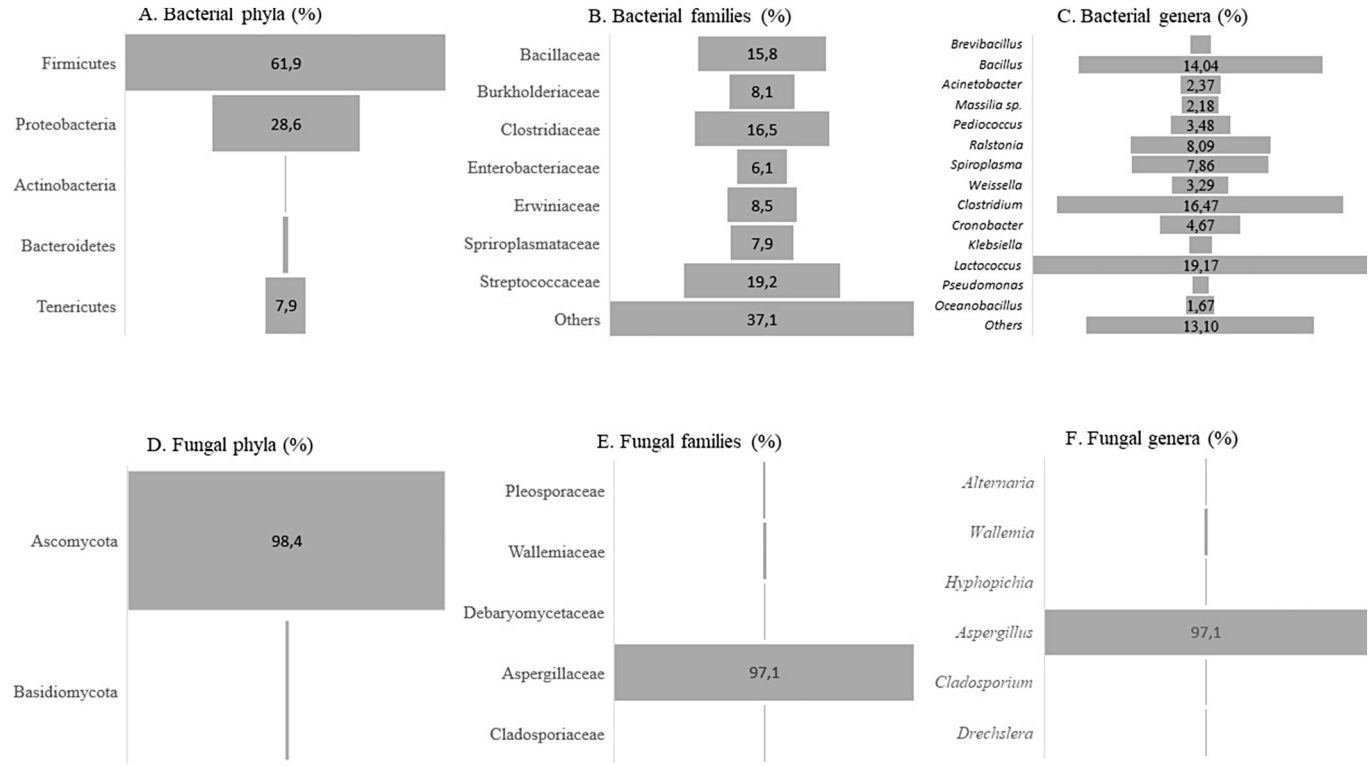

Fig. 5. Percentage of microbial taxa identified by massive parallel sequencing in *T. molitor* frass produced with diet A.

stress conditions tested occurred in the negative control and those fertilized with excrements from diet C and the highest aerial part growth is found in plants fertilized with feces from diet A as well as the positive control and with no statistical difference with those plants inoculated with excrements from diet B. Concerning root length, the application of the excrement from diet C significantly reduced root growth in comparison to the unfertilized control. All the other plants treated with fertilizers showed root lengths similar to those of the control grown under saline and waterlogging stress, and longer in drought conditions in diets A and B. Finally, the largest dry weights were obtained for plants fertilized with excrement from diet A grown under saline and waterlogging stress; the results were like those obtained for the positive control.

It was observed that drought had the most serious effect on plant survival and all unfertilized plants died. Although the fertilizers made from all three diets reduced the number of dead plants, the best result was obtained using excrement from diet A.

3.4. Analysis of the effect of the microbiome to promote plant growth and induce abiotic stress tolerance

The microbiome present in the excrement produced from diet A was the best biofertilizer for plant growth promotion and the induction of abiotic stress tolerance. To test how the microbiome may contribute to changes in growth parameters, plants fertilized with natural and sterilized excrement were compared.

In chard plants (Fig. 3), it was observed that the absence of microbiota in the sterilized feces from diet A was associated with reduced chlorophyll content in the leaves. The weight of the aerial part of fresh plant material was also significantly reduced. The bean plants grown under abiotic stress (Fig. 4) showed a significant decrease in aerial length, as well as in the dry weight of the plants grown under salinity and waterlogging stress conditions.

3.5. Analysis of the microbial community in T. molitor feces

A total of 4772 bacterial and 1225 fungal sequences were obtained from the entire dataset after quality trimming and the removal of chimeras and singletons. Bacterial sequences were clustered into 57 OTUs (Supplementary Table S1). The bacterial community was mainly comprised of Firmicutes (61.9%), followed by Proteobacteria (28.6%) (Fig. 5a). All Firmicutes belonged to the classes Bacilli (45,2%) and Clostridia (16,7%), while the Proteobacteria belonged to the Gammaproteobacteria (18,1%) and Betaproteobacteria (10,5%). On the family level, Streptococcaceae, Clostridiaceae and Bacillaceae were predominant (Fig. 5b), although *Lactococcus*, *Clostridium* and *Bacillus* were the most common genera found (Fig. 5c).

Regarding fungi, the sequences were grouped into 19 OTUs (Supplementary Table S2) and the community was dominated mainly by Ascomycota (98.4%), while Basidiomycota represented approximately 1.6% of the total number of sequences (Fig. 5d). On the family level, the vast majority of sequences belonged to Aspergillaceae (97.1%) (Fig. 5e), in particular, genus *Aspergillus* was the most abundant (Fig. 5f).

3.6. Isolation and identification of microbial strains

The list of bacteria and fungi isolated and identified in this study, according to their 16S rRNA and ITS sequences, is presented in Table 2.

A total of 19 bacterial strains and 6 fungal strains were isolated from medium MFsm; 16 bacterial strains and 9 fungal strains from MFm; 21 fungal strains from SAB; 96 bacterial strains and 4 fungal strains from NA and 16 bacterial strains and 1 fungal strain from TSA. The total number of microbial strains isolated was 188, of which 147 were bacteria and 41 were fungi.

3.7. Analysis of PGP traits of microbial isolates from T. molitor feces

The ability of the microbial strains isolated from diet A to promote plant growth was analyzed and the results are presented in Table 3.

Table 2
Identification of isolated microorganisms based on their 16S rRNA gene/ITS (bacteria/fungi) sequence. The strain names are based on their isolation medium: MFMFm = MFm; MFMFsm = MFsm; MFSAB = SAB; MFTSA = TSA, MFNA = NA.

	Identification
Bacterial strains	
MFNA56A	*Acinetobacter radioresistensis*
MFNA84	*Bacillus cereus/B. thuringiensis*
MFNA52	*Bacillus megaterium*
MFNA51, MFTSA8, MFNA36, MFNA56F, MFNA59, MFNA60, MFNA38, MFNA39B	*Brevibacillus halotolerans*
MFNA46B	*Cronobacter condimenti*
MFNA45	*Dermacoccus nishinomiyaensis*
MFNA33B	*Enterobacter kobei*
MFNA17, MFNA27, MFNA27, MFTSA14, MFTSA2A, MFTSA6B, MFNA31, MFNA34, MFNA42, MFNA66, MFNA69, MFNA70, MFNA72, MFNA73, MFNA74, MFNA75, MFNA77, MFNA78	*Enterococcus durans*
MFNA4, MFNA48	*Enterococcus faecalis*
MFTSA10, MFNA15, MFNA5, MFNA87A, MFNA90, MFNA93	*Enterococcus faecium*
MFNA13B	*Enterococcus hirae*
MFTSA1	*Enterococcus malodoratus*
MFTSA13, MFTSA3	*Enterococcus mundtii*
MFTSA2B	*Enterococcus raffinosus*
MFNA85A	*Enterococcus termitis*
MFNA22, MFNA29	*Enterococcus thailandicus*
MFNA16, MFNA8, MFTSA12, MFTSA2C, MFNA1, MFNA10, MFNA11, MFNA12, MFNA13A, MFNA14, MFNA23, MFNA47, MFNA7, MFNA76, MFNA86A, MFNA9, MFNA26, MFNA61	*Enterococcus villorum*
MFMFsm11, MFMFsm13, MFMFsm15	*Erwinia oleae*
MFMFm14B, MFMFm7	*Klebsiella oxytoca*
MFNA18, MFNA24	*Lactobacillus lactis*
MFMFm13A, MFNA32, MFNA63, MFNA86B, MFNA87B	*Lactococcus garvieae*
MFTSA4, MFNA30, MFNA40B, MFNA41A, MFNA44, MFNA54, MFNA64	*Lactococcus lactis*
MFMFsm14, MFMFsm16, MFMFsm17, MFMFsm18, MFMFsm22, MFMFsm23, MFMFsm3, MFMFsm4, MFTSA9, MFNA19, MFNA2, MFNA21, MFNA28, MFNA3, MFNA49, MFNA6, MFNA65, MFNA67	*Lactococcus taiwanensis*
MFMFm1, MFMFm11, MFMFm15A	*Leifsonia naganoensis*
MFMFm13C	*Microbacterium paraoxydans*
MFNA56D	*Micrococcus lactis*
MFNA53, MFNA89	*Micrococcus luteus*
MFNA56E	*Neomicrococcus aestuarii*
MFMFm14A	*Nocardia coeliaca*
MFMFm4, MFMFsm10	*Paenibacillus* sp.
MFMFsm12	*Paenibacillus taichungensis*
MFMFm2	*Pantoea* sp.
MFMFm5	*Pantoea vagans*
MFMFm8, MFNA83	*Pseudomonas parafulva*
MFMFm15B, MFMFm3	*Pseudomonas punonensis*
MFNA43	*Rothia aeria*
MFMFm12, MFMFm13B	*Sphingobacterium multivorum*
MFNA79, MFNA81, MFNA82, MFNA85B, MFNA91	*Staphylococcus saprophyticus*
MFNA92	*Streptomyces gougerotii*
Fungal strains	
MFMFm10	*Ulocladium dauci*
MFSAB13A, MFSAB13B	*Aspergillus pseudoglaucus*
MFSAB14, MFSAB18	*Aspergillus restrictus*
MFSAB7	*Aspergillus* sp.
MFSAB5	*Cladosporium cladosporioides*
MFSAB3, MFNA35, MFNA39A	*Cladosporium halotolerans*
MFMFsm19, MFMFsm24, MFMFsm6, MFSAB4	*Cladosporium* sp.
MFMFsm25, MFMFsm26	*Fusarium oxysporum*
MFMFm9, MFMFsm21, MFMFsm9	*Penicillium ochrochloron*
MFMFsm20, MFMFsm7	*Penicillium* sp.

Siderophore production was positive in 34.43% of the isolates. Auxins production was detected in 26 isolates (17.21%) and only 10 bacterial strains (6.62%) showed positive signs of ACC deaminase production. However, 13 bacterial strains (8.60%) were found to fix nitrogen. The halos produced from the solubilization of potassium and phosphorus were detected around 4.63% and 58.27% of the colonies, respectively.

4. Discussion

In the search for alternatives to decrease the use of highly polluting agricultural chemicals, insect excrement is currently being considered as a potential option (Currie, 2001; Fortanelli Martínez and Servín Montoya, 2002) and the use of *T. molitor* feces has already been proposed (Li et al., 2013; Liu et al., 2003). However, to the best of our knowledge, this is the first study on the influence of the insect's diet on the potential to promote plant growth and abiotic stress resistance and on the contribution of the microbiome present in feces to these processes.

Although changes in the nutritional content of excrements have already been reported in the feces of many animals, such as pigs (Jarret et al., 2011, 2012), ruminants (Codron et al., 2012), laying hens (Zhang and Kim, 2013), broiler chicks (Donsbough et al., 2010; Namroud et al., 2008) and hamsters (Villanueva et al., 2011), this is the first report in *T. molitor* frass. Our results show that excrement from diets A and B have similar nutritional contents: the excrement from these two diets contains higher amounts of P, K, Mg and Mn than that from diet C, which is probably due to a higher carbohydrate content in diets A and B. In addition, the excrement produced from diet C is high in C, N, S and Mo

Table 3
Results from *in vitro* plant growth promotion traits experiments.

Isolate	Presumptive nitrogen fixation	Phosphate solubilization	Potassium solubilization	Auxins production	Siderophore production	Production of ACC deaminase
MFMFm1	+	+		+	+	
MFMFm10	X				+	
MFMFm11	+	+		+	+	
MFMFm12	+					
MFMFm13A					+	
MFMFm13B					+	+
MFMFm13C	+				+	
MFMFm14A					+	
MFMFm14B					+	
MFMFm15A		+				
MFMFm15B	+	+		+	+	
MFMFm2		+			+	
MFMFm3	+	+		+	+	
MFMFm4		+	+		+	
MFMFm5		+		+	+	
MFMFm7	+				+	
MFMFm8		+		+		
MFMFm9	X			+		
MFMFsm10		+	+		+	
MFMFsm11		+				
MFMFsm12				+	+	
MFMFsm13				+		
MFMFsm14		+				
MFMFsm15				+	+	+
MFMFsm16		+			+	
MFMFsm17		+				
MFMFsm18		+		+	+	
MFMFsm19	X			+		
MFMFsm20	X	+			+	+
MFMFsm21	X	+			+	
MFMFsm22		+				
MFMFsm23		+				
MFMFsm24	X			+		
MFMFsm25	X			+	+	+
MFMFsm26	X			+	+	
MFMFsm3		+				
MFMFsm4		+				
MFMFsm6	X			+		
MFMFsm7	X	+			+	+
MFMFsm9	X				+	
MFNA1		+				
MFNA10		+				
MFNA11		+				
MFNA12		+			+	
MFNA13A						
MFNA13B						
MFNA14		+				
MFNA15		+				
MFNA16		+				
MFNA17		+	+			
MFNA18	+	+				
MFNA19		+				
MFNA2		+				
MFNA21		+				
MFNA22		+				
MFNA23		+				
MFNA24	+	+				
MFNA26		+				
MFNA27		–				
MFNA28		+				
MFNA29		+				
MFNA3		+				
MFNA30		+				
MFNA31	+	+				
MFNA32		+				
MFNA33B		+				+
MFNA34		+	+			
MFNA35	X			+		
MFNA36					+	
MFNA38					+	
MFNA39A	X			+		
MFNA39B						
MFNA4		+				
MFNA40B						

(*continued on next page*)

Table 3 (*continued*)

Isolate	Presumptive nitrogen fixation	Phosphate solubilization	Potassium solubilization	Auxins production	Siderophore production	Production of ACC deaminase
MFNA41A		+	+	+	+	
MFNA42						
MFNA43	+					
MFNA44						
MFNA45	+					
MFNA46B		+				
MFNA47						
MFNA48						
MFNA49						
MFNA5		+				
MFNA51						
MFNA52					+	
MFNA53						+
MFNA54						
MFNA56A		+	+		+	
MFNA56D					+	
MFNA56E			+			
MFNA56F						
MFNA59						
MFNA6		+				
MFNA60					+	
MFNA61						
MFNA63		+				
MFNA64		+				
MFNA65		+				
MFNA66		+				
MFNA67		+				
MFNA68						
MFNA69		+				
MFNA7		+				
MFNA70						
MFNA72		+				
MFNA73		+				
MFNA74		+				
MFNA75						
MFNA76		+				
MFNA77		+				
MFNA78		+				
MFNA79						
MFNA8						
MFNA81					+	
MFNA82					+	
MFNA83		+		+	+	
MFNA84		+		+	+	
MFNA85A		+				
MFNA85B					+	
MFNA86A		+				
MFNA86B		+			+	
MFNA87A		+				
MFNA87B					+	
MFNA89						
MFNA9		+			+	
MFNA90		+			+	+
MFNA91				+	+	+
MFNA92	+	+		+	+	
MFNA93					+	+
MFSAB13A	X	+			+	
MFSAB13B	X	+				
MFSAB14	X	+				
MFSAB18	X	+				
MFSAB3	X			+	+	
MFSAB4	X			+	+	
MFSAB5	X			+	+	
MFSAB7	X	+			+	
MFTSA1						
MFTSA10						
MFTSA12		+			+	
MFTSA13						
MFTSA14		+				
MFTSA2A		+				
MFTSA2B						
MFTSA2C		+				
MFTSA3						
MFTSA4		+				
MFTSA6B		+				

(*continued on next page*)

Table 3 (*continued*)

Isolate	Presumptive nitrogen fixation	Phosphate solubilization	Potassium solubilization	Auxins production	Siderophore production	Production of ACC deaminase
MFTSA8		+			+	
MFTSA9		+				

+ = PGP observed in *in vitro* assay; X = eukaryotic cells, exclusive process for prokaryotic cells.

content. Nevertheless, the C/N ratio in diet C is very low, which could hamper the transformation of organic compounds into forms that plants can assimilate (Brown et al., 2007). Finally, the higher Fe content in the excrement from diet A could be due to the source of carbohydrates, with a greater amount of photosynthetic tissues (such as leaves), rich in Fe (Rawashdeh and Sala, 2015). This element has been reported to greatly affect not only plant productivity, but also the quality of plant products (Briat et al., 2015).

Since diet A appeared to promote plant growth the best, the possible causes underlying these improvements were further addressed. Many reports confirm that microorganisms greatly influence plant growth (Menéndez and Garcia-Fraile, 2017) thus, we analyzed the effect of microbiota present in the excrement from diet A on plant growth promotion and abiotic stress tolerance. To this end, plant growth parameters were measured in plants fertilized with sterilized excrement and compared to those grown in the presence of the original frass.

As microbiota seemed to play a crucial role in plant growth promotion, the microorganisms present in the excrement were studied through massive parallel sequencing. Although to the best of our knowledge this is the first report of the microbial communities in the *T. molitor* feces, some of the genera found in this study have already been reported as members of the microbial community of the insect, such as *Bacillus*, *Weissella, Pseudomonas*, *Spiroplasma*, *Pantoea*, *Paenibacillus*, *Lactococcus*, *Staphylococcus*, *Clostridium* and *Erwinia,* among others (Jung et al., 2014; Wang and Zhang, 2015; Li et al., 2016; Osimani et al., 2018). It is important to note that the species *Spiroplasma* found in *T. molitor* by other researchers are considered unique to this insect (Jung et al., 2014).

Most bacterial isolates were classified within genera detected by massive parallel sequencing, but those identified as *Dermacoccus*, *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Leifsonia* and *Microbacterium*. On the other hand, we did not isolate bacterial strains belonging to the genera of some abundant OTUs determined by the analysis of massive parallel sequencing. It should be noted that the Illumina MiSeq platform only sequences fragments of approximately 300 bp, which is a very small fragment when trying to identify bacteria at the species or even genus level. Consequently, it is possible that some of the strains were misidentified. For instance, there is an OTU that was identified as *Leucobacter*, a genus belonging to the Microbacteriaceae family, as *Leifsonia* or *Microbacterium*. In addition, no OTUs were identified as *Lactobacillus*; however, one of the abundant OTUs was identified as *Pediococcus*, which also belongs to the family Lactobacillaceae. Finally, isolates such as *Enterobacter* and *Enterococcus* are Enterobacteria closely related to the genus *Klebsiella*, which seems to be relatively abundant according to the OTUs analysis. On the other hand, some of the microbes detected by massive parallel sequences may require specific conditions that were not used in this study for their isolation. This is the case for *Clostridium* strains which require anaerobiosis to grow.

The fungal isolates obtained from the excrement from diet A were classified into 5 different genera according to their ITS sequence: *Aspergillus* and *Cladosporium*, both also detected by massive parallel sequencing, *Fusarium*, *Penicillium* and *Ulocladium*. Instead of the three latter ones, the remaining fungal OTUs found by the Illumina sequencing were identified as *Alternaria, Wallemia, Dreschlera* and *Hyphopichia*. *Alternaria*, *Deschlera* and *Ulocladium*, fungal genera belonging to the family *Pleosporaceae* and the short fragment sequenced by the Illumina MiSeq could be the origin of a misidentification. On the other hand, *Penicillium* and *Aspergillus* are two closely related genera and strains from the genus *Penicillium* could be easily identified as *Aspergillus* based on the massive parallel sequencing.

Several of the identified microorganisms have been described as potential biofertilizers: *Bacillus* has been determined as a bacterial genus capable of promoting seed germination (Widnyana and Javandira, 2016), root development (Ab Aziz et al., 2015) and nutrient assimilation (Shi et al., 2014), and can act as a biocontrol agent (Borriss, 2015; Grosu et al., 2015; Balabel et al., 2013) and degrade toxic waste in soils (El-Helow et al., 2013). *Pantoea* has been described as being a PGPR (Plant Growth Promoting Rhizobacteria) (Feng et al., 2006; H.J. Kim et al., 2012; Selvakumar et al., 2008) and a biocontrol agent against other bacteria (Enya et al., 2007.; Hsieh et al., 2005.; Smits et al., 2010); *Klebsiella* has been identified as a PGPR (Govindarajan et al., 2007; Park et al., 2005; Rueda-Puente et al., 2004) capable of producing auxins (Sachdev et al., 2009) and degrading toxic waste (Sasikala et al., 2012); *Brevibacillus* is also a PGPR (Nehra et al., 2016), and is known to be a biocontrol agent against plant pathogen fungi (Che et al., 2015; Wei et al., 2014) and a degrader of pesticide waste (Arya and Sharma, 2015; Hernández-Ruiz et al., 2017). *Pseudomonas* has been described as a PGPR (Sivasakthi et al., 2014) and biocontrol agent (Gómez-Lama Cabanás et al., 2017; Sivasakthi et al., 2014); *Acinetobacter* has also been described as PGPR (Padmavathi et al., 2015), able to produce gibberellins (H.J. Kim et al., 2012), auxins (Zhao et al., 2013) and to act as a biological control agent (Xue et al., 2009; ZhongHua et al., 2011); *Sphingobacterium* has been described as PGPR (Ahmed et al., 2014) and *Paenibacillus* has been identified as PGPR (Ma et al., 2012; Xie et al., 2016), since it improves the nitrogen supply to plants (Puri et al., 2015), increases the abiotic stress tolerance (Sukweenadhi et al., 2015), acts as a biocontrol agent against fungi (Kim et al., 2015; Guo and Liao, 2014; Sato et al., 2014; Seo et al., 2016) and nematodes (Hong et al., 2013) and degrades pesticide wastes (Romeh and Hendawi, 2014). Among the fungi with beneficial properties for agriculture, *Aspergillus* has been described as a P solubilizing organism (Bhavsar et al., 2008; Pradhan and Sukla, 2006; Richardson et al., 2002) and as biocontrol agent (Soliman et al., 2012); Wallemia is known to promote tolerance to drought stress (Díaz-Valderrama et al., 2017); Cladosporium as mycoparasite of different plant pathogens (Assante et al., 2004) and *Dreschlera* as producer of natural herbicides (Evidente et al., 2006).

The *in vitro* testing of plant growth promotion by the strains isolated in this study showed that the excrement from diet A contains bacterial species with several of these properties. The different stains identified as *Lactococcus* had the ability to solubilize P and K and to produce auxins and siderophores, processes that have not been previously found in this bacterial genus. In the literature it is known that microbial strains of other genera also exhibit characteristics similar to those found in PGPR: *Bacillus* strains are able to solubilize P (Kim et al., 2014) and produce auxins (Talboys et al., 2014) and siderophores (Sivasakthi et al., 2014); *Pantoea* strains solubilize P (Jorquera et al., 2008; Selvakumar et al., 2008), produce auxins (Apine and Jadhav, 2017; Selvakumar et al., 2008) and siderophores (Loaces et al., 2011; Selvakumar et al., 2008); *Klebsiella* is able to fix atmospheric N (Chen et al., 2015a; Wei et al., 2014) and produce siderophores (Yu et al., 2018); *Brevibacillus* stains able to solubilize P (Yadav et al., 2013) and produce siderophores (Gupta and Gopal, 2008). In addition, several strains of *Pseudomonas* are known to fix N (Fox et al., 2016), solubilize P (Otieno et al., 2015), produce auxins (Kudoyarova et al., 2017) and siderophores (Luján et al., 2015) and *Acinetobacter* strains are able to

solubilize P and K (Shi et al., 2011; Bhattacharya et al., 2016) and produce siderophores (Zhao et al., 2013). Some strains isolated from *Paenibacillus* are known to solubilize P and K (Silva et al., 2015; Zhang et al., 2013) and produce siderophores (Sukweenadhi et al., 2015).

Regarding fungi, *Cladosporium* strains were found to have the ability to produce auxins, gibberellins and siderophores. To the best of our knowledge, this is the first report describing the ability of *Cladosporium* strains to produce auxins; however its ability to produce gibberellins (Hamayun et al., 2010) and siderophores (Collemare et al., 2014) have been previously observed. Lastly, it was observed that *Aspergillus* strains are able to produce siderophores, which is in agreement with what has already described (Machuca and Milagres, 2003; Patel et al., 2017; Schrettl et al., 2004).

5. Conclusions

Our results confirm for first time that different diets of *T. molitor* determine not only the composition of frass, but also the potential of the feces to promote plant growth and induce tolerance to abiotic stress. In this study, we showed that out of the three diets tested, diet A significantly increases several plant parameters. In addition, diet A can promote abiotic stress tolerance in plants. This is the first study to report the potential use of *T. molitor* feces to promote plant resistance to abiotic stress. Moreover, we show that the microorganisms present in the resulting feces play an important role in improving plant growth. We have shown through *in vitro* analysis that several bacterial and fungal isolates, obtained from insects fed on diet A, present PGP characteristics. Therefore, our findings indicate this frass is of great interest regarding its use as a biofertilizer. Nevertheless, further studies are required to establish the correct timing of when the biofertilizer should be applied and the specific amount of fertilizer that would achieve optimal results.

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Conflict of interest

We declare that the research was funded by a private corporation, MealFood Europe SL, which sells *Tenebrio molitor* excrement as a biofertilizer (MealFrass®); however, we ensure the research is free of bias.

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