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Short communication

Pelleting is a successful method to eliminate the presence of *Clostridium* spp. from the digestate of biogas plants

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ABSTRACT

Biogas production is increasing as a sustainable energy supply, with digestate resulting as a by-product of biogas plants. As a result, the high concentration of *Clostridium* spp. in digestate became a concern in dairy farming areas. *Clostridium* spores can contaminate soils and crops when digestate is used as fertilizer, causing a conflictual cohabitation of biogas with traditional cheese productions. In order to solve the problem, this study aimed to search for a technical solution enabling either a drastic reduction or the elimination of the content of *Clostridium* spp. within digestate. Results showed a complete elimination of *Clostridium* spp. in pelleted stored solid digestate; in addition, pelleting caused a reduction of pH and water mass fraction in terms of fresh weight, and a concentration of mineral nutrients compared to stored solid digestate. Pellet can represent a possible sustainable solution both in reducing potential risks linked to the presence of *Clostridium* spp. in digestate and in improving the transportation and distribution of high-value fertilizer. Hence, pelleting of solid digestate could offer a simple and efficient method to allow cohesistence of biogas plants and dairy farming.

1. Introduction

Digestate is either a solid or a liquid by-product of biogas plants. The physical state of digestate mainly depends on biogas production technologies implemented. Nowadays farmers use digestate either as a fertilizer [1] or as a soil amendment in current crop management [2,3]. Noteworthy, farmers cultivating forage crops for the production of aged hard cheeses such as Parmigiano Reggiano have become seriously concerned about the use of digestate in the field, due to its high concentration of *Clostridium* spp. [4,5]. High content of *Clostridium* spp. in the ratio of cows could pose serious issues to cheese manufacturers as some species, such as *Clostridium tyrobutyricum*, *Clostridium butyricum* and *Clostridium sporogenes* are the main cause of alteration during the aging phase of cheese [6]. A diffused distribution of digestate in fields might potentially spread these ubiquitous spore-forming bacteria from one farm to another, causing soil and crop contamination. Such potential economic risk related to digestate obtained from biogas plants originates from substrates that were treated in biogas plants [4]. Some studies reported that pathogenic bacteria such as *Salmonellae*, *Clostridiae* and *Listeria* might survive after anaerobic digestion [7,8], as well as viable bacteria can grow after the application of digestate to croplands [8,9]. Therefore, further treatment of the digestate was advised to obtain a more efficient reduction of pathogens [10]. High temperature treatments can reduce the amount of various bacteria within digestate, but anaerobic biogas plants work within a temperature range (35–50 °C) that does not allow the sanitation of biomass and final digestate. In fact, spores of *Clostridium* spp. were not inactivated at 35 °C or 53 °C [11]. Moreover, in order to reduce the risk of insurgence of undesired bacteria, waste material should be treated before its use as fertilizer or amendment. On the other hand, digestate contains a relatively high proportion of mineral nutrients, which grants digestate so remarkable fertilizing value that it could replace inorganic fertilizers [12]. Indeed, digestate can be used as a useful source for crop nutrition, since nutrients from

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ingestates used in digesters remain in digestate after the digestion process [13–15]. Hence, the main objective of this study was to find a technical solution to either eliminate, or at least reduce, the content of *Clostridium* spp. in the solid digestate, so that it can be safely used in dairy-farming, hard cheese-producing areas as fertilizer or amendment.

2. Material and methods

2.1. Anaerobic digestion and sampling

The present study was performed using samples of raw materials routinely used as input in the digester (ingestate) and digestate collected from the CAT (Cooperativa Agroenergetica Territoriale) biogas plant, located in Correggio, Reggio Emilia, Italy (44°45′N; 10°48′E, altitude 28 m a.s.l.). Ingestates used in digestion were maize (*Zea mais* L.) silage (43%), triticale (X Triticosecale Wittmack) silage (22%), cow slurry (27%), and grape stalks (of *Vitis vinifera* L.) (8%). Ingestate proportions were calculated according to their fresh weight. Each ingestate was stored in concrete slabs under a plastic tarpaulin. At sampling, maize silage had eight months of storage, triticale silage ten, cow slurry three and grape stalks six. Ingestates were collected right before their introduction in the digestion process. For each ingestate, 20 samples were collected from the pile at the height of 1 m above ground, the diameter of cross-section was 20 cm, and the horizontal distance was at least 1 m between each sample. The 20 samples were mixed to extract three representative samples of 1.2 kg, which were placed in sterile plastic bags and maintained cold during transportation (5 °C) to the laboratory, where they arrived within 2 h.

After a complete biogas production cycle, CAT made available six different types of digestate for this study: fresh unseparated digestate, fresh liquid digestate, fresh solid digestate, stored liquid digestate, stored solid digestate and pelleted stored solid digestate. The fresh unseparated digestate (90% of water mass fraction) was separated into liquid and solid (94 and 82% of water mass fraction respectively) by means of a helical compressor separator; liquid digestate was then stored in a closed, underground concrete tank, while solid digestate was stored in a concrete slab in open air until a water mass fraction of about 54% was reached. Pellets were produced by a PP300 Kompakt pellet press that converted stored solid digestate, dried at 15% of water mass fraction using the thermal heat produced by cogeneration of biogas plant, into pellets of 8 mm in diameter and 10 mm in length with final water mass fraction of 8%. Pellet temperature was measured immediately after production, using the thermal resistance sensor Pt100 (Type GE-PT100-DINB/SC, Geass, Turin, Italy); pellet samples were immediately put in sterile bags for further analyses.

Different forms of digestate were collected at the end of the digestion process in sterile plastic bottles; fresh samples were sampled soon after their production, before and after separation, while stored samples were collected after three months of storage.

For each material (digestates and pellets), three samples were collected following the same procedure used for the sampling of the ingestate. Two subsamples of 100 g derived from each 1.2 kg elementary sample of ingestate, digestate and pellet were used to detect total *Clostridium* spp.

2.2. Microbiological analyses

Microbiological analyses were performed on each ingestate used in digestion to characterize the total initial microbial contamination of *Clostridium* spp. Furthermore, the different forms of digestates were collected both at the end of the process (*i.e.* the “fresh” samples) and after storage (*i.e.* the “stored” samples) in order to be analyzed and evaluated for final total microbiological hazards compared to total *Clostridium* spp.

2.2.1. Sampling preparation

Microbiological sampling was performed by taking 10 g of product from the subsample in absolute sterility and placing it in 90 cm^3 of sterile physiological (saline) solution. Samples were homogenized with a Lab Blender Stomacher 400 (Type BA 7021Seward, London) and the resulting solution was subjected to a thermal shock for 10 min at 80 °C, as reported in published protocols [16–18]. This treatment was necessary to induce spores germination, thus eliminating all other vegetative forms. Subsequently, samples were used to perform serial dilutions.

2.2.2. Bacterial growth conditions

One (1) cm^3 of the last dilution was inoculated in triplicate sterile plates previously marked with the indication of the dilution. Each plate contained the reinforced clostridial agar (RCA) (Oxoid S.p.a., Milan, Italy), consisting of tryptose 10 g, meat extract 10 g, yeast extract 10 g, dextrose 5 g, sodium chloride 5 g, soluble starch 1 g, cysteine hydrochloride 0.5 g, sodium acetate 3 g, agar 15 g (total volume filled up to 1000 cm^3 with distilled water) to enumerate *Clostridium* spp. After medium solidification, a plug of agar at a concentration of 20 g L^{-1} was placed around the plates to avoid direct air contact with anaerobic cultures and to highlight the metabolic gas production; furthermore, plates were placed in anaerobic jars in order to prevent direct oxygen contact with cells. Plates were incubated in anaerobic conditions, under 90% of N_2 and 10% CO_2 as volume fraction of these gases, in a thermostated chamber at 30 °C for 24/48 h as reported by Phillips and co-workers [19]. The detection of *Clostridium* spp. was only performed on samples where spores had germinated.

2.2.3. Catalase test

Many cells synthesize different antioxidant enzymes; one of the most important is catalase, which converts H_2O_2 to H_2O and gaseous O_2. Since Clostridia do not possess the enzyme catalase, this test was considered a good discriminating factor during isolation of studied strains, as reported by Jay and co-workers [20].

2.2.4. PCR identification of Clostridium spp.

DNA extraction and PCR were performed according to Klijn et al. [6], in order to confirm the identification of some of the isolated strains. PCR for specific amplification of part of the 16S rRNA gene (nucleotides 41 to 1114) of 1070 bp was performed. Briefly, PCR analysis was carried out in a final volume of 50 mm^3 containing 1.57 kg m^{-3} Tris–HCl (pH 8.8), 2.92 kg m^{-3} NaCl, 0.29 kg m^{-3} $MgCl_2$, 1.21 kg m^{-3} deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Ampli-Taq; Perkin-Elmer, Waltham, MA, USA), and 15 ng of primers P1 (5′-GCGGCGTGCCTAATACATGC-3′) and P2 (5′-GGGTTGCGCTCGTTGCGGGA-3′). After being heated to 95 °C to eliminate all protease activity, 5 cm^3 of template DNA were added. Amplification was performed in 30 cycles of melting DNA at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and elongation at 72 °C for 2.5 min. Fragments amplified by PCR were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide; a 100 bp DNA ladder (New England Biolabs, Hitchin, United Kingdom) was used as size marker.

2.3. Chemical analyses

Stored solid and pelleted digestate were chemically and physically characterized: pH was measured with a pH meter (type Basic 20, Crison, Barcelona, Spain) using 3.0 g of homogenized fertilizer added with 50 cm^3 of distilled water and shacked for 30 min at