

MICROBIOLOGICAL CONTAMINATION OF DIGESTED PRODUCTS FROM ANAEROBIC CO-DIGESTION OF BOVINE MANURE AND AGRICULTURAL BY-PRODUCTS

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TITLE PAGE

3 **MICROBIOLOGICAL CONTAMINATION OF DIGESTED PRODUCTS FROM**
4 **ANAEROBIC CO-DIGESTION OF BOVINE MANURE AND AGRICULTURAL**
5 **BY-PRODUCTS**

7 Si. Bonetta¹, E. Ferretti¹, Sa. Bonetta¹, G. Fezia², E. Carraro^{1*}

9 ¹ *Department of Environmental and Life Science, University of Piemonte Orientale “A.*
10 *Avogadro”, Via T. Michel 11, 15121, Alessandria, Italy;*

11 ² *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d’Aosta, Via delle*
12 *Industrie 3, 15121, Alessandria, Italy*

14 **Running headline:** Microbial contamination of digestate

16 * Corresponding author: Elisabetta Carraro, Dept. of Environmental Science and Life,
17 University of Piemonte Orientale “Amedeo Avogadro”, Via T. Michel 11, 15100
18 Alessandria, Italy; tel +39 131 360261; fax +39 131 360243; e-mail:
19 elisabetta.carraro@mfn.unipmn.it

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4 **1 ABSTRACT**
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6 **2 Aims:** This study was performed to investigate the microbiological contamination of
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3 digestate product (DP) obtained from the anaerobic co-digestion of bovine manure and
4 agricultural by-products.

5 **Methods and results:** Microbiological analyses were performed on bovine manure,
6 fresh DP, liquid and solid fractions and stored liquid fraction of DP. A statistically
7 significant reduction of faecal bacterial indicator was found after anaerobic digestion
8 except for Enterococci. After liquid/solid DP separation, bacteria tend to be
9 concentrated in the solid fraction. Storage does 'not seem to influence the indicator
10 parameters, except for Enterococci. *E.coli* O157:H7 and *Yersinia* were never found in
11 any samples analysed. *Salmonella* was rarely detected in DP samples and its derivatives,
12 while *L. monocytogenes* was encountered in many samples.

13 **Conclusions:** The results obtained indicate that the hygienic quality of DP is equal or
14 even better than that of the bovine manure and suggest the need to identify specific
15 pathogen indicators related to the hygienic characteristics of digestate products.

16 **Significance and impact of the study:** This study highlights that the anaerobic co-
17 digestion of bovine manure and agricultural by-products in a field-scale biogas plant
18 does not increase human health risk respect to the use of animal manure for agricultural
19 fertilization.

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23 **Keywords:** anaerobic digestion, faecal indicator bacteria, pathogenic bacteria, bovine
24 manure, fertilizer

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1. INTRODUCTION

The global energy demand is growing rapidly and about 88% of this demand is met at present by fossil fuels. In this context, it is essential to develop sustainable energy supply systems that aim to cover the energy demand with renewable sources (Amon et al., 2007a). Biogas production from a wide range of energy crops, animal manures and organic wastes is of growing importance as it offers considerable environmental benefits and an additional source of income for farmers. Renewable energy is produced, and after anaerobic digestion the products can be used as a valuable fertilizer for agricultural crops due to the increased availability of nitrogen and superior short-term fertilization effects (Amon et al., 2007b; Weiland, 2010). Reuse of the digested products could present health concerns that must be satisfied before land application becomes an accepted practice. Different studies have shown that livestock faeces can be significantly contaminated with pathogens (Albihn and Vinnerar, 2007). In this context, the microbial quality of manure should not be neglected since many outbreaks of gastroenteritis related to livestock have been reported (Massè et al., 2011). The bacterial pathogens most important with regard to human health include, for example, *Salmonella* spp., *Escherichia coli* O157:H7, *Campylobacter jejuni* and *Yersinia enterocolitica*. *Listeria monocytogenes* has also been reported as causative agent of human infections related to livestock (Bagge et al., 2005, Massè et al., 2011).

Some studies attested that sometimes pathogens can survive anaerobic digestion (Sidhu and Toze, 2009) and the growth of the survived bacteria after the application of DP to land has been demonstrated for some bacterial species (Estrada et al., 2004; Johansson et al., 2005). Pathogen inactivation rates are lower in mesophilic than in thermophilic anaerobic digestion plants (Watcharasukarn et al., 2009).

1 Health concerns related to DP reuse include pathogen transmission to vegetable food,
2 animals and/or agricultural workers and contamination of groundwater or surface water
3 with faecal material deriving from field run-off (Islam et al., 2005; Petersen et al.,
4 2007).

5 Considering the possibility of reusing DP and its derivatives as fertilizers and the related
6 health risk the aim of this study was the evaluation of the microbiological contamination
7 of the products obtained from mesophilic anaerobic co-digestion of bovine manure and
8 agricultural by-products.

10 **2. MATERIALS AND METHODS**

11 **2.1 Biogas plant and sampling**

12 The study was performed in an anaerobic digestion plant located in the Piedmont region
13 (Italy). The plant produces energy from renewable sources such as bovine manure and
14 agricultural byproducts. The configuration of the plant and the sampling points are
15 shown in Figure 1. The biogas plant consists of a mixing tank where the input substrates
16 are mixed, two digestion tanks (1 and 2), a liquid-solid DP separator and a storage tank.
17 Samples were collected over one year starting in September 2008 and ending in October
18 2009. Sampling was performed on input substrates (point A), output material after
19 anaerobic digestion (point B), liquid and solid fractions obtained by DP separation
20 (point C and D) and DP liquid fractions after 120 storing days (point E).

22 **2.2 Microbiological analyses**

23 *2.2.1 Faecal indicator parameters*

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1 Each sample (50 g) was homogenized in sterilized 0.9% NaCl solution using a
2 Stomacher Laboratory-Blender 400 (PBI International, Milan, Italy). Serial dilutions
3 were prepared and inoculated in triplicate on specific agar media to enumerate bacterial
4 indicators: mesophilic counts on Tryptic Soy Agar (TSA, Applichem) at 37°C for 24 h;
5 *Escherichia coli* on Tryptone bile X-glucoronide medium (TBX, Biolife) at 44°C for 24
6 h; Enterobacteriaceae on Violet Red Bile Glucose Agar (VRBG, Oxoid) at 37°C for 24
7 h; faecal enterococci on Kanamycin Aesculin Azide Agar Base (KAA, Biolife) at 37°C
8 for 24-48 h. Bacterial counts were expressed as log CFU g⁻¹ of wet matter.

9 The influence of the anaerobic digestion process and of DP storage on survival of the
10 microbial indicator parameters was evaluated using analysis of variance (ANOVA,
11 SYSTAT, version 8.0).

12 The presence of *Clostridium perfringens* was determined on Tryptose Sulphite
13 Cycloserine Agar (TSC, Biolife) after anaerobic incubation at 42°C for 24 h and was
14 confirmed with the reverse CAMP test. A qualitative analysis was performed for
15 helminth eggs detection based on sample purification by flotation and microscope
16 examination.

18 2.2.2 Pathogens

19 *Salmonella* analysis (25 g sample): after pre-enrichment in Buffered Peptone Water
20 (BPW, Oxoid) (24 h at 37°C), an aliquot (100 µL) was inoculated into Rappaport-
21 Vassiliadis broth (RV, 10mL, Biolife) (18-24 h at 42°C) and another aliquot (1000 µL)
22 was inoculated into Selenite Broth base (SB, 9 mL, Biolife) (24 h at 37°C). Both RV
23 and SB broths were streaked on Bismuth Sulphite Agar (BSA, Biolife) and Xylose
24 Lysine Desoxycholate Agar (XLD, Biolife) and incubated at 37°C for 24 h. Colonies

1 with typical *Salmonella* morphology were confirmed with the agglutination test
2 (Biolife) and biochemical tests using the Biolog Microbial Identification System
3 (BIOLOG, Inc.).

4 *Listeria monocytogenes* analysis (25 g sample): after pre-enrichment in Fraser Broth
5 Half concentration (Oxoid) (30°C for 24 h), an aliquot (100 µL) of the pre-enrichment
6 broth was inoculated into 10 mL of enrichment Fraser Base Broth (Oxoid) (24 h at
7 30°C). Aliquots of preenrichment and enrichment broths were streaked on *Listeria*
8 Palcam Agar Base (Biolife) (37°C for 24 h) and ALOA Agar (Biolife) (30°C for 48 h).
9 Colonies with typical *Listeria* morphology were confirmed as *Listeria monocytogenes*
10 by Real-Time PCR (iQ-Check *Listeria monocytogenes* Kit, BioRad).

11 *E. coli* O157:H7 analysis (25 g sample): after enrichment in Tryptic Soy Broth (Biolife)
12 supplemented with novobiocin (42°C for 24 h), samples were subcultured onto
13 MacConkey Sorbitol Agar (CT-SMAC, Biolife) plates by streaking (24 h at 37°C).
14 Suspected colonies were confirmed by multiplex PCR as reported by Bonetta et al.
15 (2010).

16 *Yersinia* spp. analysis (1-10g samples): after inoculation in both *Yersinia* PSB Broth
17 (Biolife) (25°C for 5 d) and *Yersinia* ITC Broth Base (Biolife) (25°C for 48 h), samples
18 were cultured onto CIN Agar (Biolife) (30°C for 48 h). Suspected colonies were
19 confirmed with biochemical tests of the Biolog Microbial Identification System
20 (BIOLOG, Inc.).

21 The results of pathogen contamination were expressed as presence/absence.

22 23 **3. RESULTS**

24 **3.1 Faecal indicator parameters**

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5 1 The results of the bacterial indicator counts in the bovine manure, DP and its derivatives
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7 2 are reported in Table 1. Comparison of the bacterial indicator levels of the input
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9 3 substrate (bovine manure) and DP revealed a statistically significant decrease of all the
10
11 4 parameter counts after anaerobic digestion (*E. coli* $p<0.05$, mesophilic count $p<0.001$)
12
13 5 and Enterobacteriaceae $p\leq 0.001$) with the exception of Enterococci.

16 6 The liquid/solid separation of fresh DP led to higher bacterial content in the solid
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18 7 fraction with respect to the liquid one (Table 1), with the exception of Enterococci,
19
20 8 which were equally distributed between the two fractions.

23 9 Storage of the DP liquid fraction for 120 days did not reduce the mesophilic counts, did
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25 10 not influence *E. coli* or Enterobacteriaceae counts (which were already very low in the
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27 11 DP liquid fraction), but it resulted in a significant reduction of the Enterococci counts
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29 12 ($p<0.05$).

33 13 The anaerobic digestion process does not seem to reduce the percentage of positive
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35 14 sample for *C.perfringens*: 78% of fresh DP was contaminated by *C. perfringens*;
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37 15 liquid/solid separation of fresh DP and storage of the DP liquid fraction did not reduce
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39 16 *C.perfringens* positive samples percentage. Helminth eggs were never found in bovine
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41 17 manure, DP samples and its derivatives.

18 19 **3.2 Pathogens**

20 The frequency with which bacterial pathogens were detected in all the samples is
21
22 21 reported in Table 2. Neither *E. coli* O157:H7 nor *Yersinia* spp. were ever found in
23
24 22 bovine manure or in DP.

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26 23 *Salmonella* spp. and *L. monocytogenes* were rarely detected in samples of bovine
27
28 24 manure (20%). DP resulted occasionally contaminated by *Salmonella* (8%), while the

1 presence of *L.monocytogenes* was encountered in 25% of DP samples. Liquid and solid
2 fractions of DP were rarely contaminated by *Salmonella*, but always presented
3 *L.monocytogenes* contamination. In the stored liquid fraction of DP *Salmonella* was
4 never detected and *L.monocytogenes* was found only in one sample (33%).
5 All *Salmonella* strains isolated were identified as *Salmonella choleraesuis*.

6 7 **4. DISCUSSION**

8 9 **4.1 Faecal indicator parameters**

10 In general, bacterial indicator counts in bovine manure and DP samples monitored in
11 this study are in agreement with those reported in other studies (Soupir et al., 2006;
12 Watcharasukarn et al., 2009). Respect to the other indicator parameters analyzed,
13 Enterococci showed similar counts before and after anaerobic co-digestion. This finding
14 could be due to the great variability of Enterococci counts, with values ranging between
15 < 2 (detection limit) and $5.3 \text{ Log}_{10} \text{ CFUg}^{-1}$, both in bovine manure and in fresh DP
16 samples. Otherwise it could depend on an effective variability of the microbial
17 reduction efficiency by the digestion process. This trend also may reflect the
18 unsuitability of Enterococci, that is considered by the European regulation on animal
19 by-products, a reference parameter for monitoring the digestion process efficiency
20 towards the reduction of microbial contamination.

21 Considering the purpose of reusing DP as fertilizer in agriculture it is important to
22 highlight that the microbiological quality of the DP analysed in this study always
23 complied with the microbial parameter thresholds of the Italian law for fertilizers (*E.*
24 *coli* $< 1000 \text{ CFU/g}$) (D.M. 29819/2009). However, the greater part (58%) of the fresh

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1 DP samples exceeded the standard for Enterococcaceae reported in the European
2 regulation on animal by-products (Commission Regulation EC n. 208/2006).

3 Considering the results obtained after liquid/solid separation, the presence of a greater
4 bacterial content in the solid fraction has been reported also in other studies (Vanotti et
5 al., 2005; Higgins et al., 2007), and this finding has been attributed to the following
6 hypotheses: i) sample matrix effects; ii) recontamination of samples; iii) re-growth of
7 viable but not culturable microorganisms (VBNC) stressed after anaerobic digestion.

8 Although there is some controversy in the literature regarding the VBNC state, most of
9 the evidence seems to support this phenomenon (Arana et al., 2007; Higgins et al.,
10 2007).

11 The DP liquid fraction after 120 days' storage complied with the standards of the EC
12 regulation for agricultural DP reuse for Enterococcaceae.

13 The presence of *C. perfringens* contamination in the DP and its derivatives observed in
14 this study was also reported in earlier studies. Bagge et collaborators (Bagge et al.,
15 2005) observed that if there are any pathogenic spore-forming-bacteria in the incoming
16 manure they persist in the digested residues. Therefore *C. perfringens* could pose a
17 hygienic problem when DP and its derivatives are spread on land.

18 19 **4.2 Pathogens**

20 Considering the results obtained in this study, the mesophilic anaerobic digestion causes
21 a reduction in the *Salmonella* content as reported in many works (Horan et al., 2004;
22 Sidhu and Toze, 2009), but the absence of *Salmonella* in 25 g of DP should be
23 demonstrated in representative samples of the digestion residues before using DP as
24 fertilizer (D.M. 29819/2009; Commission Regulation EC n. 208/2006). However, the

1 anaerobic digestion process seems to have less ability to reduce *Listeria monocytogenes*
2 contamination. This finding is in contrast with the results obtained by Horan et al.
3 (2004) in a study performed in a lab-scale digester. Probably, as recently noted by other
4 authors, microorganism dynamics during anaerobic digestion process are likely
5 different between lab-scale and field-scale digesters (Wagner et al., 2008).

6 The absence of *Salmonella* in 25 g of material is considered the standard for its use as
7 fertilizer as a guarantee of bacterial pathogen absence. However, the results obtained in
8 this study indicate that *Listeria monocytogenes* can be present without *Salmonella*
9 contamination; this situation suggests the need to reconsider the usefulness of
10 *Salmonella* as the sole indicator of bacterial pathogen presence. Moreover a long
11 storage time seems to have the greatest effect on pathogen reduction, as verified in other
12 studies (Cote et al., 2006). Considering that *Salmonella* is the parameter used to control
13 fertilizer safety, only the stored DP liquid fraction should be used as fertilizer for land
14 application, but considering that this fraction was contaminated (33%) by *Listeria*
15 *monocytogenes* consumer health risks cannot be excluded.

16

17 **5. CONCLUSIONS**

18 In conclusion, the results obtained in this study indicate that the hygienic quality of DP
19 is equal or even better than that of the input material (bovine manure). An analogous
20 conclusion has been reached by EFSA in an evaluation of the biological risk of the
21 mesophilic process of biogas and compost treatment of animal by-products (EFSA,
22 2007). Therefore, in comparison with the use of animal manure for agricultural
23 fertilization, the use of digestate produced by bovine manure and agricultural biomass

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1 co-digestion may not result in new routes of pathogens and disease transmission
2 between animals and humans via environmental matrices.

3 However, this conclusion should take into account that this study was performed in an
4 anaerobic digestion plant where the sources and quality of the input substrates were
5 constant, and the ratio among the input substrates was steadily maintained. Thus, under
6 these conditions, the anaerobic co-digestion of bovine manure and agricultural by-
7 products does not seem to increase human health risk. Moreover, the results obtained in
8 this survey suggest the need to reconsider the usefulness of *Salmonella* as a bacterial
9 pathogen indicator and to identify specific pathogen indicators related to the hygienic
10 characteristics of the digestion plant input materials.

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1 Table 1. Mean, minimum and maximum values (expressed as log₁₀ CFU g⁻¹) of bacterial
 2 indicator parameters in input and output materials of a biogas digestion plant

	Mesophilic count			<i>E.coli</i>			Enterobacteriaceae			Enterococci		
	mean	min	max	mean	min	max	mean	min	max	mean	min	max
Bovine manure	8,0	6,4	8,5	5,0	<2	5,7	5,3	<2	5,8	4,6	<2	5,0
Fresh DP	6,4	5,3	6,8	1,9	<2	3,0	2,5	<2	3,3	4,6	<2	5,3
Solid fraction	8,0	6,2	8,4	4,4	3,4	4,8	5,1	3,5	5,3	6,0	<2	6,4
Liquid fraction	6,3	5,7	6,6	<2	<2	<2	<2	<2	<2	5,6	4,5	6,1
Stored liquid fraction	6,4	6,1	6,5	<2	<2	<2	<2	<2	<2	<2	<2	<2

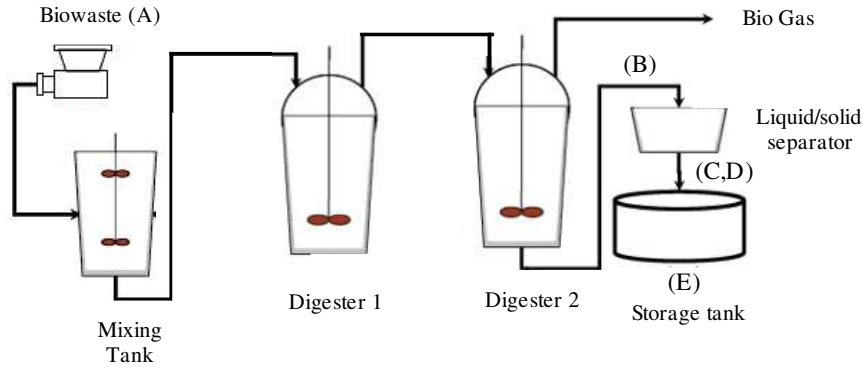
1 Table 2. Frequency (%) of bacterial pathogens in the different types of samples
 2 analysed.

Pathogens	Bovine manure	Digestates	Solid fraction	Liquid fraction	Stored liquid fraction
<i>Salmonella</i>	20 (1/5)	8 (1/12)	25 (1/4)	33 (1/3)	0 (0/3)
<i>L. monocytogenes</i>	20 (1/5)	25 (3/12)	100 (4/4)	100 (3/3)	33 (1/3)
<i>E.coli</i> O157:H7	0 (0/5)	0 (0/12)	0 (0/4)	0 (0/3)	0 (0/3)
<i>Yersinia</i> spp.	0 (0/5)	0 (0/12)	0 (0/4)	0 (0/3)	0 (0/3)

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1 Figure 1. Flow diagram of the biogas plant and sampling points.



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A - Input substrates: cattle slurry (n=5), agricultural biomass (n=5); B - fresh DP (n=12); C - liquid fraction of DP (n=3); D - solid fraction of DP (n=3); E - 120 days harvested liquid fraction of DP (n=3).

For Peer Review