THE EFFECT OF ANAEROBIC DIGESTION AND STORAGE ON INDICATOR MICROORGANISMS IN SWINE AND DAIRY MANURE

Annamaria Costa[1], Claudia Gusmara[2], Davide Gardoni[1], Mauro Zaninelli[3], Fulvia Tambone[4], Vittorio Sala[2], Marcella Guarino[1]

[1]Department of Health, Animal Science and Food Safety, Faculty of Veterinary Medicine, University of Milan

[2]Department of Veterinary Science and Public Health, Faculty of Veterinary Medicine, University of Milan

[3] Università San Raffaele, Rome

[4] Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy, University of Milan

Corresponding author:

Annamaria Costa, Department of Health, Animal Science and Food Safety, Faculty of Veterinary Medicine, University of Milan, Via Celoria 10. 20133, Milano (Italy); phone: +39-02-503-17904, e-mail: annamaria.costa@unimi.it

Abstract.

The aim of this experimental study was to evaluate the influence of anaerobic digestion and storage on indicator microorganisms in swine and dairy excreta. Samples were collected every 90 days for 15 months at 8 farms, 4 pig and 4 dairy farms, 4 of them having a biogas plant. Moreover, to evaluate storage effects on samples, 20 L of manure and slurry taken at each farm (digested manure only in farms with a biogas plant) were stored in a controlled climatic chamber at 18°C, for six months. The bacterial load and the chemical-physical characteristics of excreta were evaluated at each sampling time, stored slurry and manure were sampled and analyzed every two months. A high variability of the concentration of bacteria in the different excreta types was observed during the experiment, mainly depending on the type and time of treatment. No sample revealed either the presence of *Escherichia coli O157:H7* or of *Salmonella*, usually linked to the temporary rearing of infected animals in facilities. Anaerobic digestion and storage affected in a significant way the reduction of indicator bacteria like lactobacilli, coliforms and streptococci. Anaerobic digestion lowered coliforms in pig slurry (-2.80 log, $P<0.05$), streptococci in dairy manure (-2.44 log, $P< 0.001$) and in pig slurry (-1.43 log, $P<0.05$), lactobacilli in pig slurry (- 3.03 log, $P<0.05$). Storage lowered coliforms and the other indicators counts, in particular in fresh wastes, while clostridia did not show a reduction in concentration.

Capsule abstract

The present study is aimed to evaluate the effect of anaerobic treatment and storage time on bacteria concentration reductions in swine and dairy manure.

Keywords. *Dairy wastes, Pig slurry, Anaerobic digestion, Storage, Microbial Load, Pathogens, Indicators.*

INTRODUCTION

In the Lombardy Region in Northern Italy, livestock farming represents a significant portion of the local economy. In 2010, about 1.5 million cows and 4.8 million pigs (representing, respectively, 27% and 50% of the national total amount), distributed on an agricultural area of about 1 million hectares, were surveyed (ISTAT, 2010). This high concentration of animals poses serious concerns regarding the production of slurries and manure, their impact on groundwater, ammonia and greenhouse gas emissions, and food security resulting from the potential presence of zoonotic pathogens.

In recent years, there has been an increasing interest in the spreading of zoonotic pathogens, their persistence in soils and the correlation between the presence of pathogens and the safety of agricultural products (Hutchison et al., 2005; Pachepsky et al., 2006; Ziemer et al., 2010; Rogers et al., 2011; Toth et al., 2013). This concern is even more present in Europe and North America, where the availability of “pathogen free” products is a sensitive topic for public opinion (Bicudo and Goyal, 2003; Cummings et al., 2009; Newell et al., 2010; Krause and Hendrick, 2011). The recycling of these kind of wastes to agricultural land creates the risk of pathogens, contaminating the environment, entering the food chain, or infecting livestock (Martinez and Burton, 2003). Pandey et al. (2014) highlighted the great risk coming from pathogens and related to wastewater effluents for public health.

A clear example is the *Escherichia coli* O104:H4 outbreak occurred in Germany during the spring of 2011 (3816 cases, including 54 deaths), in which the consumption of bean sprouts was identified as the most likely vehicle of infection (Frank et al., 2011). Other verotoxin-producing strains of *Escherichia coli*, such as strain O157:H7, able to survive under adverse conditions (Pell, 1997), whose reservoir is identified in dairy farms (Wells et al., 1991; Hancock et al., 1994; Zhao et al., 1995), can induce serious symptoms as hemorrhagic colitis, hemolytic uremic syndrome, and

66 thrombocytopenic purpura. Many of the available publications about health risks linked to animal
67 waste disposal are addressed to study *Escherichia coli* O157:H7 and *Salmonella* (Huston et al., 2002;
68 Murinda et al., 2002; Blau et al., 2005; Cho et al., 2006; Semenov et al., 2011), while several other
69 pathogens have also been investigated, including *Campylobacter jejuni*, *Listeria monocytogenes*,
70 *Cryptosporidium parvum*, *Giardia lamblia*, *Enterococcus faecalis*, and *Clostridium* spp. (Hutchinson
71 et al., 2004; Watcharasukarn et al., 2010).
72 There is a higher risk of pathogen transfer into the food chain when fresh manure is applied to the
73 land than when stored manure is applied, because in the former case there is no storage or
74 treatment period to decrease pathogen numbers (Watanabe et al, 1997). As a consequence, the
75 minimization of the sanitary impact of slurries and manure in the environment has to be considered
76 as a primary objective in livestock farming.
77 Storage is a traditional practice that consists in storing animal excreta for long periods in order to
78 reduce the organic and bacterial loads. Prolonged isolated storage for 3–6 months before land
79 spreading is still the most common practice in Italy. This approach allows the number of pathogens
80 in manure to decrease but not to totally disappear.
81 Anaerobic digestion performed in biogas plants is a recent alternative way to handle animal wastes
82 for the production of energy and of fertilizers to be spread on cultivated land, limiting the risk for
83 human health and reducing greenhouse gas emission. The usefulness of treatments like digestion,
84 and, traditionally storage, to destroy, or limit, infectious microorganisms in animal waste for land
85 application is well known.
86 In a recent study, Biswas et al. (2016) evaluated the performance of limited aerobic and anaerobic
87 storage conditions in decay of pathogens in dairy manure at four temperatures under minimal
88 mixing. Results showed that the effects of both limited aerobic and anaerobic storage conditions on
89 pathogen reductions were almost similar in the minimal mixing condition potentially due to poor

aeration of dairy manure. *Escherichia coli* survival was longer than *Salmonella* and *Listeria monocytogenes* in all temperature conditions. *Salmonella* and *Listeria monocytogenes* levels were reduced to non-detectable level in both limited aerobic and anaerobic storage conditions within 3 days of incubation.

The temperature and hydraulic retention time are crucial factors for pathogenic bacteria survival during anaerobic digestion (Dumontet et al., 1999). Anaerobic digestion can be performed either at 30–38 °C (mesophilic) or thermophilic at 50–55 °C and bacterial inactivation due to temperature is strictly related to time (Olsen and Larsen, 1987). Gibbs et al. (1995) and Larsen et al. (1989) found that the time required for a 90% reduction of viable counts of a population of microorganisms (T90) for many bacteria can be counted in hours in thermophilic digestion and in days in mesophilic digestion, compared to weeks and months in conventional treatment (storage). Gibbs et al., in 1995, reported at least a T90 of 2 weeks for *Escherichia Coli* and *Salmonella typhimurium*, of 2.7 weeks for enterococci in storage at 18°C. Enterococci showed a T90 of 21.4 weeks at a storage temperature of 6-15 °C.

However, pathogens represent a rather limited fraction of the bacteria in the feces of animals, with the exclusion of the acute phases of enteric diseases. Pathogen bacteria are released into the environment on a non-continuous basis, in relation to the health and the immune status of the subjects, and they are not ideal indicators for monitoring the different maturation processes of sewage. The evaluation of more common bacteria, ubiquitous in manure, could be used as "indicators" of the pathogenic potential of the different categories of bacteria that might be present in the feces, because of their similar biochemical and respiratory needs (Bicudo and Goyal, 2003). The use of indicator organisms (e.g., fecal coliforms, *Escherichia coli*) for evaluating pathogen levels has been widely discussed; however, the use of indicator organisms is likely to continue for assessing pathogen levels in water resources potentially for the lack of an alternative reliable

114 solution (Pandey et al., 2014). The use of indicator microorganisms as surrogate for pathogenic fecal
115 organisms in both fate and transport was performed in past studies performed by Wang et al.
116 (2004), Ogden et al. (2001), Mubiru et al. (2000). In the last decades, the goodness of indicator
117 organism evaluation for assessing pathogen levels in ambient water bodies on the basis of the
118 similar decay is confirmed by many studies (Malakoff 2002; Pandey et al. 2012a; Pandey et al.
119 2012b; Pandey and Soupir 2013). Smith al. (1973) found that *Salmonella* decay in streamwater was
120 similar to that of fecal coliforms. In Denmark, fecal streptococci (FS) - method is used for quality
121 assurance of digested residues for common pathogens (*Salmonella, Listeria, Campylobacter,* and
122 *Yersinia*; Espensen, 1996). This method, however, present the limitation when the temperature in
123 the treatment process exceeds 55° C, because fecal streptococci are quickly reduced and are
124 impossible to quantify above this temperature (Bendixen and Ammendrup, 1992). De Luca et al.
125 (1998) found fecal streptococci to be the only indicator bacteria with a statistically significant
126 correlation to *Listeria monocytogenes*.
127 In general, the decrease of the counts of microbial indicators also corresponds to a lower
128 concentration of pathogens, this happens in the case of Coliforms for *Salmonella* spp. and, also for
129 verotoxigenic *Escherichia coli*, which is metabolically similar (Vanotti et al., 2005).
130
131 For the above mentioned reasons, the present study was aimed at evaluating the effect of
132 anaerobic treatment (at least 6 complete digestion cycles during the trial) and of storage time on
133 bacteria concentration reductions and on the physical characteristics of livestock wastes.
134 The effects of storage time (0, 2, 4 and 6 month) on the bacteria concentrations of the eight manure
135 samples (4 cattle manures and 4 pig slurries, 2 samples for each categories were digestates) stored
136 in tanks at 18°C in a climatic cell to avoid undesired environmental additional effects.
137

MATERIAL AND METHODS

Four cattle farms and four pig farms were considered in this study as representatives of Italian intensive cattle and pig husbandry. Four of them, two cattle and two pig farms, had a mesophilic biogas plant. Manure and slurry were spread on land for corn and alfalfa productions.

Animals and farms

Pig farms

Four pig farms were involved in the study. The first farm is a full cycle piggery (from birth to slaughtering), with 12000 pigs in total (650 sows), the manure is collected under the pit for *vacuum system* removal and moved to the biogas plant, a mesophilic plant working at 43°C with a hydraulic retention time (HRT) of 56 d. The plant consist in a primary, a secondary digestion plant and an ultimate “cold” tank to recover the residual biogas from digested manure.

The second pig farm is a full cycle with 8000 pigs reared from weaning to slaughter (from 35 kg to 160 kg of live weight). The farm has a slatted floor with vacuum system for manure removal. Manure is collected and moved to a primary tank and then to the mesophilic digestion tank, with a temperature of 37°C for 40 d of HRT.

The third farm is a full cycle farm with 400 sows, the manure is separated and moved to the tank for 180 d of storage.

The fourth farm is a full cycle farm with 250 sows, the manure is collected into the deep pit and then sent to the tank for 180 d of storage.

Dairy cattle farms

The first farm is a dairy cattle farm with 300 Friesian Holstein dairy heifers, the manure is removed through scrapers and under the pit, then, it is moved to the mesophilic digestion plant (set up in a

primary and a secondary digestion plant) working at a temperature of 48°C, HRT of 90 d.

The second dairy cattle farm reared 600 Friesian Holstein dairy cows, the manure is removed through scrapers and under the pit, then, then it is moved to the mesophilic digestion plant(set up in a primary and a secondary digestion plant) working at a temperature of 48°C, HRT of 90 d. The plant in this farm is identical to the plant adopted by the first dairy farm.

The third dairy cattle farm reared 150 Friesian Holstein dairy cows, the manure falls in to a pre-tank placed under the perforated floor and moved to tank for 120 d of storage.

The fourth dairy cattle farm reared 400 Friesian Holstein dairy cows, the manure is removed through scrapers and under the pit, then it separated into solid/liquid fractions and stored for 120 d.

Sampling in real conditions

The manure samples were taken in the farms for 15 months every 90 days (6 times in the study) to evaluate their physical, chemical and microbiological characteristics. In the farms with storage pits, the manure was taken directly from the pits, or under the slatted floors. In the farms with anaerobic plants, the samples were taken before and after the digestion process, at the end of HRT period. Manure was mixed in the lagoons and in the pits, then, 5 tanks of 10 L were collected from various zones (at middle height of the tank, one sample was taken in the central zone and four in the lateral zones). Then the collected manure samples were mixed together and 3 samples of 100 g for each manure type was collected and taken to the laboratory for microbiological (50 g) and chemical (50 g) analyses.

Sampling of stored manures in controlled climatic conditions

185 In each farm, 20 L of excreta (fresh manure/slurry for farms with storage tank and digestate product
186 for farms with anaerobic plant) were stored for six months at 18°C to study the effect of storage on
187 bacterial load in manure kept at constant temperature.
188 At the beginning of the two cycles, the manure was collected in every farm, as follows: manure was
189 mixed in the lagoons and in the pits, then, 5 tanks of 10 l were collected from various zones (at
190 middle height of the tank, one sample was taken in the central zone and four in the lateral zones).
191 Then the manure collected in the tanks was mixed together and 20 l were taken to the climatic cell
192 for storage. For the analysis at 0, 2, 4 and 6 months of storage, 3 samples of manure for each 20 L
193 tank (slurry and/or manure type) were withdrawn at the bottom, in the middle and in the high part
194 of the tank. The samples (100 g each) were taken to the laboratory for bacterial counts (50 g) and
195 chemical (50 g) analyses within two hours from sampling.
196 The climatic control was achieved through a conditioning system and the temperature was
197 monitored every minute with a datalogger system (HOBO UX100, ELCAM SpA). Microbial
198 concentrations were measured every two months, for six months, at time 0 = first sampling day,
199 time 1 = 2nd month, time 2 = 4th month, time 3 at the 6th month. This trial was performed twice in
200 the experimental period.
201

202 **Microbiological analysis**

203 The presence of the selected “indicator-bacteria” coliforms (Gram-negative, aerobic/facultative
204 anaerobes), enterococci (Gram-positive, facultative anaerobes), lactobacilli (Gram-positive,
205 facultative anaerobes) and clostridia (Gram-positive, sulphite- reducing anaerobes) was evaluated.
206 These micro-organisms are indicators of the survival of potentially dangerous pathogens of the
207 same genus. In addition, qualitative bacteriology was also performed to verify the presence and the
208 possible survival of some pathogen bacteria (*Escherichia Coli* O157:H7 just for dairy samples, and

209 *Salmonella* species) in the tested conditions.

210

211 **Quantitative bacteriology**

212 One gram of each sample was mixed in 9 mL of sterile distilled water and thoroughly homogenized.
213 A series of 10-fold dilutions (from 10^{-1} to 10^{-7}) were then prepared. 0.1 mL of each dilution was used
214 to inoculate 3 plates for each dilution of four agar selective media using the spread-technique.
215 MacConkey agar was used for the enumeration of Coliform species, Slanetz-Bartley agar for
216 *Enterococcus* species, Rogosa agar for *Lactobacillus* species and Iron Sulphite agar for *Clostridia*
217 species. The water content was determined in 1G of each sample, testing it by an infrared moisture
218 meter before and after drying in a vacuum oven at 105°C. The plates for Coliforms were incubated
219 aerobically at 37°C, 24 hours; plates for *Enterococcus* spp fat 37 °C, for 72 hours. Plates for sulphite-
220 reducing anaerobes were incubated in anaerobiosis at 37°C for 24 hours, and those for *Lactobacillus*
221 spp. were incubated for 48 hours at 45°C. After incubation, the presence of bacterial colonies on the
222 plates was examined. Only plates whith a number of colonies between 15 and 150 were counted
223 and the results were expressed as Colony Forming Units (CFU) per gram of wet feces.

224

225 **Qualitative bacteriology**

226 Qualitative assays were performed on the manure samples, before and after treatment, and at
227 different sampling times, to determine the presence of two enteropathogenic bacteria: *Salmonella*
228 spp. in samples from pigs and cattle and *Escherichia coli* O157: H7 in samples from cattle.
229 The sensitivity of the method used for the detection of *Salmonella spp*. (derived from ISO 6579:
230 2005) has been estimated at 87% of pathological material from the pig (Mainar-Jaime et al., 2013).
231 For *Escherichia Coli* O157: H7, validation studies of the method ISO 16654 - 2001 indicate a
232 sensitivity of 96.4% of plant materials (Tozzoli and Morabito, 2014).

For *Escherichia Coli* O157: H7, 10 g of each fecal sample was mixed with 90 ml of buffered peptone water (BPW) and incubated overnight at 37°C. The colonies in 1 ml of this culture medium were concentrated using immunomagnetic specific anti-O157 beads in an automated system, according to the manufacturer's recommendations (Dynal, Oslo, Norway). Briefly, the retrieved beads were inoculated on sorbitol MacConkey agar containing cefixime and tellurite (SMACct), then incubated overnight at 37°C. From each plate five sorbitol-negative colonies were isolated and identified with biochemical systems and by direct latex agglutination directly with a commercial kit (Oxoid).

For the selective bacteriology of *Salmonella* spp., 1G of each fecal sample was inoculated in culture pre-enrichment in buffered peptone water and incubated overnight at 37°C. 1 ml of this culture was transferred to a 10 ml tube of selective broth Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn), then incubated at 37°C for 24 hours. Finally, this culture was inoculated on XLT4 agar and incubated for 24 hours at 37°C.

Chemical analyses.

All samples were dried for 24 h at 40°C and then for another 24 h at 105°C (APHA et al., 2005), shredded in a blender and passed through a 1-mm mesh. Ammonia (NH_3–N) and total nitrogen (TKN) were detected on fresh samples. Fresh matter (FM), total solids (TS) and volatile solids (VS) were determined following standard procedures (APHA et al., 2005). Total P and K contents were determined by inductively coupled plasma mass spectrometry (Varian, Fort Collins, USA). Standard samples (National Institute of Standards and Technology, Gaithersburg, MD, USA) and blanks were run with all samples to ensure precision in the analyses. P and K detection was preceded by acid digestion (EPA, 1998) of the biomass samples. Total alkalinity or buffer capacity (TAC) and total volatile fatty acids (FOS) concentrations were determined in the bulk samples by a 5-times-diluted solution of 2.5 g of wet sample, filtered to 0.45 µm, according to the acid titration method (Lahav,

257 2002).

258

259 **Statistical analysis**

260 Before the statistical analysis, all the microbiological counts were transformed into log_{10}, data are
261 expressed as log_{10} CFU/g. The bacterial counts of samples collected every three months in livestock
262 farms were submitted to variance analysis (Proc GLM of the SAS statistical package 9.2, 2013) in
263 order to evaluate the effect of the collecting season on physical characteristics of slurry and on the
264 microbial concentrations.
265 Microbiological data related to samples before and after anaerobic digestion were processed
266 through variance analysis (Proc GLM of the SAS statistical package 9.2, 2013) to test the effect of
267 type of waste (dairy *vs.* swine) and of the anaerobic treatment on bacteria concentration
268 reductions, the interaction type for treatment was considered in the model.
269 A third variance analysis was performed (Proc. GLM of the SAS statistical package 9.2, 2013) on
270 samples stored in the climatic cell (4 cattle manures and 4 pig slurries, 2 samples for each categories
271 were digestates). The variance analysis evaluated the effect of type of waste (dairy *vs.* swine),
272 treatment (raw manure *vs.* digestate), storage time (0, 2, 4 and 6 month) on bacteria
273 concentrations. The interactions types - treatment - storage time, were included in the model.
274 In the variance analysis, the significance level was considered at least for $P<0.05$.
275 A Pearson correlation procedure (Proc CORR of SAS statistical package, 9.2, 2013) was performed
276 among all the variables to highlight potential correspondences between physical-chemical
277 characteristics and bacterial counts.

278

279 **RESULTS**

Pathogens investigated in the trial (*Salmonella* and *Escherichia coli* O157:H7) were not ever detected at any sampling time, indicating that no clinical or subclinical dissemination of these pathogens had occurred during the research period.

No effect of collecting season was found on the samples for all the studied bacteria.

Evaluation of the effect of anaerobic digestion

Figure 1 shows the mean values of the microbial load of dairy manure and pigs slurry (clostridia, coliforms, streptococci and lactobacilli), expressed in log_{10} CFU/g, sampled before and after the anaerobic digestion treatment during the experimental study in real conditions.

Figure 1. Microorganisms concentrations in cattle manure and pig slurry before and after anaerobic digestion

Streptococci and Lactobacilli concentrations were significantly lower ($P<0.05$) in dairy raw manure and digestate in comparison to pig wastes.

The anaerobic digestion treatment had a significant overall effect on the decrease of coliforms ($p>0.01$), streptococci ($p<0.001$), and lactobacilli ($p<0.05$). This microbial abatement was evident during the whole sampling campaign.

Clostridia concentration decreased slightly according to the anaerobic treatment in cattle manure from 4.95 log_{10} CFU/g to 4.70 log_{10} CFU/g. The anaerobic digestion induced an increase in Clostridia population in pig slurry (5.28 log_{10} CFU/g vs 6.02 log_{10} CFU/g), although not in a significant way.

The coliforms count significantly decreased in pig slurry from 5.61 log_{10} CFU/g to 2.81 log_{10} CFU/g ($P<0.05$) after the anaerobic treatment. The variation of coliforms in dairy digestate was measured in – 2.19 log in comparison with the fresh manure.

304 Streptococci counts differed significantly in relation to the manure type (dairy *vs.* swine, P<0.001)
305 and after the anaerobic digestion in comparison with the fresh manure (P<0.001).
306 In cattle manure, Streptococci count was reduced from 4.67 log_{10} CFU/g to 2.23 log_{10} CFU/g
307 (P<0.001) after the treatment, in pig slurry from 5.43 log_{10} CFU/g to 4.00 log_{10} CFU/g, P<0.05.
308 Lactobacilli concentrations showed overall effects of manure type (dairy *vs.* swine, P<0.01), and by
309 the digestion treatment (P<0.05). Pig slurry showed a significant decrease of this concentration in
310 digestate (7.92 log_{10} CFU/g *vs.* 4.89 log_{10} CFU/g, -38 %; P<0.05).
311

312 **Evaluation of storage**

313 Figure 2 shows the mean values of the microbial load of clostridia, coliforms, streptoccocci and
314 lactobacilli in digested and fresh dairy manure at month 0, 2, 4 and 6 of storage in controlled
315 climatic conditions (18°C).
316 Clostridia concentrations did not show an overall effect of time of storage in dairy manure, fresh or
317 digested. In pig digested slurry, clostridia population increased during storage time, with a
318 significant growth from month 0 to month 6. This increase was probably due to the observed
319 reduction of the competitor microorganisms that in normal conditions can inhibit the revitalization
320 of *Clostridium* spores.
321

322 Figure 2. Microorganism concentrations in digested and fresh cattle manure, in pig slurry during the
323 six months of storage in controlled climatic conditions
324

325 Coliforms concentrations in dairy were affected by manure type (fresh vs. digested, P<0.001) and
326 storage time (P<0.05), an interaction type for storage time was detected (P<0.01). Similar counts
327 were measured at the end of storage time for dairy manure and at the beginning of digestate

328 storing time.

329 This concentration did not vary significantly during the six months of storage of the digested
330 manure (2.16 $\log_{10}$ CFU/g *vs.* 2.32 $\log_{10}$ CFU/g), while the Coliforms concentration measured in fresh
331 manure decreased significantly at the end of storage time (5.50 $\log_{10}$ CFU/g at month 0 and 2.01
332 $\log_{10}$ CFU/g at month 6; $P<0.001$). Coliforms concentrations was lowered significantly ($P<0.01$) by
333 storage in pig raw slurry, from 4.26 $\log_{10}$ CFU/g at month 0 to 1.69 $\log_{10}$ CFU/g at months 4 and 6.
334 *Streptococci* concentration in dairy differed significantly in the type of manure (digested *vs.* fresh
335 manure, $P<0.001$) and according to the month of storage ($P<0.05$).
336 *Streptococci* concentration in digested manure did not vary in a significant way, while they were
337 reduced significantly in fresh manure from month 0 (6.10 $\log_{10}$ CFU/g) to month 2, month 4
338 ($P<0.01$) and at the end of storage (4.31 $\log_{10}$ CFU/g; $P<0.05$). In swine slurry, streptococci
339 decreased significantly (5.59 $\log_{10}$ CFU/g *vs.* 1.84 $\log_{10}$ CFU/g; $P< 0.001$), as in digestate samples
340 (4.39 $\log_{10}$ CFU/g *vs.* 1.70 $\log_{10}$ CFU/g; $P< 0.001$).
341 The statistical analysis revealed an overall significant effect of dairy manure type (fresh *vs.* digested,
342 $P<0.001$) and storage time ($P<0.05$) on lactobacilli.
343 Lactobacilli concentration in fresh manure was measured in 4.81 $\log_{10}$ CFU/g at the month 0 and
344 2.13 $\log_{10}$ CFU/g at month 6 ($P<0.001$), although they showed a non - linear trend. In digestate, this
345 concentration did not vary during all the periods of storage in digested cattle manure.
346 Pig slurry and digestate concentrations of *Lactobacilli* were affected by time of storage.
347
348 The chemical characteristics of the stored slurries were also monitored. Results (Table 1) showed, as
349 it was expected, a remarkable increase of the total solids due to the physiological dehydration of
350 slurry during the storage. The volatile solids amount was higher in dairy wastes and decreased in
351 time.

352

353 Table 1. Chemical properties of the dairy cows and pigs slurries during the storage

354

355 The FOS/TAC ratio (FOS are the Volatile Organic Acids, expressed as mg L^{-1} of CH_3COOH; TAC is the
356 buffer capacity, expressed as mg L^{-1} of $CaCO_3$) decreased rapidly, showing the degradation of the
357 volatile acids probably due to a slow biological degradation, pH increased over time.
358 The Pearson correlation coefficient analysis (Table 2) confirmed that the reduction of coliforms,
359 streptococci and lactobacilli could be linked to the pH and the FOS/TAC ratio. A diminishing
360 concentration of *Streptococci* resulted inversely proportional to pH ($r = -0.48$, $P<0.001$), showing
361 that when pH lowered, streptococci concentration increased. On the contrary, clostridia resulted
362 directly proportional to pH ($r=0.33$, $P<0.05$), their concentration increased with raising pH values.

363

364 Table 2. Pearson correlation coefficients among chemical and microbial characteristics of slurry
365 samples

366

367 **DISCUSSION**

368 In this study, the results demonstrate an overall significant effect of the anaerobic digestion on the
369 bacterial load of the microbial concentration of indicator microorganisms, except for clostridia.
370 Anaerobic mesophilic digestion increased clostridia population in pig digested slurry in time
371 ($P<0.01$), with a significant increase from the month 0 to the month 6 ($P<0.01$). Anaerobic
372 mesophilic digestion did not reduce clostridia levels in cattle digestates, in agreement with
373 Abdelgadir et al. (2014), who found that even thermophilic anaerobic digestion successfully reduced
374 *Salmonella* spp., and *Escherichia coli* but not *Clostridium perfringens* spores.
375 Their resistance probably depends on their capability of producing endospores, while the observed

increase was probably due to the spore re-germination linked to the lowering of the concentration of other bacteria. Similar results were reported by Kearney *et al*. (1993), Watanabe *et al*. (1997), and Sahlström (2003).

Due to their spore forming capacity, *Clostridium* spp. as well as other spore forming bacteria are very resistant. Spores can survive for many years in the environment, many severe diseases are caused by *Clostridium* spp, such as tetanus (*Clostridium tetani*), botulism, (*Clostridium botulinum*) and blackleg (*Clostridium chauvoie*) (Hirsh and Zee, 1999).

The failure in clostridia reduction after anaerobic digestion and storage should be particularly considered since two bacterial genera, *Eubacterium* and *Clostridium*, are most likely the major contributors to odorous volatile fatty acids: It is actually difficult to obtain an effective reduction of clostridia through a simple microbiological process, in agreement with studies performed by Zhu, 2000, Chauret et al., 1999.

Coliforms and the other indicators were considerably reduced by anaerobic digestion treatment, in agreement with Sobsey (1998). In our study, a greater reduction of the investigated bacteria, with the exception of Clostridia, was observed in stored wastes in comparison with digested samples, in particular way in pig slurry, considering the initial bacteria concentrations and the final reduction values after the two treatments. These results are in agreement with findings by Pandey et al. (2015), that showed that aerobic processes can be more effective in eliminating pathogens, in comparison with anaerobic digestion. However, in our study, bacteria were reduced but not eliminated. Elimination of bacteria depends on several factors, pH, temperature, availability of nutrients and also on their initial amount in the waste (Strauch, 1991).

The beneficial effects of the anaerobic treatment on the environment should also be taken into account for the reduction of emissions of greenhouse gases, such as methane and nitrous oxide (Møller et al., 2009). In addition it contributes to reduce global warming, not only from the

400 substitution of fossil fuel by biogas but also from carbon storage in the soil and inorganic fertilizer
401 substitution (Møller et al., 2009).
402 Storage results highlighted its efficiency to lower the concentration of different microorganisms,
403 especially in fresh manure and slurry, with the exception of *Clostridium*.
404 Storage applied after anaerobic digestion lowered Lactobacilli and Streptococci counts, but only in
405 swine digestates, probably for the already lower counts of these bacteria at the beginning of storage
406 in cattle digestates after the higher temperature of the anaerobic treatment in the cattle farms
407 (Wang et al., 2004).
408 The substantial reductions of coliforms concentration (2.56 log for pig slurry and 3.43 log for dairy
409 manure) are in agreement, although in a less satisfactory way, with a study performed by Coté et al.
410 (2006b), who found that a 1-month batch storage of liquid swine manure was sufficient to obtain a
411 90 % reduction of *Escherichia Coli* populations. A storage of 2-4 months can easily reduce fecal
412 indicator microorganisms reduction in pig slurries and digestates. Gibbs et al., in 1995, reported at
413 least a T90 of 2 weeks for E. Coli, of 2.7 weeks for Enterococci in storage at 18°C.
414 Our results confirmed that prolonged isolated storage for 3–6 months before land spreading,
415 usually performed in Italy, allows the number of pathogens in manure to decrease but not to totally
416 disappear. These limited, although beneficial results, are in agreement with studies of Gibbs et al.
417 (1995) and Martinez et al (2009).
418 The correlation coefficient analysis revealed a significant positive relationship between pH and the
419 bacteria concentrations included in this trial, except for clostridia: coliforms, streptococci,
420 lactobacilli resulted significantly lowered by pH increase (r=-033, r=-0.48, and r=-0.44 respectively),
421 as it was expected. According to a study performed by Pearson et al. (1987), fecal coliforms in waste
422 ponds reduce more rapidly as the pH increase above 8.50, a particularly large increase in their die-
423 off usually occur when the pH raises from 8.50 - 8.75 to pH 9.0.

Other researchers showed that extremes in pH are detrimental to organism survival, Parhad and Rao (1974) observed that *Escherichia coli* counts, in stabilization ponds, declined rapidly at pH above 9.3. More generally, a neutral pH environment seems to favor extended bacterial survival; and acid and alkaline conditions in water can greatly increase fecal coliforms decay rates (Mc Feters and Stuart, 1972). Clostridia concentration seemed to grow with pH raising (r=0.33). The FOS/TAC ratio was directly correlated with coliforms, streptococci, lactobacilli concentrations. No references are available with this finding, so further studies are needed to evaluate the relationship of these bacteria levels and FOS/TAC ratios.

Considering the purpose of reusing digested and stored manure and slurry as fertilizers in agriculture, it is important to highlight that the microbiological quality of the samples analyzed in this study did not comply with the microbial parameter thresholds of the Italian law for fertilizers (*Escherichia Coli*< 1000 CFU g^{-1}, D.M. 29819/2009).

At this point, an accurate supervision can allow a safe agronomic utilization both of the treated solid and liquid fractions, limiting the spreading of potentially dangerous materials and improving a sustainable agriculture (Nicholson et al., 2005; Côté et al., 2006b).

CONCLUSIONS

Anaerobic digestion and storage of dairy and swine manures are confirmed to be effective techniques to limit the presence of coliforms, streptococci and lactobacilli, with exception of clostridia. Storage was particularly effective on bacteria reduction in fresh manure, also affecting several chemical-physical parameters. Correlations were identified between these parameters and microorganisms levels. Further studies are needed to examine in depth the possibility of modelling the fate of indicators and pathogens as a function of the physical-chemical parameters, such as pH and FOS/TAC ratio.

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Table 1. Chemical properties of the dairy cows and pigs slurries during the storage.

	Month	Total Solids		Volatile Solids		pH		Electrical Conductivity		TKN		NH3–N		P tot		K tot		FOS/TAC	
		g kg^{-1} FM	*SD*	*g kg^{-1} TS*	*SD*		*SD*	*mS cm^{-1}*	*SD*		*SD*		*SD*	*g kg^{-1} FM*	*SD*		*SD*		*SD*
Digested manure/slurry	**Dairy**																		
	0	55.4	14.20	744.3	200.40	8.5	0.29	19.4	2.03	3.8	0.78	2.0	0.19	0.3	0.11	3.0	1.10	0.1	0.01
	2	85.2	13.60	753.5	330.03	8.5	0.16	13.2	2.43	2.6	0.62	1.4	0.26	0.3	0.09	5.7	1.14	0.1	0.01
	4	74.6	14.81	714.3	230.54	7.8	0.04	17.1	4.05	2.9	0.74	1.3	0.33	0.3	0.12	8.2	1.86	0.2	0.01
	6	98.9	26.91	697.8	210.99	8.7	0.31	19.00	6.98	4.0	1.00	1.0	0.43	0.8	0.16	6.7	1.29	0.1	0.03
	Swine																		
	0	36.3	11.28	558.1	170.11	7.9	0.01	26.3	3.71	3.9	0.35	2.9	0.25	0.7	0.14	2.2	0.45	0.1	0.02
	2	24.9	10.54	523.1	160.16	8.8	0.21	18.3	2.26	2.0	0.64	1.2	0.32	0.1	0.01	4.4	1.01	0.1	0.01
	4	41.9	8.22	510.4	260.40	8.3	0.36	19.3	3.23	2.4	1.05	0.9	0.22	0.4	0.13	6.2	1.51	0.1	0.01
	6	84.4	17.98	614.3	260.14	8.7	0.51	19.6	5.86	1.1	0.37	0.6	0.11	1.3	0.23	5.0	1.56	0.1	0.02
Fresh manure/slurry	**Dairy**																		
	0	59.0	20.55	790.6	300.83	6.9	0.19	10.5	2.69	2.7	1.18	1.1	0.28	0.4	0.16	2.4	0.55	0.7	0.03
	2	42.2	15.67	736.2	330.57	7.8	0.36	12.6	1.24	2.2	0.77	1.0	0.17	0.3	0.11	2.4	0.76	0.1	0.02
	4	67.9	19.87	747.9	410.73	7.5	0.05	14.4	1.28	2.0	1.62	0.8	0.24	0.3	0.09	4.6	0.40	0.1	0.03
	6	75.1	31.30	734.4	340.71	8.2	0.55	10.5	3.27	2.5	1.25	0.8	0.20	0.6	0.18	4.1	0.79	0.2	0.05
	Swine																		
	0	29.0	7.98	653.8	149.77	7.2	0.50	12.9	3.90	2.8	0.40	1.8	0.30	0.7	0.26	1.5	0.23	0.3	0.07
	2	45.5	11.19	362.4	80.89	8.9	0.01	7.5	0.49	0.8	0.11	0.5	0.07	0.1	0.01	1.6	0.20	0.0	0.01
	4	49.1	27.26	607.3	101.64	7.9	0.37	7.0	2.48	2.2	0.25	1.1	0.40	0.8	0.22	1.8	0.34	0.10	0.01
	6	57.1	26.03	528.1	105.95	8.3	0.06	9.3	2.15	2.3	0.72	0.6	0.19	1.1	0.38	2.8	0.62	0.1	0.01

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Table 2. Pearson correlation coefficients among chemical and microbial characteristics of slurry samples.

	Storage period	***Coli-forms***	***Strepto-cocci***	***Lacto-bacilli***	***Clostridia***	**TS**	**VS**	**pH**	**CE**	**TKN**	**NH_3–N**	**NH_3–N/TKN**	**Ptot**	**Ktot**	**FOS/TAC**
Storage period	1.00	-0.25	-0.30			0.29		0.38			-0.48	-0.37		0.32	-0.38
		P<0.05	P<0.05			P<0.05		P< 0.01			P< 0.001	P< 0.01		P<0.05	P< 0.01
Coliforms		**1.00**	**0.73**					**-0.33**							**0.41**
			<.0001					**P< 0.05**							**P< 0.01**
Streptococci			**1.00**					**-0.48**							**0.69**
								P< 0.001							**P< 0.01**
Lactobacilli				**1.00**	**1.00**			**-0.44**							**0.40**
					<.0001			**P< 0.01**							**P< 0.001**
Clostridia					**1.00**			**0.33**					**0.29**		
								P< 0.05					**P<0.05**		
TS						1.00	0.51			0.69		-0.50	0.74	0.57	
							P< 0.001			<.0001		P< 0.001	P< 0.001	P< 0.001	
VS							1.00	-0.37		0.43		-0.32			0.37
								P< 0.01		0.0015		P<0.05			P< 0.01
pH								1.00						0.28	-0.72
														P<0.05	P< 0.001
CE									1.00		0.30				
											P<0.05				
TKN										1.00	0.70	-0.38	0.63		
											P< 0.001	P< 0.01	P<0.001		
NH_3–N											1.00		0.32		
													P<0.05		
NH_3–N/TKN												1.00	-0.35		
													P<0.05		
Ptot													1.00		
Ktot														1.00	
FOS/TAC															1.00

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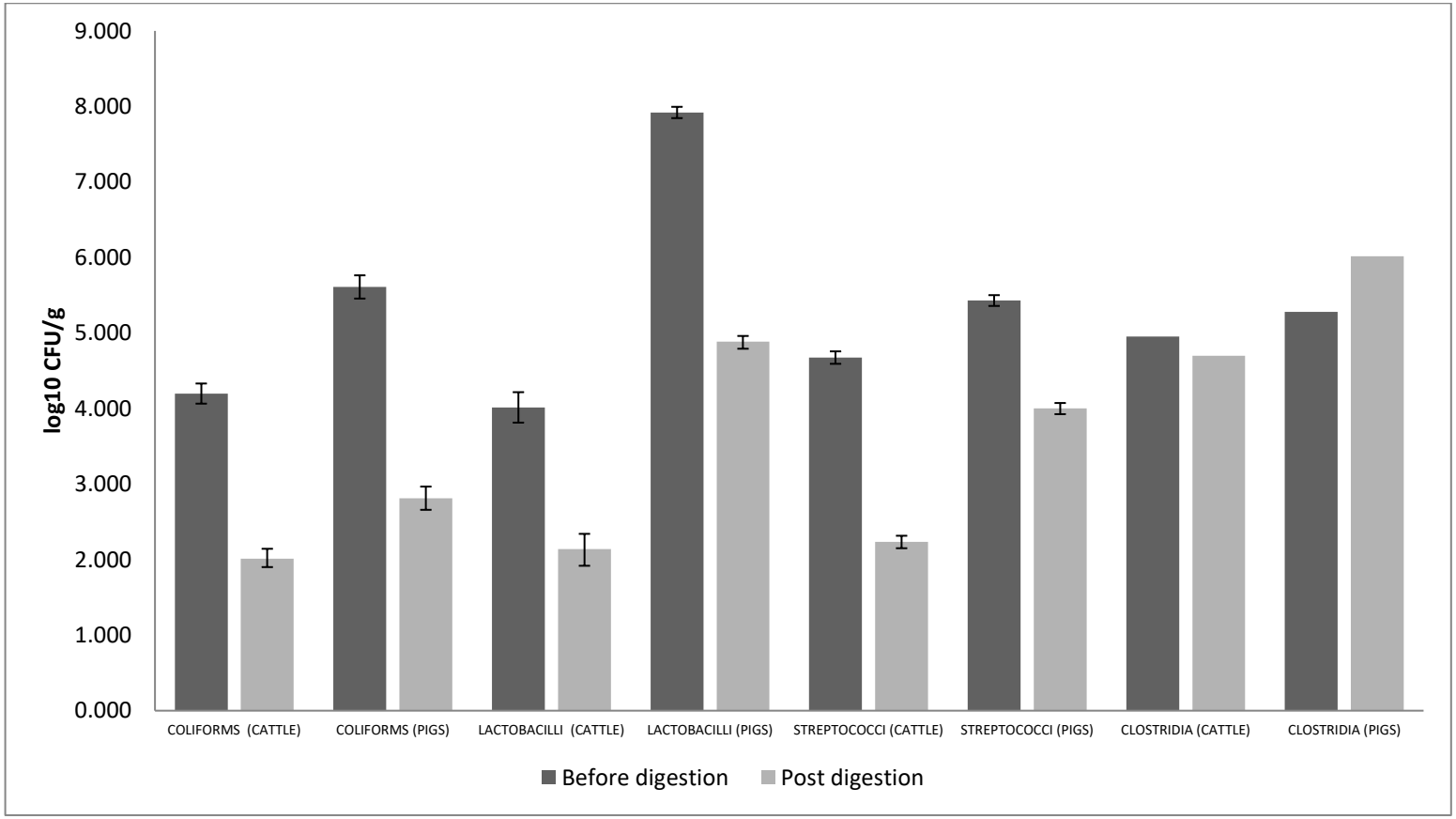

Figure 1. Microorganisms concentrations in cattle manure and pig slurry before and after anaerobic digestion

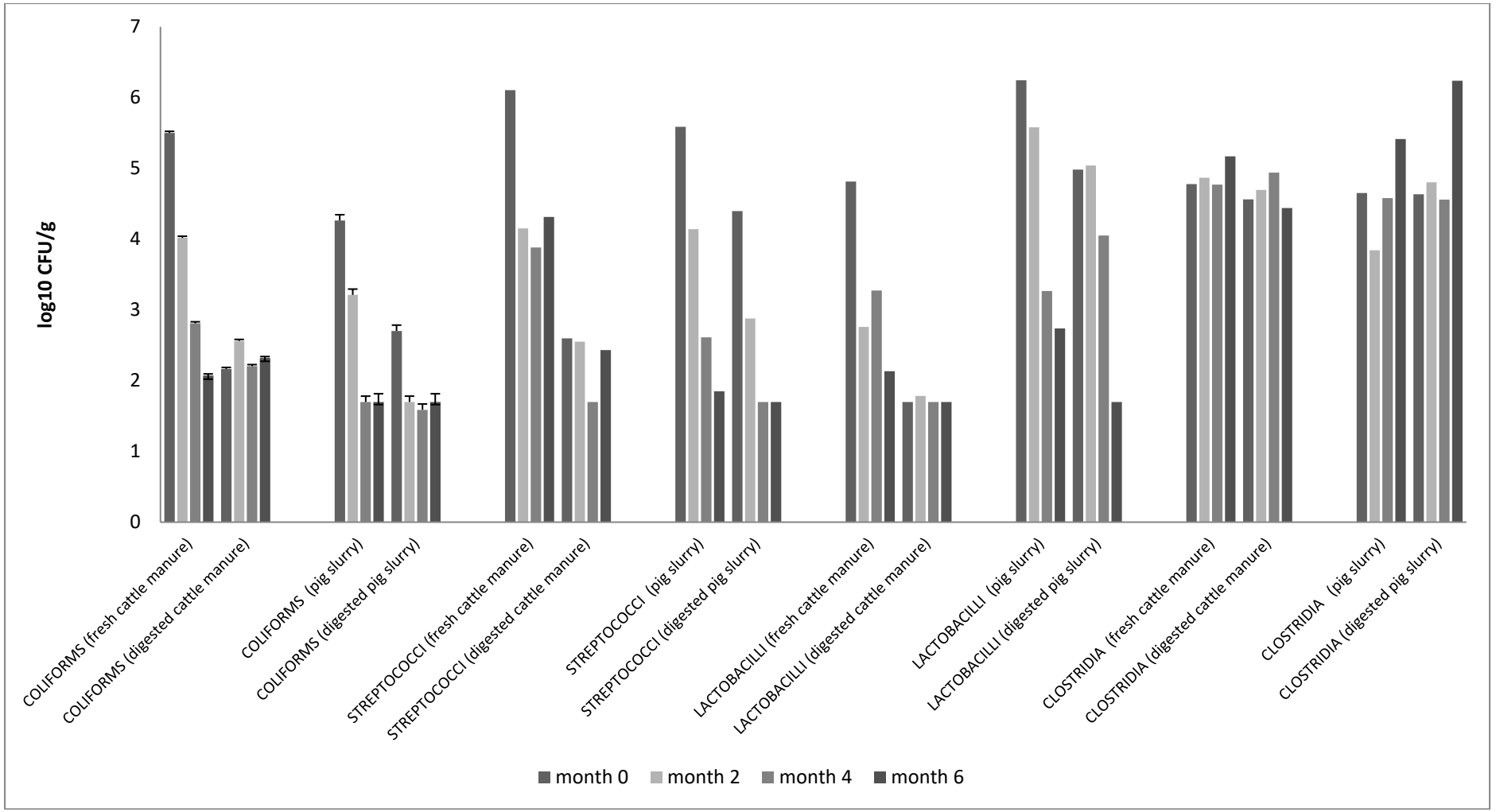

Figure 2. Microorganism concentration in digested and fresh cattle manure, in pig slurry during the six months of storage in controlled climatic conditions

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