Survival of *Mycobacterium avium* subsp. *paratuberculosis* in the intermediate and final digestion products of biogas plants

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Abstract

Aims: To evaluate the survival of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) during anaerobic digestion (AD), we studied two different biogas plants loaded with manure and slurry from *paratuberculosis*-infected dairy herds.

Methods and Results: Both plants were operating under mesophilic conditions, the first with a single digester and the second with a double digester. *Mycobacterium avium* subsp. *paratuberculosis* detection was performed by sampling each stage of the process, specifically the prefermenter, fermenter, liquid digestate and solid digestate stages, for 11 months. In both plants, MAP was isolated from the prefermenter stage. Only the final products, the solid and liquid digestates, of the one-stage plant showed viable MAP, while no viable MAP was detected in the digestates of the two-stage plant.

Conclusions: *Mycobacterium avium* subsp. *paratuberculosis* showed a significant decrease during subsequent steps of the AD process, particularly in the two-stage plant. We suggest that the second digester maintained the digestate under anaerobic conditions for a longer period of time, thus reducing MAP survival and MAP load under the culture detection limit.

Significance and Impact of the Study: Our data are unable to exclude the presence of MAP in the final products of the biogas plants, particularly those products from the single digester; therefore, the use of digestates as fertilizers is a real concern related to the possible environmental contamination with MAP.

Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the aetiological agent of a chronic proliferative enteritis in ruminants known as *paratuberculosis* (PTB) or Johne’s disease.

In recent decades, PTB has been frequently linked with Crohn’s disease in humans due to certain clinical similarities between these two enteric pathologies (Hermon-Taylor and Bull 2002); however, the role of MAP in Crohn’s disease has not been definitively demonstrated (Chioldini et al. 2012; Sechi and Dow 2015). Furthermore, MAP has also been linked with other human diseases, such as type I diabetes, Hashimoto’s thyroiditis, multiple sclerosis and others (Scanu et al. 2007; Sisto et al. 2010; Dow 2012; Frau et al. 2013; Waddell et al. 2015).

Paratuberculosis is distributed worldwide in ruminants, and the prevalence of infected farms in countries with advanced animal husbandry is growing rapidly. In this regard, numerous investigations have reported its wide distribution in Europe (Nielsen and Toft 2009) and Italy (Pozzato et al. 2011), with herd prevalence estimates over 50% (Nielsen and Toft 2009).

Animals are most frequently infected at a young age through the ingestion of faeces or contaminated feed, fodder, milk and colostrum (Sweeney 2011). The major
Symptoms of the disease, which are visible only in adults over 2–3 years of age, are diarrhoea and progressive wasting, leading to death or animal culling.

Infected animals, especially clinical cases, may shed large amounts of MAP through the faeces (over 10^6 MAP cells per gram) (Eamens et al. 2007). MAP survives for prolonged periods in the environment (Whittington et al. 2004, 2005): 163 days in river waters, 270 days in stagnant water and 11 months in bovine faeces and soil (Salgado et al. 2011, 2013; Moravkova et al. 2012). Under experimental conditions, MAP has been shown to survive in cattle slurry for 28 days at 30°C, 98 days at 15°C and up to 252 days at 5°C (Jørgensen 1977; Lombard et al. 2006; Pillars et al. 2009).

For this reason, there is great concern about the use of fresh manure from PTB-infected farms as fertilizer, since this may heavily contribute to the spread of MAP in the environment (Salgado et al. 2011, 2013; Moravkova et al. 2012), leading to maintenance of the source of infection for the animals, particularly through the consumption of contaminated fodder.

Moreover, the potential risk to human health due to exposure to a contaminated environment cannot be excluded. In a geographical survey of MAP distribution carried out in the United Kingdom, MAP was found in 10.5% of soil cores, both in farming and nonfarming areas (Rhodes et al. 2013). MAP also spreads to surface waters (lakes and rivers) through runoff from contaminated pastures (Pickup et al. 2005, 2006). In this regard, a study carried out in the United Kingdom (Pickup et al. 2006) showed that approximately 70% of water samples from a river abstracted for the domestic water supply were MAP-positive by IS900 PCR. Moreover, in the United States, MAP DNA was detected in approximately 18% of drinking water (56 of 304 samples), suggesting possible human exposure to MAP (Beumer et al. 2010).

Among the possible technologies that reduce the environmental impact of animal farming, biogas production plants are gaining increasing interest. In these plants, animal manure and other organic biomasses are used for biogas production and electrical cogeneration. After treatment, the products of anaerobic digestion (AD) are commonly used as fertilizers and animal bedding.

This process is essentially based on the action of a mesophilic microbial flora growing at temperatures between 20–45°C, which transforms the organic matter in biogas (primarily methane and carbon dioxide), thus reducing the emission of ammonia and nonmethane volatile organic and odorous compounds. Despite these advantages, the technology still raises health-related concerns due to the potential ability of some pathogens to resist the AD process and remain vital in the digestate, particularly micro-organisms such as MAP that are highly resistant to extreme environmental conditions.

The literature reports that the AD process is potentially able to reduce bacterial load, but the data related to this observation are still fragmentary (Sahlin 2003; Holm-Nielsen et al. 2009; Slana et al. 2011).

Moreover, most studies concerning pathogen contamination in biogas plants have examined indicator bacteria (such as Enterobacteriaceae, Enterococcus faecalis, Clostridium sp., etc.) or parasite eggs (Plymforshell 1995; Gantzer et al. 2001; Watcharasukarn et al. 2009). The only reports currently available in the literature regarding MAP survival during AD treatment are an experimental in vitro study carried out in laboratory batch reactors under mesophilic (30–35°C) and thermophilic (53–55°C) conditions (Olsen et al. 1985) and a field study carried out in the Czech Republic (Slana et al. 2011) with a farm-scale biogas plant equipped with a double-digester system and supplied with manure from a PTB-affected herd.

To gain additional knowledge of MAP survival in biogas products, the aim of this work was to evaluate MAP survival in two different types of biogas plants: a farm-scale plant based on a single digester and a large-scale plant with double-digester technology.

Materials and methods

Two distinct biogas plants were considered in this study: a single farm-scale plant (#1) located in northern Italy and a large-scale biogas plant (#2) serving a consortium of 29 bovine herds in central Italy.

Plant #1

The farm consisted of 240 cattle, including 120 dairy cows in which PTB had been repeatedly diagnosed. The annual incidence of clinical cases of PTB recorded throughout the last 3 years was 2–3%, which was confirmed by laboratory diagnostic tests (PCR from faeces and ELISA from blood serum).

To more accurately estimate the prevalence of PTB within the herd, serum ELISA tests were carried out twice: at the beginning and at the end of the study on all cattle older than 36 months of age. The ELISA tests showed apparent serological prevalences ranging from 6-0% (beginning of the study) to 14-3% (end of the study).

Moreover, faecal cultures were performed on the same animals once during the central study period, revealing that 11-6% of animals were positive.

During the study, no animals were purchased, and all infected animals, if they were asymptomatic, were
maintained in the herd. Only three cows that showed clinical symptoms were culled. The biogas plant was established in July 2009 and schematically included a pre-tank (PT), a single-stage digester (F), a trench for the storage of the solid digestate (SD) and a tank for the liquid digestate (LD) (Fig. 1).

In the PT, approximately 25 m$^3$ of raw material, which was composed of manure (80–85%), wash water and waste milk (8–10%), rainwater and/or recycled LD (8–10%), especially during particularly dry periods, were loaded daily.

From the PT, approximately 18 m$^3$ of material (90%) and 2 m$^3$ of other biomass (10%), consisting of waste corn or ryegrass silage, were loaded daily into the F (1300 m$^3$ of total volume). This material remained in the F, at 38–40°C, under continuous horizontal agitation. An equivalent amount of digestate was discharged from the bottom of the mass, pumped to a separator and transformed into the SD and LD. The SD was stored in a trench and periodically used as fertilizer. The LD was collected in a tank that was emptied approximately every 2–3 months for crop fodder irrigation. The schematic of the plant, duration of the steps and temperatures are shown in Fig. 1.

**Sampling scheme**

A total of 114 samples were collected during the study (11 months), according to the following scheme:

i  **PT**: 48 samples (one to two samples per week) collected directly from the PT (150 ml each);

ii  **F**: 48 samples (one to two samples per week) collected from an outlet valve placed 1.5 m from the tank bottom (150 ml each). Before every sampling, the valve was emptied and refilled to obtain fresh material.

iii  **SD**: 11 composite samples (one sample per month), each consisting of a pool of seven subsamples (50 g each) that were picked from different points of the mass;

iv  **LD**: seven composite samples (one every 2 months) for SD, which were collected from seven distinct points 50 cm below the surface of the tank.

**Plant #2**

The second part of the study was carried out in a large-scale biogas plant serving a consortium of farms in central Italy. The 29 cattle herds (for a total of 2250 animals) that supplied manure and slurry were located in a geographical area where bovine PTB was widely present, as demonstrated by previous investigations (Lillini et al. 2005; Papa et al. 2014). In particular, seven of these farms were regularly monitored for PTB and repeatedly showed positive results based on serum ELISA (ID Screen® Paratuberculosis Indirect—ID Vet Innovative Diagnostics, Grabels, France) and faecal cultures.

Based on the above considerations, we suspected that manure and slurry were likely to be contaminated by MAP. As such, we decided to directly sample PT material to assess MAP occurrence.

The plant, established in 2012, included a PT, a primary fermenter (F1), a postfermenter (F2), trenches for storage of the SD and a lagoon for the storage of the LD. Manure and slurry from the farms were stored in the PT (approximately 600 m$^3$ of volume).

Approximately 100 m$^3$ of total raw material (approximately 90 tons of material from PT and 23 tons of biomass, consisting of waste silage and ryegrass silage) were loaded daily into the F1 (5000-m$^3$ volume), where the mass remained for 45–50 days for bioactivation. Afterwards, in F2, the material completed its AD cycle in 20–30 days. In F1 and F2, the mass was maintained at a temperature of approximately 40°C under continuous stirring.

At the end of this process, there was a separation into the SD and LD; the SD (15% of the mass) was stored in the trenches, while the LD (85% of the mass) was maintained in a lagoon (20 000 m$^3$ total volume). The

![Diagram of biogas plant #1](image)

**Figure 1** Diagram of biogas plant #1: PT: pretank, F: digester, SD: solid digestate, LD: liquid digestate. Time of permanence and temperature in different sectors.
digestate was maintained for approximately 6 months before being delivered to farmers to be used as fertilizer.

The schematic of the plant, duration of the steps and temperatures are shown in Fig. 2.

**Sampling scheme**

A total of 248 samples were collected during the 11 months of the study, according to the following scheme:

i. **PT**: 36 samples (one to two samples per week in the first 2 months and then one sample on alternate weeks until the 9th month) were collected from the connection pipe between PT and F1 (400 ml each);

ii. **F1**: 70 samples (one to two samples per week from months 1 to 9) were collected from a connection pipe between F1 and F2 (400 ml each);

iii. **F2**: 70 samples (one to two samples per week from months 2 to 10) were collected from a connection pipe between F2 and the tank where the digestate was separated into the LD and SD (400 ml each).

Before every sampling, the connection pipes were emptied and refilled to obtain fresh material, which was maintained under continuous agitation.

iv. **SD**: 36 composite samples were collected in the last 2 months of the study (before the delivery of SD), each consisting of a pool of seven subsamples (50 g each) that were gathered from different points of the mass;

v. **LD**: 36 composite samples were collected in the same period for the LD, each consisting of a pool of seven subsamples (50 g each), gathered at seven distinct points, from the small tanks (approximately 40 m³ of capacity) used to load the LD from the lagoon for transport to the farms.

**Culture assay**

All samples were transferred to the laboratory after being collected and immediately processed or stored at −80°C.

Cultures were performed according to the procedure described by Taddei *et al.* (2004). Briefly, 10 g of sample were suspended in 100 ml of hexadecylpyridinium chloride 0.75% and mixed by horizontal shaking for 30 min at room temperature (RT). The samples were allowed to settle for 5 min, 35 ml of supernatant was transferred to a new 50-ml V bottom-shaped tube and allowed to settle overnight at RT again. After 18–24 h, 0.2 ml of sediment was inoculated into each selective solid medium. Each sample was inoculated in four agar slants, two tubes of Herrold’s egg yolk medium (HEYM) with mycobactin J (Mj) (2 mg l⁻¹), nalidixic acid (50 mg l⁻¹) and vancomycin (50 mg l⁻¹) and two tubes of HEYM with Mj (2 mg l⁻¹) and chloramphenicol (30 mg l⁻¹). Tubes were incubated at 37°C in a horizontal position for 5 days with loose caps to allow evaporation of residual moisture. Tubes were returned to an upright position, caps were tightened and incubation of samples continued for a total of 16 weeks. Tubes were examined weekly. Typical colonies were confirmed based on Ziehl–Neelsen staining for acid resistance, mycobactin dependence and molecular confirmation (IS900-PCR) (Taddei *et al*. 2008). For each positive sample, the total number of MAP colonies observed from the four tubes was recorded. In case of severe contamination, if the number of unreadable tubes was more than two of the four inoculated tubes, the sample was excluded from the data processing.

The analytical sensitivity of the method was determined by spiking duplicate negative faecal samples (from PTB-free herds, negative based on repeated faecal cultures and ELISA tests on all adult animals) with a suspension of MAP ATCC 19698 to obtain contamination levels ranging from 10⁶ to 10⁸ CFU per gram of faeces. The strain used for spiking was cultivated in Middlebrook 7H9 broth supplemented with OADC, Tween 80 (0.05%), sodium pyruvate (0.4%) and Mj (2 mg l⁻¹) for 1 month at 37°C under continuous agitation. The suspensions were serially diluted (1 : 10) in a physiological sterile solution and declumped by forcing the solution through a 26 G needle syringe several times. The amount of MAP present in each dilution was estimated by streaking 0.1 ml of suspension

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**Figure 2.** Diagram of biogas plant #2: PT: pretank; F1: primary fermenter; F2: secondary fermenter; SD: solid digestate; LD: liquid digestate; DI: digestate (SD and LD). Time of permanence and temperature in different sectors.

| Sector | Time     | Temperature |
|--------|----------|-------------|
| PT     | 1–2 days | Ambient     |
| F1     | 45–50 days | ~ 42°C |
| F2     | 20–30 days | ~ 40°C |
| DI     | Up to 6 months | Ambient |

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on Petri plates containing HEYM with mycobactin, with three replicates. The analytical sensitivity was estimated to be approximately $10^3$ CFU per gram of faeces, a value similar to that reported for the double-incubation method (Stabel 1997; Fecteau et al. 2013), which is considered the OIE reference method for the recovery of MAP from faeces (Anon. 2015).

**Statistical analysis**
The results of the study were evaluated with a univariate analysis (OR and confidence intervals, CI 95%). A $P$-value ≤0.05 calculated using the chi-square test or Fisher’s test was considered significant. For each sector, the mean and median numbers of colonies were calculated.

**Results**

**Plant #1**

*Mycobacterium avium* subsp. *paratuberculosis* was detected in 36 of 48 samples from PT and 26 of 48 samples in F. MAP was also detected in the SD (1/11 samples) and the LD (three of six samples). The timing of the sampling, the positive samples and the number of colonies detected for each sector of the plant are reported in Table 1. The rate of contaminated tubes in samples from PT and F was 11 and 26.5% respectively. In the SD, 38.6% of culture tubes were contaminated while in the LD 35.7% was contaminated. The contamination rate of tubes is referred only to partial contaminated samples with at least two readable tubes. If samples were totally contaminated they were not included in data processing.

**Statistical analysis**

A limited but statistically significant reduction in MAP load was observed during progressive steps of digestion. The PT sector was more likely to contain MAP than the F and SD sectors (OR = 2.54, CI95% 1.07–6.03, $P = 0.035$, and OR = 30.00, CI95% 3.47–259.35, $P = 0.002$ respectively). Moreover, the difference between SD and F was statistically significant (OR = 11.82; CI95% = 1.40–99.71, $P = 0.023$) because MAP is more likely to be present in the fermenter during the process than in the SD. Finally, no significant differences between the LD and the other sectors were recorded, although a reduction in colony numbers was observed.

**Plant #2**

Overall, MAP was detected in 24 samples out of 36 in PT, 14 of 70 samples in F1 and only 1 in 70 samples in F2. All samples from the SD and LD were negative. The timing of the sampling, the positive samples and the number of colonies detected for each sector of the plant are reported in Table 2. The rate of contaminated tubes in samples from PT, F1 and F2 was 22.2, 12.1 and 14.3% respectively. The degree of contamination was 30.5% in the SD and 18% in the LD. Often tubes contaminated were almost all readable, but in case of severe contamination, with more than two tubes contaminated and unreadable, the sample was excluded from data processing.

**Statistical analysis**

Additionally, in plant #2, the amount of MAP recovered decreased according to the progression of the digestion steps. The differences between PT and F1 and between PT and F2 were statistically significant (OR = 8, CI95% 3.23–19.81, $P = 0.000$, and OR = 138.00, CI 95% 17.03–1118.22, $P = 0.000$ respectively). The difference between F1 and F2 was statistically significant (OR = 17.25; CI95% 2.20–135.23; $P = 0.007$). MAP was never recovered from the SD and LD sectors.

**Discussion**

Biogas production is an emerging technology based on a mesophilic AD process, in which manure, slurry and

| Month | Sector | PT | F | SD | LD |
|-------|--------|----|---|----|----|
| 1st   |       | 3/4| 1/4| 0/1| 0/1|
| 2nd   |       | 7/8| 3/8| 1/1| 1/1|
| 3rd   |       | 7/7| 5/7| 0/1| 1/1|
| 4th   |       | 2/2| 1/2| 0/1| 0/1|
| 5th   |       | 3/5| 2/5| 0/1| 0/1|
| 6th   |       | 1/2| 2/2| 0/1| 1/1|
| 7th   |       | 1/1| 1/1| 0/1| 1/1|
| 8th   |       | 3/3| 3/3| 0/1| 1/1|
| 9th   |       | 6/6| 3/6| 0/1| 1/1|
| 10th  |       | 2/7| 4/7| 0/1| 1/1|
| 11th  |       | 1/3| 1/3| 0/1| 0/1|

| CFU per sample | Ranging from 1 to 10 | Ranging from 1 to 4 | Ranging from 1 to 3 |
|----------------|----------------------|---------------------|---------------------|
| Mean CFU       | 8.4                  | 1.5                 | –                   |
| Standard deviation | 12.3               | 2.1                 | –                   |
| Median CFU     | 3                    | 1                   | –                   |

### Table 1 Results of sampling in plant #1. Range CFU (colony forming unit), mean, standard deviation and median number of colonies. PT: pretank, F: digester, SD: solid digestate, LD: liquid digestate.
other farming products are used as raw materials and the final digestate product is used as fertilizer and animal bedding.

The effects of the process on the survival of animal pathogens have been only partially investigated, and consequently there are rising concerns regarding the possibility of environmental contamination. This risk may be particularly high for micro-organisms that are highly resistant to inactivating environmental agents, such as MAP. As reported in the literature, PTB-infected animals shed MAP in their faeces, leading to the contamination of pastures, soil, surface waters and rivers (Pickup et al. 2005, 2006; Beumer et al. 2010; Glenn et al. 2013; Salgado et al. 2015). The use of manure from PTB-infected farms in biogas plants may pose a serious risk in spreading the microbe in the environment, particularly if the digestion processes are unable to inactivate MAP. The efficiency of MAP inactivation appears to be strongly influenced by the technologies adopted in the plant, as suggested by our study.

A previous in vitro study (Olsen et al. 1985) that was carried out under mesophilic conditions (30–35°C) showed a MAP cell reduction of one log₁₀ in 5–6 days, and it was not possible to recover viable MAP after 21 days. Interestingly, at higher temperatures (53–55°C), the authors reported the complete depletion of MAP after 24 h.

In a second study (Slana et al. 2011), a farm-scale plant for biogas production based on double-digester technology and continuous loading was examined using culture and qPCR. Manure used in the plant was from a single herd with 15% culture-positive animals. Viable MAP cells were detected for up to 2 months in the intermediate products, while the presence of MAP DNA was demonstrated for only up to 16 months in the final product of the secondary fermenter. Since residual MAP DNA may belong to dead cells or come from viable mycobacteria that are not detectable by the culture method, the authors concluded that further investigations are required to establish if MAP survives the AD process.

Our data showed that MAP largely occurred in manure and slurry entering the plants.

For plant #1, the presence of MAP in fresh slurry was confirmed by the high number of positive samples from PT (36 of 48, 75%), and this contamination was regularly recorded throughout the study, likely due to the persistence of infected cattle on the farm. Only three infected animals were eliminated from breeding, while all other infected cows were maintained in the herd. On the other hand, no significant changes were observed in the percentage of positive samples in PT, despite the increase in the serological prevalence (from 6 to 14.3% at the end of the study). A possible explanation may be that not all newly serologically positive animals shed MAP in their faeces.

Additionally, in plant #2, MAP was frequently observed in PT samples (66%), confirming the high prevalence of disease in the farms of the consortium and, in general, in the geographical area (Lillini et al. 2005; Papa et al. 2014).

In plant #1, the percentage of positive samples decreased during various processing steps, declining from 75.0% (PT) to 54.2% (F), which was also confirmed by the reduction in the number of colonies detected (from 1–46 CFU per gram for PT to 1–10 CFU per gram for F1). However, the reduction in the number of CFU from PT to F was <1 log₁₀. According to a previous hypothesis (Olsen et al. 1985), the daily input of fresh manure in the single fermenter did not permit proper digestion because it resulted in a continuous and open process with the risk of recontamination of the digestate output, which was potentially highly similar to the incoming material.

Moreover, MAP was also recovered from the SD and LD (9.0 and 50.0% respectively).

In plant #2, a more evident decline in the percentage of positive samples throughout the process was recorded:

### Table 2 Results of sampling in plant #2. Range CFU (colony forming unit), mean, standard deviation and median number of colonies. PT: pretank, F1: primary fermenter; F2: secondary fermenter, SD: solid digestate; LD: liquid digestate

| Month | Sector | PT | F1 | F2 | SD | LD |
|-------|--------|----|----|----|----|----|
| 1st   | 7/11   | 3/11 | –  | –  | –  | –  |
| 2nd   | 5/7    | 2/9  | 0/6 | –  | –  | –  |
| 3rd   | 2/4    | 0/6  | 0/6 | –  | –  | –  |
| 4th   | 3/4    | 1/9  | 1/9 | –  | –  | –  |
| 5th   | 2/2    | 3/5  | 0/5 | –  | –  | –  |
| 6th   | 1/2    | 1/6  | 0/6 | –  | –  | –  |
| 7th   | 1/2    | 0/10 | 0/10| –  | –  | –  |
| 8th   | 2/2    | 0/8  | 0/8 | –  | –  | –  |
| 9th   | 1/2    | 4/6  | 0/6 | –  | –  | –  |
| 10th  | –      | –    | 0/14| 0/15| 0/10| 0/21|
| 11th  | –      | –    | –   | 0/21| 0/26| 0/26|
|       |        |      | 24/36 (66-6%) | 14/70 (20.0%) | 1/70 (1.4%) | 0/36 | 0/36 |
| CFU per sample | Ranging from | 1 to 16 | Ranging from | 1 to 30 | 1 | – | – |
| Mean CFU | 4.1 | 0.9 | – | – | – | – |
| Standard deviation | 4.8 | 3.9 | – | – | – | – |
| Median CFU | 2 | 0 | – | – | – | – |
20-0% in F1, 1-4% in F2 and no positive samples in the SD and LD, suggesting the higher efficiency of MAP killing than that of plant #1.

A possible explanation may be the presence of a second digester, which led to longer exposure to mesophilic anaerobic conditions. This potentially improved the effectiveness of the digestion process. The material remained in the F1 sector for approximately 45–50 days, in the F2 sector for another 20–30 days and under mesophilic conditions for a total of approximately 65–80 days, which is a total fermentation period higher than that in the first plant (approximately 60 days). Moreover, after being filled, the F2 sector remained closed for 20–30 days, avoiding possible contamination of the final product with fresh faeces.

Mesophilic temperatures are permissive for the growth of MAP, which has been cultivated under laboratory conditions at 42°C (Slana et al. 2011). On the other hand, other factors such as pH, the composition of volatile organic acids and competitive microbial flora influence MAP survival during the AD process (Grewal et al. 2006).

Although a decline in the number of positive samples, as well as in the number of MAP colonies, was clearly evident for both plants, false-negative results cannot be excluded when contamination levels were very low given the analytical sensitivity of the culture method used (approximately 10^7 CFU per gram of faeces/slurry). Therefore, according to our results and previously published studies (Olsen et al. 1985; Slana et al. 2011), the theoretical risk of the presence of viable MAP in the final products of the biogas plants cannot be excluded.

Finally, since the principal aim of the study was to evaluate MAP viability during different phases of the process, we used a culture assay rather than qPCR, which is faster but unable to differentiate between live and dead bacteria. Notably, a study by Slana et al. (2011) previously reported how MAP DNA remained detectable for up to 16 months despite an inability to recover any viable MAP after 2 months.

Under conditions mirroring those described in plant #1 (temperature, times and flows), the risk of spreading viable MAP through the digestate is consistent, while more advanced technologies (equipped with two or more fermenters) appear to be more effective for MAP killing.

Thus, higher digestion temperatures or additional steps, such as pasteurization, should be applied to the final products before separation (SD/LD) to reduce the risk of MAP spreading, particularly in single-digester plants.

Since thermophilic conditions, consisting of 53–55°C for at least 24 h, have been demonstrated to completely inactivate MAP within 24 h (Olsen et al. 1985), we believe these conditions should be adopted in continuously managed biogas plants.

A final important factor is the amount of MAP entering the biogas plant. High concentrations of MAP should be suspected in known PTB-affected herds. Slana et al. (2011) suggested the adoption of control plans for PTB in herds loading biogas plants. The adoption of control plans for PTB, such as prescribing the identification and removal of MAP faecal shedders from the herd (especially high shedders), will reduce the risk of introducing this pathogen into biogas plants. In cases involving consortia, biogas plant managers should require a health certification for each herd, limiting the risk of delivering contaminated manure to negative/low-risk herds.

The implementation of all suggested measures may lead to a decrease in environmental contamination, including that of surface water and soils (Pickup et al. 2005, 2006; Glenn et al. 2013; Salgado et al. 2015), thus reducing the potential risk of MAP exposure for humans.

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Conflict of Interest

None of the authors have conflicts of interest, financial or otherwise to declare.

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