Waste Management 119 (2021) 215–225

Contents lists available at ScienceDirect

Waste Management

journal homepage: www.elsevier.com/locate/wasman

New insight into microbial degradation of mycotoxins during anaerobic digestion

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ARTICLE INFO

Article history:
Received 19 June 2020
Revised 4 September 2020
Accepted 24 September 2020

Keywords:
Aflatoxins
Fumonisins
Bioremediation
Carboxylesterase
Biogas

ABSTRACT

Anaerobic digestion represents an interesting approach to produce biogas from organic waste materials contaminated by mycotoxins. In this study a shotgun metagenomic analysis of lab-scale bioreactors fed with mycotoxin-contaminated silage has been carried out to characterize the evolution of microbial community under the operating conditions and the key enzymatic activities responsible for mycotoxin degradation. The study was conducted at two different level of contamination for fumonisins and aflatoxin B_1. After 15 days biogas production was not influenced by the presence of mycotoxins. Metagenomic analysis revealed that a high contamination rate of mycotoxins interfere with microbial diversity. Degradation of mycotoxins accounted in about 54% for aflatoxin B_1 and 60% for fumonisins. The degradation activity of fumonisins resulted in the presence of partially hydrolyzed forms in both tested contamination levels. Accordingly, metagenomic functional analysis revealed the presence of two new carboxylesterase genes belonging to *D. bacterium* and *P. bacterium* putatively involved in fumonisin degradation.

1. Introduction

Cereals are the most important agricultural crops in the world, for both its productivity and its nutritional value. Approximately 195 million hectares of maize, and 214 million hectares of wheat are cultivated yearly worldwide (FAOStat, 2018). However depending on conductive pre- and post-harvest conditions, cereals may be infected with multiple species of toxigenic fungi, and most fungal strains produce more than one type of mycotoxin. Mycotoxins are fungal secondary metabolites with diverse chemical structures that contaminate many of the most frequently consumed food and feed commodities worldwide (Binder et al., 2007; Miller and Greenhalgh, 1988). Mycotoxins were mainly reported in wheat and maize showing the highest concentrations of fumonisins (FBs), deoxynivalenol (DON), aflatoxins (AFs), and zearalenone (ZEN). In particular, Aflatoxin B_1 (AFB_1) was classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1), and recognized as one of the most potent liver genotoxic carcinogens (IARC, 2002). Fumonisins B_1 and B_2 (FB_1, FB_2) were classified in Group 2B, compounds considered carcinogenic to animals and possibly carcinogenic to humans (IARC, 2002). IARC recently also associated AFs and FBs dietary exposure with high levels of stunting and growth impairment in children. Moreover, additive, synergistic, or antagonistic effects have also been associated with the co-exposure to multi-mycotoxin (Grenier and Oswald, 2011). Therefore in such scenario, the toxic effects of some mycotoxins could be deleterious even at levels below the recommended levels. Because of high level of mycotoxin contamination many cereal lots are no longer usable for food or animal feed and represent a food waste. In recent years, the search for renewable energy resources to replace fossil fuels has received increasing attention worldwide. Anaerobic digestion (AD) represents an interesting approach in this regard, in order to produce biogas (a renewable energy carrier) from different organic waste materials and dedicated energy crops (Weiland, 2010; Appels et al., 2011; Nkoa, 2014). Moreover, the AD process results in a by-product, the digestate, that may have great agronomic value due to its high content of mineral nitrogen, and its relatively stable organic matter (Cucina et al., 2017; Tambone et al., 2010). However, the use of contaminated material as feedstock for biogas production should consider the possible negative effect on AD process, biogas yield, fate of mycotoxins and digestate quality. Recently several studies have addressed these aspects, revealing a reduction of several mycotoxin from 12% up to 99% under mesophilic and thermophilic conditions in both batch test and semi-continuous reactors (Salati et al., 2014; De Gelder et al., 2018; Giorni et al., 2018). Moreover, the presence of the mycotoxins did not affected

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https://doi.org/10.1016/j.wasman.2020.09.048
0956-053X/

biogas production. The benefits derived from the AD of contaminaed cereal lots as an alternative to destruction by incineration are starting to be appreciated in the policy actions aimed to the management of contaminated waste.

It is well established that several mycotoxins are prone to biological degradation (Vanhoutte et al., 2016), also in the rumen similarly harboring anaerobic fermentative conditions (Upadhaya et al., 2010). The degradation of mycotoxins has always been of great interest. Indeed, some efforts to find organisms or enzymes, able to metabolize and degrade mycotoxins and thus, providing a biotechnological solution to the problem, have been undertaken. The advent of high throughput next generation sequencing (NGS) and the improvement of bioinformatics for NGS-based metagenomics and genomewide data analysis have provided a significant contribute in deciphering the complex microbial community engaged in AD process, unraveling the network of bacterial interactions and the applicability potential of the derived knowledge. However, to our knowledge, no data are available on the effect of contaminated feedstock on microbial communities and on their direct activity in degrading mycotoxins during AD process. Indeed, the main objectives of this research was to gain a better understanding of the response of the microbial community to the presence of mycotoxins in feedstock used for AD and to investigate the possible biological processes involved in mycotoxin biodegradation.

2. Materials & methods

2.1. Anaerobic digestion

The AD process was conducted in 2 L batch-flasks. Four batch-flasks were prepared, two for each thesis. The batch-flasks were set up by mixing 80 g of silage with 1 L of digestate inoculum in stable methanogenic conditions, sampled from the bioreactor of an industrial scale biogas plant (AUSTEP, Dorno, IT). A first batch experiment (C1) was set up by using the digestate from biogas plant without adding any other mycotoxins since it was naturally contaminated by FB_1 = 241.5 μg kg^{-1}, FB_2 = 86.5 μg kg^{-1} and FB_3 = 42.5 μg kg^{-1}. Considering the occurrence of FBs and AFs in silage (Ogunade et al., 2018), a second batch experiment (C2) was set up by increasing fumonisins content up to FB_1 = 13,874 μg kg^{-1}, FB_2 = 3,877 μg kg^{-1} and FB_3 = 3,591 μg kg^{-1}. The methanolic extract used for the artificially contamination of the digestate was obtained by inoculating maize grain with *F. verticilliodes* (ITEM 3418). Moreover an artificial contamination with AFB_1 was performed at final concentration of AFB_1 = 251 μg kg^{-1} with an AFB1 stock solution (Sigma Aldrich, Saint Louis, USA).

The bioreactors were incubated for 15 days at 43 °C. Operational conditions were monitored after 15 days by monitoring of pH, electrical conductivity (EC); percentage of Total solid (%TS), percentage of Volatile Solid (%VS) and VS/TS, according to European standard procedures (EN 13037, 1999; EN 13038, 1999; EN 13039, 1999). Partial and total pressure was measured continuously with an ANKOM Gas Production System (ANKOM Technology, USA). The biogas produced during AD was collected in 10 L Multi-Layer Foil Gas Sampling Bags and the biogas composition was monitored using an Optima 7 portable biogas analyser (MRU GmbH, Germany). Biogas production was measured by converting the accumulated gas produced in Nm^3/ton of silage according to Valero et al. (2016).

2.2. Sample collection, DNA and RNA extraction, cDNA synthesis

Samples (150 g) were collected in sterile containers from each batch-flasks of C1 and C2 thesis after 15 days of AD. Total DNA was extracted in triplicates from about 0.5 g of material using the PowerSoil DNA Isolation Kit (MO BIO laboratories, USA). Moreover, total RNA was extracted with Direct-zol RNAMiniPrep Plus (Zymo Research) from 0.3 g of contaminated samples. The quality and the quantity of the extracted DNA and RNA were determined using NanoDrop (ThermoFisher Scientific, USA), Qubit fluorometer (Life Technologies, USA) and 1.0% (w/v) agarose gel electrophoresis. The cDNA was synthetized from 1 μg of total RNA using the SuperScript IV VILO Master Mix (ThermoFisher Scientific, USA) containing the ezDNaseEnzyme for complete DNA contamination removal.

2.3. Shotgun libraries preparation and bioinformatic analysis

Total DNA (1 μg) extracted from each sample was sheared using a Covaris S2 system (Covaris, USA). Shotgun libraries were prepared from 100 ng of sheared DNA using the Ion Xpress Plus gDNA Fragment Library kit (Life Technologies, USA), following the manufacturer's instructions. Size selection of libraries (~400 bp) were performed by agarose gel electrophoresis using 2% E-Gel SizeSelect Agarose Gels (Life Technologies, USA). After purification, libraries were quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, USA), pooled and sequenced on an Ion 530 Chip using an Ion S5System (ThermoFisher Scientific, USA). Raw sequences were subjected to quality check using the FastQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reads were than processed using PRINSEQ for removing low quality reads (Q < 30) and filtered by length (>100pb) (Schmieder and Edwards, 2011). High quality shotgun reads were aligned against nr-protein database (NCBI release 2018) (Benson et al., 2005) using DIAMOND (Buchfink et al., 2015), in BLASTx mode, with E-value cutoff set to 10^{-5} and sensitive mode activated. Taxonomic and functional binning was performed with MEGAN software (MEGAN 6 v6.6.3, Huson et al., 2016). For the LCA algorithm the following settings were used: minScore = 100, maxExpected = 0.01, minIdentity = 0.01, topPercent = 10 and minSupportPercent = 0.03.Additionally, the 'Weighted LCA' assignment (80.0%) on Taxonomy was applied. The functional annotation of the reads was performed using the eggNOG (Evolutionary genealogy of genes: Non-supervised Orthologous Groups). Reads were normalized between samples by using MEGAN 6 built-in normalization tool for comparative analysis among samples.

2.4. Determination of aflatoxin B_1 in the digestate sample

Aflatoxin B_1 content was quantified in digestate samples from inoculum, after artificial contamination and at the end of AD. A total of 50 g of digestate sample plus 2.5 g sodium chloride was extracted with methanol/water solution (60:40, v/v). The extraction was carried out by blending at high speed for 3 min with a SorvallOmnimixer (Dupont Instruments, New town, CT, USA). The extract was centrifuged for 10 min at 2,500 × *g*, and 10 mL of supernatant were mixed with 40 mL of PBS buffer pH 7 and filtered through Whatman n.4 filter paper. The remaining solid fraction after centrifugation was dried and 5 g were extracted with 50 mL methanol/water solution (80:20, v/v) by blending for 2 min using an orbital shaker, then centrifuged for 10 min at 2,500 × *g* and 10 mL were diluted with 40 mL PBS buffer pH 7 and filtered through filter paper. Diluting extracts 10 times before loading onto immunoaffinity columns was necessary to avoid saturation of the Afla-antibody binding sites. Five millilitres volume (equivalent to 0.04 g test portion) was passed through AflaTest immunoaffinity column, and after washing the column with 10 mL water (2 times), aflatoxins were eluted with 2 mL methanol and collected in a 4 mL amber silanized vial. The eluted extract was diluted with 2 mL of water and analysed by UPLC/FLD. Five microliters volume was

injected into the UPLC apparatus. The fluorometric detector was set at wavelengths of 365 nm (excitation) and 435 nm (emission). The mobile phase consisted of a mixture of water:acetonitrile: methanol (64:18:18, v/v/v) at a flow rate of 0.4 mL min^{-1} . The temperature of the column was maintained at 40 °C. The $AFB_1$was quantified by measuring peak areas at the retention time of aflatoxins standard solutions and comparing these areas with the relevant calibration curve. With this mobile phase, the retention time of AFB_1 was about 3.7 min. The LOQ of the method was 0.1 μg kg^{-1} for AFB_1 based on a signal to noise ratio of 3:1.

2.5. Determination of fumonisins in the digestate

Fumonisins content was quantified in digestate samples collected from inoculum, after artificial contamination and at the end of AD. A total of 50 g of digestate sample was extracted with acetonitrile/water solution (50:50, v/v). The extraction was carried out by blending at high speed for 2 min with a SorvallOmnimixer (Dupont Instruments, New town, CT, USA).The extract was centrifuged for 10 min at 2,500 × *g*. Ten milliliters of supernatant were diluted with 40 mL of PBS buffer pH 7 and filtered through glass microfiber paper. The remaining solid fraction after centrifugation was dried and 5 g were extracted with 30 mL acetonitrile/water solution (50:50, v/v) by shaking for 60 min using an orbital shaker, then centrifuged for 10 min at 2,500 × *g*. Ten milliliters were diluted with 40 mL PBS buffer pH 7 and filtered through glass microfiber paper.

Two point five-milliliters volume (equivalent to 0.275 g and 0.08 g test portion for liquid and solid fraction, respectively) was passed through a FumoniTest immunoaffinity column, and after washing the column with 5 mL PBS followed by 2 mL water, fumonisins were eluted with 2 mL methanol followed by 2 mL water and collected in amber silanized vial. The eluted extracts were evaporated to dryness under air stream at 50 °C, dissolved in 1 mL acetonitrile/water (30:70, v/v) and analysed by HPLC/FLD after derivatization with *o*-phtaldialdehyde reagent (OPA) solution by using HPLC autosampler (VarianInc., Palo Alto, CA, USA). Fifty microliters of the derivatized solution were injected into the HPLC/fluorescence detection system (Varian Inc.) by full loop at exactly 3 min after adding the OPA reagent. The analytical column was a Gemini C18 150 × 2.0 mm, 5-μm (Phenomenex, Torrance, CA, USA) preceded by a Gemini C18 guard column (4.0 × 2.0 mm-5 μm) and the mobile fase consisted of a binary gradient applied as follows. The initial composition of the mobile phase, 60% of (A) acetonitrile/water/acetic acid (30:69:1, v/v/v) and 40% of (B) acetonitrile/water/acetic acid (60:39:1, v/v/v), was kept constant for 5 min, then (B) solvent was linearly increased to 88% in 21 min and kept constant for 4 min. Finally, to wash the column the amount of acetonitrile was increased to 100% and kept constant for 4 min. The column was thermostated at 30 °C. The flow rate of the mobile phase was set to 0.2 mL min^{-1}.

The fluorometric detector was set at wavelengths, ex = 335 nm, em = 440 nm. Retention time for FB_1 was about 26 min and for FB_2 36 min. Fumonisins (FB_1 and FB_2) were quantified by measuring peak areas, and comparing them with a calibration curve obtained with standard solutions, $FB_3$was quantified based on the FB2 calibration curve. The limit of detection of the analytical method was 10 μg kg^{-1} for each fumonisin.

2.6. LC-HRMS/MS analysis of fumonisin derived products

The investigation was focused only on derived products of FB_1 and FB_2, namely partially hydrolyzed FB_1 ($PHFB_1$) and both structural isomers of partially hydrolyzed FB_2 ($PHFB_{2a}$ and $PHFB_{2b}$), since their detection could escape from HPLC-FLD due to the lack of appropriate purification protocol. Digestates were extracted from samples (consisting of digestate and silage) collected before AD (T_0) and after 15 days incubation (T_f) according to the procedure described in section 2.5 slightly modified. Specifically, to identify fumonisins modified forms derived from degradation activity, digestate extracts were submitted to a coarse purification by using C18 column Sep-pak C18 Vac RC cartridges (500-mg) to remove most matrix interferences minimizing the loss of target compounds. Before loading, column was conditioned with 5 mL of methanol and 5 mL of ACN/ H_2O (1:9, v/v), then 10 mL of digestate diluted with PBS and passed through glass microfiber paper were added to the column and left pass thoroughly. After washing with 5 mL of ACN/ H_2O (1:9, v/v), mycotoxins were eluted with 20 mL of ACN/H_2O (1:1, v/v). Obtained eluate was dried at 50 °C under gentle stream of air and then dissolved in 1 mL of MeOH/ H_2O 80:20 (v/v) containing 0.5% acetic acid. Samples were then analysed by LC-HRMS. Each digestate sample (at T_0 and T_f) was extracted in duplicate and injected twice into LC-HRMS instrument.

Analysis were accomplished on a Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer coupled to a Ultra-High-Performance Liquid Chromatography (UHPLC) pump system (Thermo Fisher Scientific, San José, CA, USA). Mycotoxins were separated on a Gemini C18 (150 × 2.0 mm, 5-μm particles, Phenomenex) analytical column, preceded by a Gemini C18 guard column (4 × 2.0 mm, 5-μm particles, Phenomenex) according to the LC conditions described by De Girolamo et al. (2014). Volume of injection was set to 20 μl.

MS spectra were acquired in the mass range of 80–1000 *m*/*z* by running the instrument in FullMS/All Ion fragmentation (FullMS/AIF) acquisition mode and only positive ions were taken into account. Full MS analysis were accomplished by setting resolution at 140,000 full width at half maximum (FWHM), automatic gain control (AGC) at 3e6 and maximum injection time (IT) at 250 ms. The parameters for MS2 fragmentation experiments were set as following: resolving power 70,000 FWHM, microscan of 1, AGC target $2e^5$, maximum IT 60 ms and normalized collision energy (NCE) at 20, 30 and 40 eV by activating the stepped option. The HESI source settings are here reported: sheath and auxiliary gas flow rates 30 and 25 arbitrary units, respectively; spray voltage 3.6 kV; probe heater temperature 280 °C, capillary temperature 250 °C, and S-lens RF level 50. The level of PHFBs, detected in digestate samples withdrawn at the beginning (T_0) and at the end of the process (T_f) and extracted twice, was quantified by interpolating each PHFBs peak area recorded in the different samples with the respective calibration curves obtained with standard solutions of hydrolyzed fumonisin B_2 (HFB_2), partially hydrolyzed fumonisins ($PHFB_1$ and $PHFB_2$) purified according to De Girolamo et al. (2014). The values recorded between the two time points (T_0/T_f) were statistically treated by means of ANOVA test to highlight any statistical significant difference (p-value < 0.05).

2.7. Carboxylesterase reads assembly and gene analysis

Reads assigned to carboxylesterase function in EggNog functional map were extracted from contaminated sample datasets and assembled with MEGAHIT (Li et al., 2015), using the default parameters. Contigs shorter than 500 bp were discarded. The obtained contigs were aligned against nr-database by using BLASTx algoritm. Only hits with a percentage identity and query coverage higher than 90% were considered for further analyses. The nucleotide sequence of best hit was aligned with the respective contig sequence using ClustalW algoritm. Reverse transcription PCR (RT-PCR) was used to verify the presence of carboxylesterase transcripts related to the obtained contigs and specific primers were designed based on best blast hit sequence. Primer were designed to amply the full-length gene from cDNA obtained from

contaminated. PCR were carried out using the Platinum SuperFi II PCR Master Mix (Invitrogen, San Diego, CA). The primer pairs listed in Table S1 were used under the following conditions: 98 °C for 30 sec, followed by 35 cycles of 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 1 min, and then a final extension step at 72 °C for 5 min. PCR amplicons were checked by 1.5% (w/v) agarose gel electrophoresis and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA). The deduced amino acid sequence was determined using the EMBOSS Sixpack tool (https://www.ebi.ac.uk/Tools/st/emboss_sixpack/). Protein sequences were aligned by using muscle algorithm and the functional domain was detected by SMART (http://smart.embl-heidelberg.de/) and Prosite (https://prosite.expasy.org/) analysis.

2.8. Statistical analysis

Statistical analysis was applied to metagenomic data. Rank-abundance data, alpha-diversity indexes and beta-diversity were estimated using Microbiome Analyst (Dhariwal et al., 2017). Moreover, principal coordinate analysis (PCoA) was also calculated using the Bray-Curtis index to compute dissimilarities among different samples. Hypothesis testing was done by Analysis of Molecular Variance (AMOVA) test (p-value < 0.05). Statistical comparison of the relative abundance of taxonomic categories was performed using STAMP software (Parks and Beiko 2010). The Analysis of Variance (ANOVA) was applied as statistical test among all samples combined with the Tukey-Kramer method as post-hoc test. If the p-value for the 95% confidence interval was below 0.05, differences were considered significant. Statistical analysis between groups was performed using Welch's *t*-test in STAMP software (White et al., 2009). The Benjamini–Hochberg False Discovery Rate (FDR) method (q-value) was used to correct the multiple comparisons (Benjamini and Hochberg, 1995). The statistical cutoff of the p-value < 0.05 (Welch's two-sided test) and q-value < 0.05 (FDR) were set as the significance threshold.

3. Results and discussion

3.1. Microbial community analysis

After sequencing and quality filtering a total of 5,652,605 reads were generated. The sequence datasets are available at NCBI SRA under BioProject ID PRJNA635885 (www.ncbi.nlm.nih.gov/sra). Considering the diversity indexes, the alpha diversity index Chao1 and abundance-based coverage estimators (ACE) indicated that richness of C2 samples was higher than the C1 samples (Fig. 1a). The reduction of OTUs abundances is also related to a reduced microbial diversity (Shannon and Simpson) indexes in not contaminated samples. Principal Coordinates Analysis (PCoA) showed that the microbial communities in all samples were distinct to each other (Fig. 1b). The results were also validated with the analysis of molecular variance (AMOVA).

At the phylum level, the most dominant bacterial phyla in all samples were assigned to *Firmicutes*, followed by *Bacteroidetes* (Fig. 2a). It has been reported that *Firmicutes* and *Bacteroidetes* are both responsible for acetogenesis and hydrolysis in the AD process (Sun et al., 2015). Remarkably, the overall proportion of *Firmicutes* and *Bacteroidetes* did not change significantly. Reads assigned to *Firmucutes* accounted in 51.8% and 50.4% in C1 and C2 samples, respectively. For *Bacteroidetes* 31.8% and 31.5% for C1 and C2 samples, respectively. The third most abundant phylum was *Euryachaeota* represented by 10.2% and 12.9%, followed by *Synergistetes* were 2.2% and 2.1% , and reads assigned to *Proteobacteria* were 1.4% and 1.0% for C1 and C2 samples, respectively. The relative abundance of *Euryachaeota* was significantly higher in C2 than C1samples ($p < 0.05$).

At genus level, considering the *Firmicutes* phylum (Fig. 2b), the most abundant genus was *Peptococcaceae,* 49.6% and 47.4%, followed by *Syntrophaceticus* 9.1% and 6.9%, *Sedimentibacter* 3.9% and 3.1%, *Streptococcus* 0.9% and 8.6%,*Clostridium* 2.9% and 2.4%, *Hungateiclostridium* 3.1% and 2.9%, *Tepidanaero bacter* 2.4% and 2.1%, *Thermoclostridium* 2.2% and 1.9%, *Bacillus* 1.3% and 1.2%, *Paenibacillus* 1.3% and 1.2%, *Herbinix* 1.6% and 1.1% in C1 and C2 samples, respectively. Difference in mean proportions for *Peptococcaceae, Syntrophaceticus, Streptococcus* and *Sedimentibacter* genera were statistically different ($p < 0.05$).

Peptococcaceae are chemoorganotrophic bacteria that ferment proteins or carbohydrates, with lower fatty acids as the main fermentation products (Zhu et al., 2019). *Sedimentibacter* are implicated in anaerobic digestion of cellulose (Li et al., 2014), while *Syntrophaceticus* are mesophilic, anaerobic bacteria capable of oxidizing acetate to CO_2 and H_2 in intimate syntrophic relationship with methanogenic partner. However, due to strict cultivation requirements and difficulties in reconstituting the thermodynamically unfavorable acetate oxidation, the physiology of this functional group is poorly understood (Manzoor et al., 2016).

Moreover, in artificially contaminated samples a considerable increase of *Streptococcus* was observed. *Streptococcus* and other *Lactobacilli* are the predominant taxa in the ensilaging process, and since bioreactors were fed with maize silage, their occurrence is not surprising. Infact, microorganisms belonging to this genus are involved in the hydrolytic process by releasing hydrolytic enzymes (i.e., cellulase, amylase, lipase, protease, and xylanase) to break down the complex compounds into soluble organic substances (sugar, fatty acids, amino acids) that can be used by bacteria to perform fermentation (Liew et al., 2020). We could speculate that they could have contributed to pH variation observed in sample C2, since they are able to ferment carbohydrates with exclusive formation of lactic acid. However, their significant increase in artificially contaminated sample is difficult to rationalize and therefore further investigation are required. Within *Bacteroidetes* phylum, at genus level (Fig. 2c), the most abundant genus was *Dysgonamonadaceae* 31.0% and 38.2%, followed by *Petrimonas* 13.4% and 14.4%, *Bacteroides* 15.6% and 11.2%, unclassified *Bacteroidetes* 8.5% and 7.4%, unclassified *Bacteroidia* 3.4% and 3.6%, unclassified *Porphyromonadaceae* 3.6% and 3.2%, *Fermentimonas* 2.7% and 2.5%, *Proteiniphilum* 2.2% and 2.4%, *Prevotella* 4.0% and 2.1%, *Geofilum* 1.7 and 2.1%, *Anaerophaga* 1.1% and 1.2%, *Tangfeifania* 0.9% and 1.1% in C1 and C2 samples, respectively. Difference in mean proportions for *Dysgonamonadaceae, Fermentimonas, Proteiniphilum, Geofilum* and *Anaerophaga* were statistically different ($p < 0.05$). Within *Bacteroidetes*, a marked increase of *Dysgonamonadaceae* in artificially contaminated samples was observed. This taxa would have a role in the degradation of cellulose but its contribute in biomass degradation is not yet well understood (Tian et al., 2019).

The third main phylum that was altered by a high contamination rate was the *Euryachaeota* that significantly increased in artificially contaminated samples. This phylum is mainly represented by methanogenic *Archaea*. Methanogens are classified into three broad categories based on the compounds they use for methanogenesis: hydrogenotrophic, methylotrophic and acetoclastic (Ziganshin et al., 2016). The analysis of methanogenic population in digestate samples revealed that *Methanoculleus* were predominant in both thesis and represented by 97.2% and 93.9% of assigned reads in C1 and C2 samples, respectively (Fig. 2d). Moreover, a significant increase ($p < 0.05$) of reads assigned to *Methanomassiliicoccus* was observed in the presence of higher mycotoxin contamination (2.9%) respect to naturally contaminated samples

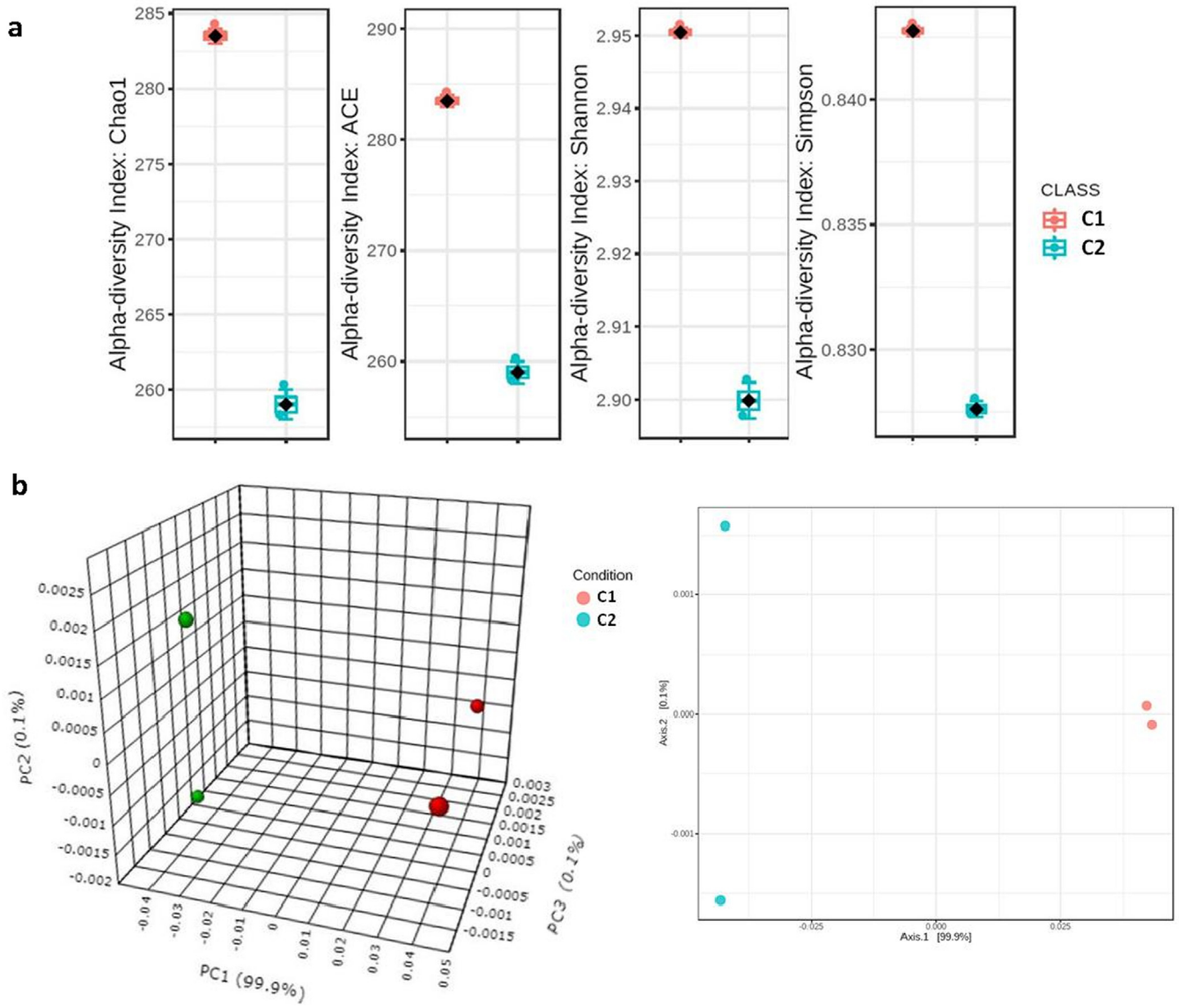

Fig. 1. Diversity indexes of microbial community samples (a) and Principal Coordinates Analysis (PCoA) of microbial consortia (b) in naturally contaminated (C1) and artificially contaminated (C2). PCoA was calculated using the Bray-Curtis index to compute dissimilarities among different samples. Hypothesis testing was done by Analysis of Molecular Variance (AMOVA) test (p-value < 0.05). Mean observed OTUs were 282 and 258 for C1 and C2 samples, respectively.

(0.4%). This genus, which belong to the novel order *Methanomassiliicoccales*, has the capacity to use methylamine substrates for methanogenesis by H_2-dependent methylotrophic pathway (Lang et al., 2015; Moissl-Eichinger et al., 2018). However, its contribution to biogas and methane production seemed to be not significant, if compared to the naturally contaminated samples.

3.2. Biogas production

Operational conditions for C1 and C2 bioreactors are reported in Table 2. Total biogas production was about 172 ± 4.24 and 178 ± 2.12 Nm^3/ton of silage, respectively in C1 and C2 bioreactors. Methane production was not severely hampered by mycotoxins contamination. Methane fraction in the biogas after 15 days of AD was 56% for C1 and 55% for C2 bioreactor. Based on these evidences, the high content of mycotoxins did not influenced the overall production of biogas, and in particular of methane production, if compared to the naturally contaminated samples. This evidence suggests a safe use of contaminated feedstock for biogas production, although studies conducted on the fate of mycotoxins in contaminated feedstoks for biogas production have shown that toxins are degraded at different rate during the anaerobic fermentation process (Salati et al., 2014; De Gelder et al., 2018; Giorni et al., 2018; Tacconi et al., 2018).

3.3. Mycotoxins analysis

Results of fumonisins and AFB_1 analysis in different bioreactors fed with contaminated silage, prior and after 15 days of anaerobic digestion, are reported in Table 1. Since in naturally contaminated samples (C1) AFB_1 was not detected, an artificial contamination was performed in samples C2 with a standard solution. The samples of unprocessed digestion investigated were representative of either naturally contaminated silage or silage inoculated with a fungal extract of fumonisins producing *Fusarium* strains and AFB_1 standard. With respect to the AFB_1 after anaerobic digestion pro-

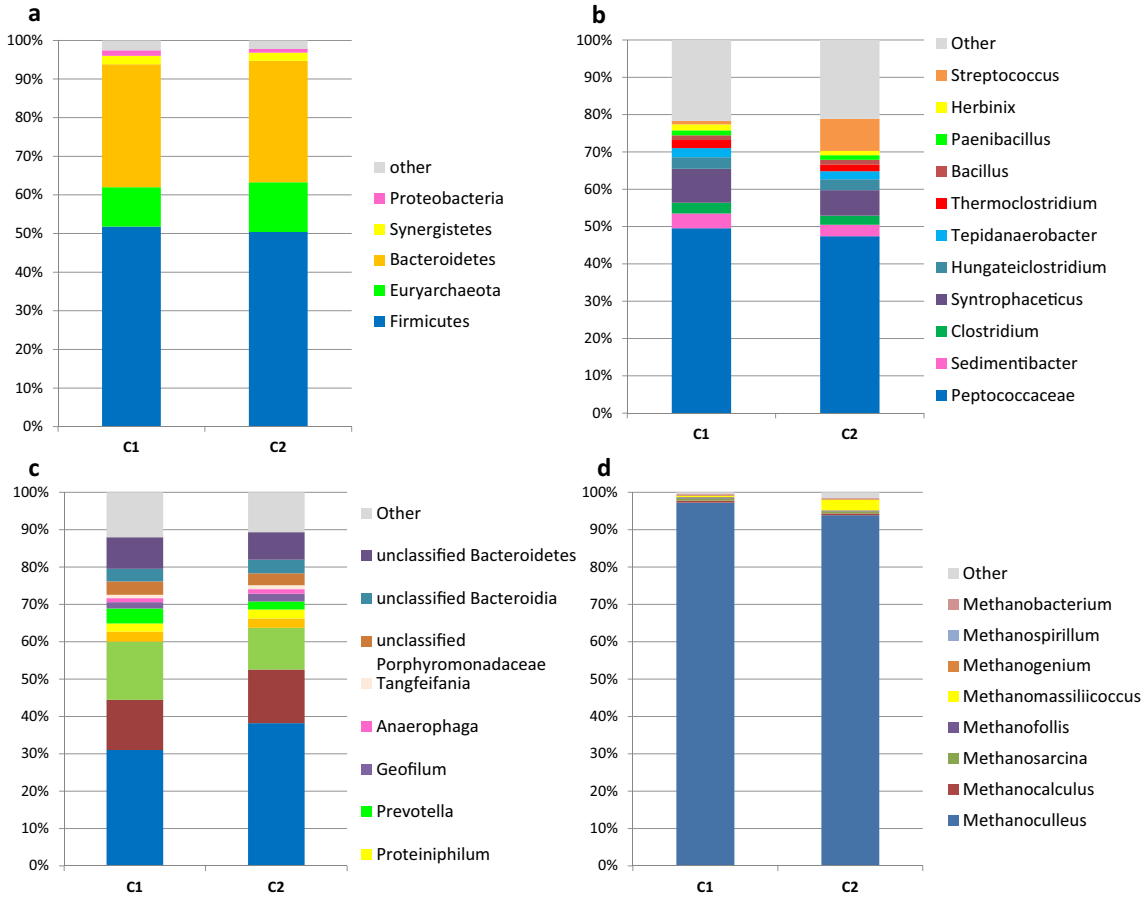

Fig. 2. Relative abundance and taxonomic assignments of microbial flora at phylum (a), *Firmicutes* (b) and *Bacterioidetes* (c) and methanogenic bacteria (d) level in naturally contaminated (C1) and artificially contaminated (C2) samples. Minor taxa having < 1% relative abundance are represented as "Other".

Table 1
Levels of mycotoxins in digestate samples at initial time (T_0) and after 15 days (T_f) of anaerobic digestion in naturally contaminated (C1) and artificially contaminated (C2) samples. Superscript letters [a] and [b] indicate technical replicates.

Sample	FB_1 (μg kg^{-1})	FB_2 (μg kg^{-1})	FB_3 (μg kg^{-1})	AFB_1 (μg kg^{-1})
$C1T_0^a$	278 ± 12	102 ± 14	41 ± 25	n.d.
$C1T_0^b$	205 ± 23	71 ± 7	44 ± 31	n.d.
$C2T_0^a$	13784 ± 1602	3877 ± 610	3591 ± 91	250 ± 7
$C2T_0^b$	11518 ± 1249	3014 ± 550	3628 ± 775	252 ± 17
$C1T_f^a$	228 ± 7	62 ± 3	20 ± 0	n.d.
$C1T_f^b$	156 ± 28	61 ± 7	25 ± 8	n.d.
$C2T_f^a$	5310 ± 104	2038 ± 57	2387 ± 9	116 ± 23
$C2T_f^b$	4780 ± 126	1864 ± 61	2225 ± 47	112 ± 7

Table 2
Operational conditions after 15 days of anaerobic digestion in naturally contaminated (C1) and artificially contaminated (C2) bioreactors. EC = electrical conductivity; TS = Total solid; VS = Volatile Solid.

Bioreactor	pH	EC (mS cm^{-1})	%TS	%VS	VS/ST
C1	8.17 ± 0.02	13.46 ± 0.06	8.56 ± 0.23	83.62 ± 0.09	9.52 ± 0.01
C2	7.90 ± 0.07	13.48 ± 0.75	8.28 ± 0.82	84.08 ± 1.12	10.17 ± 1.12

cess, levels of AFB_1 dropped to 54.6% with coefficients of variation (CV) of 9%.

A similar result was observed by Giorni et al. (2018) that found under mesophilic conditions a reduction of AFB_1 between 12% and 95% at different AFB_1 contamination rates (2, 70 and 470 μg kg^{-1}). Additionally, Salati et al. (2014) found a degradation of AFB_1 for 69–87% after 60 days in batch tests at 37 °C and a degradation up to 90% was observed also under thermophilic conditions (De Gelder et al., 2018). Although in general the contamination with AFB_1 of feedstocks used for AD has no significant effect on the

biogas quantity and quality (De Gelder et al., 2018; Salati et al., 2014), a daily addition of AFB_1-contaminated feedstock (100 μg kg^{-1}) to a semi continuous AD reactor affected biomethane yield, reducing the efficiency of the process (Tacconi et al., 2018). Considering the high level of artificial contamination, our experimental evidence in batch-test trials did not show this negative effect. This difference could be related to the addition of the toxins only at the beginning of AD, instead of a daily supplementation. The different rate of degradation could be related to several factors, among which the inoculum source and the incubation time are in our opinion the most important. We could speculate that the use of digestate as source of inoculum belonging to an industrial biogas plant daily fed with AFB_1 contaminated material, would potentially enhance the degradation process and the overall degradation rate. As well as the incubation time could influence the degradation efficiency since biological degradation of mycotoxins is not a crucial biochemical process for microbial survival and longer time of exposure could result in a higher degradation rate. Indeed, considering our results and the data available from the current literature (Salati et al., 2014; De Gelder et al., 2018; Giorni et al., 2018; Tacconi et al., 2018), we could hypothesize that even in our experimental conditions a high rate of degradation of AFB_1 could be achieved by increasing the incubation time. Focusing on FBs, our results showed a different rate of degradation between naturally and artificially samples after 15 days of anaerobic digestion at 43 °C. Specifically, degradation values in naturally contaminated samples were of 20, 29 and 6% with CV of 8, 10 and 14% for FB_1, FB_2 and FB_3 respectively. On the contrary, a much higher degradation rate was observed in artificially contaminated samples, with values up to 60% for FB_1, 43% for FB_2 and 36% for FB_3 with CV of 6, 10 and 6%, respectively. This different behavior could be likely ascribed to the different nature of the samples, namely artificially or naturally contaminated, where mycotoxin-matrix interactions should occur at different extent. It is worthy to be mentioned that in fortified samples mycotoxins are externally added and left to interact with the food matrix for a variable time period before its processing. This in addition to the high level of mycotoxin spiked into the matrix, might account for a different behavior displayed in terms of interactions with the matrix, consequently leading to mycotoxin entrapment or binding with matrix components much weaker compared to what observed in naturally contaminated samples as already described in the literature (Dall'Asta et al., 2009; Berthiller et al., 2013; De Angelis et al., 2014). Indeed, FBs in fortified samples should occur in a more free-matrix state resulting much more exposed and susceptible to the degradation mechanisms exerted by microrganisms or by the chemical environment of AD, thus leading to a final FBs content dramatically reduced with respect to the naturally contaminated sample.

In solid fraction of digestate, the percentage of fumonisins retained was from 20 to 67% for FB_1, from 17 to 19% for FB_2 and 18% for FB_3 in naturally contaminated samples. Conversely, in artificially contaminated samples the percentage of fumonisins in the solid fraction of the digestate was about 18% for each fumonisins amount. The variation of FB_1 retention in solid fraction may be due to a saturation of solid matrix due to the high level of mycotoxin in the digestate. Our evidence is in line with previously reported results reporting a 70% and 85% degradation of FB1 for mesophilic and thermophilic digestion, respectively (De Gelder et al., 2018).

3.4. Fate of mycotoxins upon biogas production by LC-HRMS/MS analysis

Dedicated experiments accomplished by LC-HRMS/MS were carried out to assess the extent of production of FB_1 and FB_2 hydrolyzed or modified forms upon 15-days of AD. As reported in Table S2, among mycotoxin derived products, only partially hydrolyzed forms were found in the MS traces of digestates and, they were mainly detected in the protonated form $[M + H]^+$ with experimental masses of 564.3744 m/z for $PHFB_1$ and 548.3795 m/z for $PHFB_2$. In the case of $PHFB_2$, both structural isomers $PHFB_{2a}$ and $PHFB_{2b}$ were detected. Compound identification was confirmed by comparing the fragmentation patterns of native toxins with those of relevant hydrolyzed derivatives. Typical extracted ion chromatograms referred digestate sample and filtered on the m/z value of the FB_1 and FB_2 partially hydrolyzed forms are reported in Fig. S1. As appearing from the Fig. 3, high levels of both $PHFB_1$ and $PHFB_2$ were found at T_0 both in naturally (Fig. 3a) and artificially contaminated (Fig. 3b) samples whereas a markedly different trend was observed after fermentation depending on the mycotoxin considered and on the type of contamination whether natural or artificial. Specifically, in naturally contaminated samples, a general increase up to 31% and 48% were observed for $PHFB_1$ and $PHFB_2$ levels, respectively, after 15 days of AD. These results directly correlate with the parallel degradation found for native FB_1 and FB_2 as ascertained by HPLC analysis. Interestingly, despite what observed in naturally contaminated samples, PHFBs levels were found to decrease after AD incubation of spiked sample and a reduction of approximately 24% and 27% were calculated respectively for $PHFB_1$ and $PHFB_2$. This different trend could be related to the limited time of incubation. The naturally contaminated samples belong to an industrial biogas plant daily fed with agricultural waste and the degradation activity could have already started in the industrial bioreactor. Additionally we could not exclude that the partially hydrolyzed forms of FBs were further metabolized as precursors of other microbial pathways.

3.5. Carboxylesterase gene analysis

The degradation of fumonisins has always been of great interest. Indeed, some efforts to find organisms or enzymes, able to metabolize fumonisins and thus, providing a biotechnological solution to the problem have been undertaken. Duvick et al. (1998) reported the first microbes capable of metabolizing FB_1. These organisms were isolated by enrichment from different maize tissues and identified as *Exophiala spinifera*, *Rhinocladiella atrovirens* and a bacterium, assumed to belong to the genus *Sphingomonas* or *Xanthomonas*. Benedetti et al. (2006) screened microorganisms from soil and isolated a bacterium, supposed to be related to the *Delftia*/*Comamonas* group, NCB 1492, which was shown to be able to hydrolyze and deaminate FB_1, but no sequences information of the responsible genes have been made available so far. In 2010 Heinl and co-authors demonstrated the degradation of fumonisin B_1 by the consecutive action of two bacterial enzymes from *Sphingomonas* sp. MTA144 (Heinl et al., 2010).

The above-mentioned evidences of biological degradation of FB_1 by microorganism have the same limitation factor represented by the use of culture dependent methodologies to screen and isolate microorganism able to metabolize and degrade mycotoxins.

Metagenomics has emerged as an alternative approach to conventional microbial screening that allows exhaustive screening of microbial genomes in their natural environments. In this study for the first time, we use a function-based screening of a microbial community involved in biogas production under anaerobic condition in order to find enzyme potentially involved in mycotoxin degradation. The known fumonisin biodegradation pathways in black yeast strains (Blackwell et al., 1999) and bacteria (Duvick et al., 2003; Heinl et al., 2010) is linked to the hydrolytic release of the tricarballylic acid (TCA) moieties, catalyzed by a carboxylesterase, resulting in partially hydrolyzed or hydrolyzed FBs

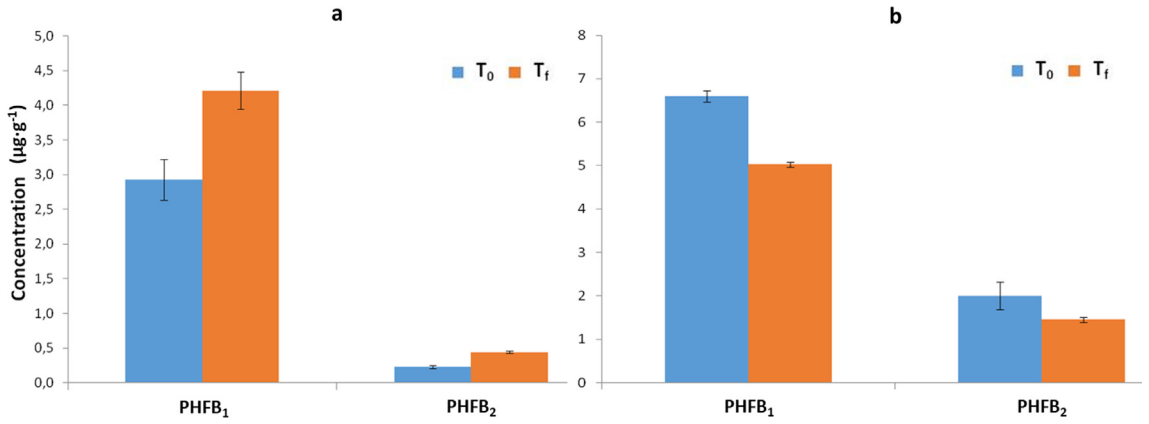

Fig. 3. Fate of $PHFB_1$ and $PHFB_2$ during biogas production process. Mycotoxin levels) were recorded by HPLC-HRMS in naturally contaminated samples (a) and in artificially contaminated (b) samples collected at beginning (T_0) and after 15 days of AD (T_f). $PHFB_2$ level was calculated as sum of $PHFB_{2a}$ and $PHFB_{2b}$ isomers. Two independent replicates were analyzed for each sample.

derivates. In this regard, we focused our attention on reads mapped on carboxylesterase protein sequences. The overall counts of reads matching with a carboxylesterase activity were not equally distributed between the dataset derived from the two bioreactors and appeared to be more abundant in artificially contaminated samples. Functional analysis of sequenced reads resulted in 1,197,521 reads assigned in EggNog functional map. The total amount of reads assigned to carboxylesterase function in the two replicates of C1 and C2 samples were 265 and 698, respectively.

The assembly of reads assigned to carboxylesterase gene extracted from C2 sample datasets resulted in 6 contigs with a length ranging from 617 to 939 bp and only 3 contigs with a length ranging from 624 to 733 bp for C1 samples. BLASTx analysis of contigs from C2 samples revealed a high similarity of contig_6 (939 bp) with a carboxylesterase protein (WP_101487746.1) from *Dysgonamonadaceae bacterium* (Fig. S2) and of contig_2 (668 bp) with a carboxylesterase protein (KLU39904.1) from *Peptococcaceae bacterium* (Fig. S3). The analysis of contigs from C1 samples confirmed a high similarity of contig_2 (650 bp) with the same carboxylesterase protein (KLU39904.1) of *Peptococcaceae bacterium* (data not shown). None of the other contigs from C1 samples matched with *D. bacterium* carboxylesterase protein sequence WP_101487746.1.

As shown by taxonomic analysis of microbial communities, the *Peptococcaceae* and *Dysgonamonadaceae* were the most abundant genera revealed within *Firmicutes* and *Bacteroidetes* phylum, respectively. RT-PCR analysis revealed the presence of carboxylesterase gene transcript for both target strains. In details, the carboxylesterase gene of *D. bacterium and P. bacterium* was successfully amplified from cDNA obtained from C2 samples (Fig. 4).

The obtained amplicons were sequenced and the relative nucleotide sequence were deposited at Genbank database with the accession number MT547562 and MT547563 for CE1 and CE2, respectively. Full-length sequence analysis confirmed the expected amplicon size of 1653 bp and 1539 bp for *D. bacterium* CE1 and *P. bacterium* CE2, respectively. The deduced amino acid sequences resulted in 2 protein sequence of 550 aa and 512 aa for CE1 and CE2, respectively. SMART analysis of protein sequences confirmed the presence of a COesterase domain in both sequence, and according to Prosite domain scan A the presence of a Carboxylesterases type-B serine active site was detected (Fig. 5).

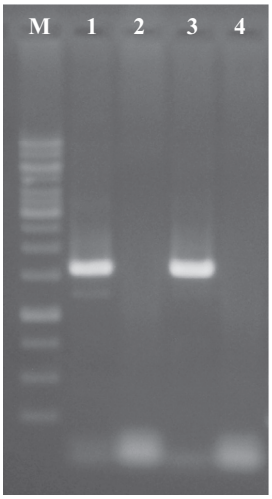

Fig. 4. RT-PCR of carboxylesterase gene of *Peptococcaceae bacterium* CE2 (1) and *Dysgonamonadaceae bacterium* CE1 (3) from cDNA obtained from artificially contaminated samples. Marker 1 kb (M); no template control (2,4).

This evidence is in agreement with the analysis reported by Heinl et al. (2010) and indicated a possible involvement of these two new carboxylesterase genes in the degradation of fumonisins. This evidence is supported by HRMS analysis. After anaerobic digestion the presence of $PHFB_1$, $PHFB_{2a}$ and its isomer $PHFB_{2b}$, was detected in both thesis. Although the degradation of mycotoxins during AD has been already demonstrated, this is the first time that the presence of metabolites resulting from the hydrolysis of fumonisins has been confirmed, suggesting the presence of such enzymatic degradation during the anaerobic digestion of fumonisins contaminated feedstocks. Our experiment did not show the presence of completely hydrolyzed form of FBs after 15 day of incubation in both thesis. Compared to other studies related to FBs degradation during AD, most of paper reported a degradation of FBs after at least 30 days of incubation. Indeed, considering the shorter time of incubation and the very high level of contamination rate achieved in thesis C2, an extension of incubation period up to 30 days or longer could have led to a complete hydrolysis of FBs.

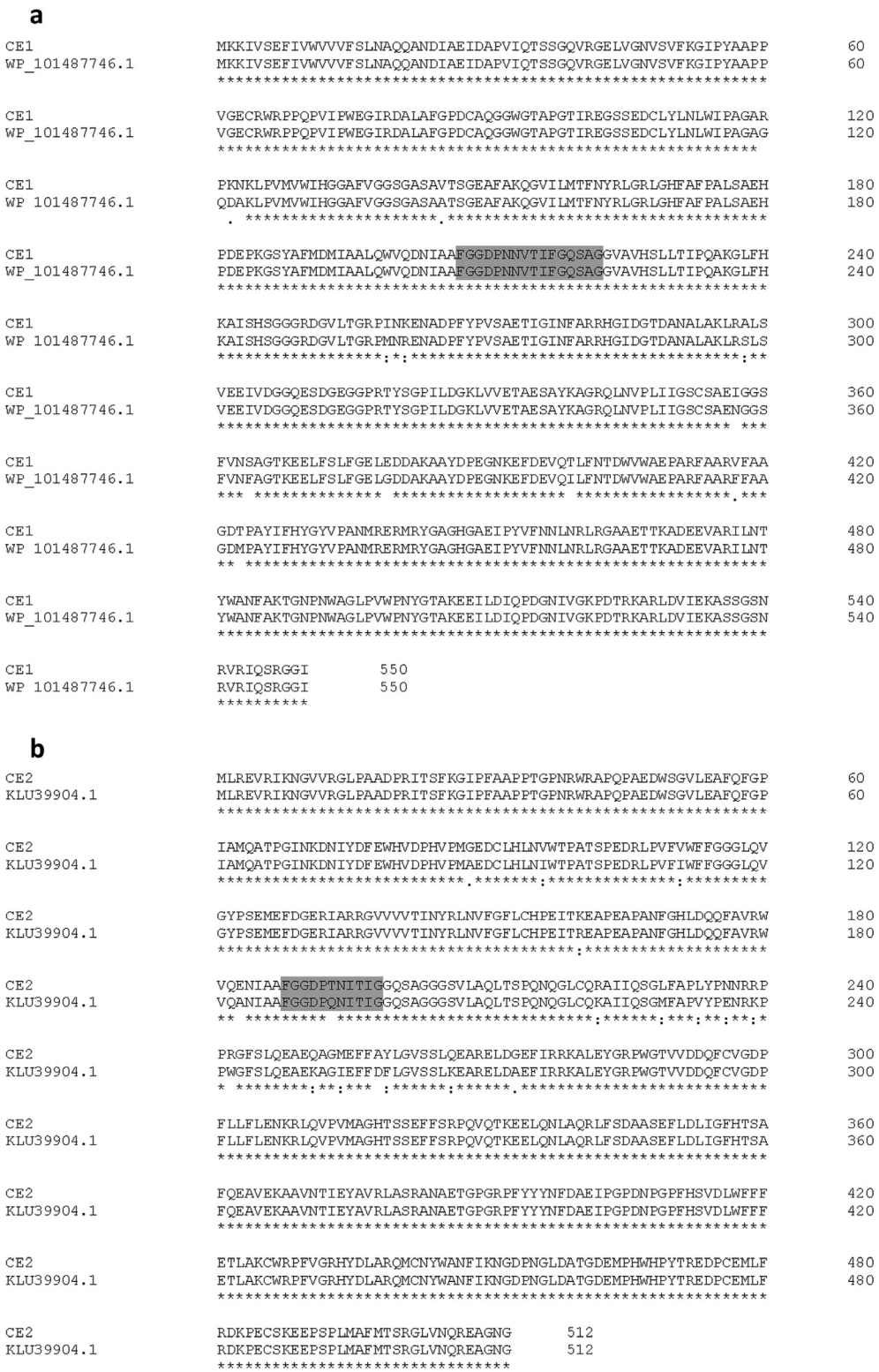

Fig. 5. Pairwise alignment of full-length amino acid sequence of carboxylesterase CE1 (a) and CE2 (b) with the respective carboxylesterase reference protein of *Dysgonamonadaceae bacterium* (WP_101487746.1) and *Peptococcaceae bacterium* (KLU39904.1). The Carboxylesterases type-B serine active site is highlighted in a grey box.

4. Conclusion

This study point out for the first time the effect of mycotoxin contaminated feedstock on microbial population during anaerobic digestion and biogas production. The reduction of the mycotoxin concentration in the digestate represents a remarkable advantage if compared to other processes such as the bioethanol production both of them involved in the energy exploitation. In the latter case, the mycotoxin concentration does not decrease but instead it generally results concentrated in the by-products obtained, thus facing

again the problem of a contaminated matrix to be handle. The possibility to reduce the initial mycotoxin concentration in the substrate through the AD allows to evaluate further uses of the by-products in a sustainable way. Moreover, we demonstrated that a function-driven approach based on metagenomic analysis represents a powerful tool to investigate and discover potential new mycotoxin degrading enzymes. Specifically for fumonisins our experimental evidences revealed a biological degradation exerted by microorganisms involved in degradation of organic matter during anaerobic digestion. The two identified carboxylesterase enzyme belonging to *D. bacterium* and *P. bacterium* need to be further investigated in order to better understand their specific activity and their possible other application for remediation approaches.

Author contributions

M.F. designed, set up and performed the experiments, analyzed the data, and wrote the paper; M.H. conducted HPLC analyses, analyzed the data and wrote the paper; E.D. and L.M. conducted HRMS analyses, analyzed the data and wrote the paper; M.D. and A.P. performed chemical analysis and analyzed the data; A.F.L. and G.M. designed experiments and wrote the paper.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

The authors acknowledge Biogas Servizi S.r.l. for providing digestate, Dr. Annalisa De Girolamo for providing standards for LC-HRMS/MS analysis and Mr Francesco De Marzo and Mr Domenico Genchi of the Institute of Sciences of Food Production (CNR) for the skilled technical support provided during the realization of this work. This work was supported by H2020-EU3.2-678781-MycoKey-"Integrated and Innovative Key Actions for Mycotoxin Management in the Food and Feed Chain".

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2020.09.048.

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