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Son Thi Thanh Dang, Dung van Truong, Henry Madsen, Anders Dalsgaard. Survival of faecal indicator bacteria in treated pig manure stored in clay-covered heaps in Vietnam. *Veterinary Microbiology*, 2011, 152 (3-4), pp.374. 10.1016/j.vetmic.2011.05.004 . hal-00724206

HAL Id: hal-00724206

<https://hal.science/hal-00724206>

Submitted on 20 Aug 2012

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Accepted Manuscript

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PII: S0378-1135(11)00268-9
DOI: doi:10.1016/j.vetmic.2011.05.004
Reference: VETMIC 5301

To appear in: *VETMIC*

Received date: 26-11-2010
Revised date: 3-5-2011
Accepted date: 4-5-2011

Please cite this article as: Dang, S.T.T., Van Truong, D., Madsen, H., Dalsgaard, A., Survival of faecal indicator bacteria in treated pig manure stored in clay-covered heaps in Vietnam, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2011.05.004

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1 **Survival of faecal indicator bacteria in treated pig manure stored in clay-covered**
2 **heaps in Vietnam**

3

4

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27

28 **Abstract**

29 Small-scale pig farmers in Vietnam typically store manure in heaps covered by a
30 layer of clay. However, the hygienic quality of manure applied to agricultural soil is
31 uncertain as storage time is determined by the need for soil fertilizer. This study aimed
32 to assess how the storage of pig manure added straw, lime stone or urea in heaps
33 covered by clay affected the survival of faecal bacteria. *E. coli* was reduced from 4 log₁₀
34 CFU/g to below the detection limit within two weeks with 2% w/w urea (CO(NH₂)₂)
35 whereas other amendments were associated with limited *E. coli* reductions.
36 *Enterococcus* spp. were not reduced in any heaps indicating possible re-growth and that
37 enterococci should be used as hygiene indicators with caution. Follow-up research
38 should document the practical conditions under which the farmer practice of storing
39 manure with a clay cover can be utilized to obtain a bactericidal effect of ammonia and
40 a safe product with high fertilizer value.

41

42 **Key words:** Pig manure, urea, ammonia, *E. coli*, survival, Vietnam.

43

44 **1. Introduction**

45 The pig population in Viet Nam is expected to reach more than 33 million by 2011
46 (Huynh et al. 2007) with small-scale pig farming accounting for about 80% of pig
47 production (Lapar et al. 2003). Current management practices of pig manure in Vietnam
48 as well as other developing countries, i.e. collection, storage, transportation, treatment
49 and application, show that pig manure is mainly used as fertilizer in agricultural fields
50 (e.g. rice, maize) and gardens (root and leafy vegetables) or is discharged into aquatic
51 recipients, including fish ponds (Guan and Holley 2003;Unc and Goss 2004;Vu et al.
52 2007). Little knowledge is available about environmental hygiene and veterinary public
53 health hazards of such practices, e.g. occupational risks and food safety, including type
54 and transmission of main pathogens.

55 Vietnamese farmers typically add rice straw, plant waste, ash or lime stone to
56 manure to absorb liquid and to make it easier to handle and process, i.e. in small manure
57 heaps typically covered with a layer of clay that prevents dogs and other animals to
58 access and destroy the heaps (Vu, Tran, & Dang 2007). Composting and storage periods
59 vary between 3-4 months depending on crop growth periods and needs for fertilizer, but
60 with little if any awareness of hygiene impacts.

61 The use of a layer of clay on manure heaps implies reduced aeration, which is
62 likely to reduce the temperature development in the manure because the aerobic and
63 heat-generating composting process is inhibited. As temperature and exposure time are
64 key factors determining pathogen survival (Guan and Holley 2003;Nicholson et al.
65 2005), the hygienic safety of manure treated by current management practices is
66 uncertain. On the other hand, the combined use of clay cover and addition of lime stone
67 and possible urea may increase pH and ammonia concentrations to levels that could
68 significantly inactivate pathogens so the manure can safely be disposed off or used as
69 fertilizer (Vinnerås, 2007). The objective of this study was to assess how the storage of
70 pig manure added straw, lime stone, or urea in heaps covered by clay as practiced by

71 Vietnamese farmers affect hygienic quality as determined by the survival of the faecal
72 indicator bacteria *E. coli* and *Enterococcus* spp..

73

74 **2. Materials and methods**

75 *2.1. Treatment and storage of pig manure*

76 The study was conducted from January to March, 2008, at a small-scale farm located in
77 peri-urban Hanoi. This period is the coldest in northern Vietnam with night
78 temperatures down to 8-15°C. Survival of faecal bacterial indicators and pathogens are
79 usually longer at such low temperatures compared to the higher temperatures found
80 during the rest of year. Three heaps were made by mixing 500 kg of fresh solid pig
81 manure with 50 kg rice straw only, 50 kg rice straw + 2% lime stone (CaCO_3), or 50 kg
82 rice straw + 2% w/w urea ($\text{CO}(\text{NH}_2)_2$), respectively; and the heaps were then covered
83 by a 3-cm thick mud layer (Table 1). The addition of rice straw or other organic straw
84 material as well as lime stone is a normal practice of Vietnamese farmers. Lime stone is
85 traditionally added to the manure when making the heap partly to absorb liquid (i.e.
86 urine and water) but mainly because farmers use lime stone as fertilizer in the rice field
87 (Vu et al. 2007). Vietnamese farmers do not have any tradition of using slaked lime and
88 the associated increased pH levels as a sanitizer of organic wastes. The urea was added
89 to test effect on survival of faecal bacterial indicators at elevated ammonia levels, which
90 is produced when urea hydrolyses in the manure. All material in each heap was mixed
91 well with a shovel before covered by mud. Six glass thermometers were placed in six
92 different locations inside each heap to measure possible differences in development of
93 temperature. Those locations involved dividing each of the composting heaps into three
94 vertical parts (top, center, and bottom). Samples and temperature measurements were
95 collected at the right and left inside of the heap for all three parts of the heap. The
96 bottom radius of the heaps was about 2 m and they had a height of about 1.3 m.

97

98 2.2. *Sample collection and enumeration of fecal bacterial indicators*

99 Samples were collected at days 0, 14, 28 and 45 during storage of the manure. At these
100 four sampling times, sub-samples (100 g/sample) were collected from each of the six
101 locations in the heap. The samples were collected by carefully digging a sterile hard
102 plastic tube into the different locations in the heap. The mud crust was initially removed
103 from the area in which the plastic tube was to be inserted. Immediately after collecting
104 the sample, mud crust was placed to cover the hole made thereby minimizing the entry
105 of air into the heap. Also, it was noted that the hole made during sampling most often
106 quickly disappeared due to replacement of heap material. The samples were transported
107 to the laboratory in an insulated box with cooling elements and then analyzed the same
108 day for numbers of *E. coli* and *Enterococcus* spp. as well as total coliforms. A 10-g sub-
109 sample was weighed and added 90 ml peptone (Oxoid, Cambridge, England) diluents
110 containing 0.9% NaCl to make a stock solution (10^{-1}). This solution was serially diluted
111 from 10^{-1} to 10^{-8} , and 100 μ l samples were subsequently taken from each dilution and
112 spread on Selective *E. coli*/coliform Chromogenic medium agar plates (Oxoid) and
113 Slanetz & Batley agar plates (Oxoid) for enterococci enumeration. Each dilution was
114 analyzed in duplicate and agar plates were incubated at 44 °C for 24 h before bacterial
115 colonies were enumerated. The number of CFU (cell forming units)/g stored manure of
116 each indicator bacteria were calculated based on enumerations in two sequential
117 dilutions.

118

119 2.3. *pH and moisture content measurements*

120 The pH of manure samples was determined by pH meter (Precisa model pH 900,
121 Zürich, Switzerland) one hr after the samples were mixed with distilled water (1:5 v: v).
122 Samples were stored in sterile glass vials with closed lids. The moist manure samples
123 were weighed and dried in an oven at 100-105 °C for 6-8 hrs. The weight of the initial

124 moist sample was subtracted the remaining dry weight and the moisture content was
125 then expressed as a percentage of the wet weight.

126

127 2.4. Statistical analysis

128 Bacterial counts were logarithmically transformed (base 10) after adding 1 and these
129 were then analyzed using multiple linear regressions with location of manure sample in
130 the heap, method of treatment and storage time as categorical variables. Model
131 assumptions, normality and homogeneity of variance, were assessed by plots of
132 residuals, i.e. histogram and residual versus fitted plots, respectively. Temperature,
133 moisture content and pH were also added as explanatory factors in the model. This was
134 done one at a time due to the small sample size. P-values of <0.05 were considered
135 significant.

136

137 3. Results and discussions

138 3.1. Bacterial elimination in manure heap added straw and urea

139 The addition of 2% urea to the manure was associated with a fast and significant
140 reduction in numbers of *E. coli* and total coliforms (Figs. 1 and 2). None of the bacterial
141 counts varied significantly among locations within the heap on day 0. The pH was
142 slightly alkaline (mean value of 8.5) and increased over time ($p<0.001$). It was higher in
143 the middle and bottom section of the heap than in the top layer ($p<0.01$). No apparent
144 temperature increase was seen with a maximum measured temperature of 33 °C (Table
145 1). At day 0, the mean \log_{10} value concentrations of *E. coli* and total coliforms were
146 4.22 and 5.11 (CFU/g), respectively. Two weeks later numbers of *E. coli* and total
147 coliforms were below the detection limit of <5 (CFU/g) manure. At day 45, total
148 coliforms were found at low concentration 0.72 \log_{10} CFU/g which is likely due to re-
149 growth of some of the non-faecal bacterial species represented in the group of total
150 coliform bacteria, e.g. *Citrobacter* spp. or *Arizona* spp..

151 The fast reduction of *E. coli* and Gram-negative rods in the presence of ammonia
152 in animal manure is supported by earlier studies where *Salmonella* was eliminated
153 within two weeks at 14 °C in cow manure added 2% w/w urea (Ottoson et al. 2008).

154 The concentration of *Enterococcus* spp. in this treatment did not vary
155 significantly by location within the heap and varied during the experiment ($p < 0.05$) with
156 an initial concentration of 3.97 log₁₀ CFU/g at day 0 and a similar concentration at day
157 45, i.e 3.62 log₁₀ CFU/g (Fig. 3). The genus *Enterococcus* consists of several species of
158 which some, e.g. *E. faecium* and *E. faecalis*, are strictly of faecal origin. However, some
159 species, e.g. *E. casseliflavus* and *E. mundtii*, are not of faecal origin and may be found
160 in the environment, e.g. as plant-associated species (Petersen and Dalsgaard 2003). It is
161 unknown to what extent the added rice straw may have contributed with such non-faecal
162 *Enterococcus* spp. as initial analyses of rice straw for enterococci were not done. The
163 apparent lack of reduction in numbers of enterococci may be explained by survival and
164 multiplication of non-faecal *Enterococcus* spp., despite a reduction of *Enterococcus* spp.
165 of strictly faecal origin. Preliminary studies of *Enterococcus* spp. survival in stored
166 human urine did show elimination of *E. faecium* and *E. faecalis*, but clonal re-growth of
167 *E. gallinarum* (unpublished data). Our findings are supported by (Ottoson, Nordin, von
168 Rosen, & Vinnerås 2008) that also found only limited reductions in numbers of
169 enterococci in cow manure containing 2% w/w urea stored at 14 °C. Here it was
170 suggested that Gram-positive cocci with their lower cell-permeability as well as surface-
171 to-volume ration are less susceptible to ammonia than Gram-negative rods. Further
172 studies should investigate possible re-growth of certain *Enterococcus* spp. by
173 determination of species and genotypes involved as well as the explanatory factors
174 associated with a possible reduced susceptibility of *Enterococcus* spp. to ammonia. In
175 conclusion, *E. coli* seems to be a more appropriate bacterial indicator as compared with
176 *Enterococcus* spp. for monitoring hygienic quality of manure treated with urea.

177

178 3.2. Bacterial elimination in manure heaps added straw or straw + lime stone

179 The addition of straw only significantly reduced numbers of *E. coli* only (Fig. 1).
180 Counts of *E. coli* differed between these two treatments ($p < 0.05$) and varied over time
181 ($p < 0.001$), while there was no effect of location. The interaction between the two
182 treatments and time was not significant (location was dropped from this model).
183 Concentration of *E. coli* in the heap added straw only was reduced from $4.20 \log_{10}$
184 CFU/g on day 0 to $1.63 \log_{10}$ CFU/g after 45-days of storage. The mean temperature in
185 this manure heap was $34 \text{ }^{\circ}\text{C}$ with a maximum temperature of $47 \text{ }^{\circ}\text{C}$ registered at days 2,
186 3 and 4 and pH was neutral (7.5) with little changes measured (Table 1). These findings
187 indicate that *E. coli* and therefore possible also other Gram-negative rods, e.g.
188 *Salmonella*, are reduced significantly (2-log units) in clay-covered heaps with pig
189 manure amended rice straw following six weeks of storage. However, it should be noted
190 that a similar reduction in *E. coli* numbers was not seen in manure heaps added straw
191 together with lime (Fig. 1). It is difficult to explain this apparent lack of *E. coli*
192 reduction in the heap with lime, but it may partly be explained by little if any
193 temperature development in this heap (mean temperature of $29 \text{ }^{\circ}\text{C}$). It should also be
194 noted that the addition of lime stone had limited effect on pH which was only slightly
195 alkaline (pH=8.4)

196 Numbers of total coliforms were not reduced during storage of manure added
197 straw only and lime stone/straw with CFU/g ranging from 5-6 \log_{10} (Fig. 2).
198 Interestingly, however, total coliform counts were significantly higher at the bottom of
199 the heaps than in the top and the middle part ($p < 0.001$). *Enterococcus* spp. counts did
200 not differ significantly among locations in the heaps but differed between the two
201 treatments ($p < 0.05$) and varied among time points ($p < 0.001$). *Enterococcus* spp. counts
202 were higher in the treatment with lime than in the treatment with only straw at days 15,
203 30 and 45. An increase in CFU/g was seen at day 15 ($p < 0.001$) followed by a decline
204 until day 45 (Fig. 3).

205

206 *3.3. Influence of temperature, pH, and moisture content*

207 Temperature varied significantly over time ($p < 0.001$) and differed between the three
208 different manure heaps ($p < 0.001$) after adjusting for variation over time and location in
209 the heaps. Temperature did not vary significantly among the three locations within the
210 heaps. Also pH varied over time ($p < 0.001$) and between the three types of manure heaps
211 ($p < 0.001$) and variation among locations within heaps was not significant. Moisture
212 content only varied significantly over time, i.e. a gradual decline ($p < 0.001$). pH was
213 negatively associated ($p < 0.01$) with total coliforms counts after adjusting for main
214 effects of the composition of the heap (straw only versus straw/lime) and time.
215 Similarly, pH was negatively associated with enterococci counts ($p < 0.05$) after
216 adjusting for main effects of all three compositions of manure heaps and sampling
217 times.

218 The practice of almost all small-scale Vietnamese pig farmers in Northern
219 Vietnam is to cover manure heaps with clay. The readily availability of urea at low cost
220 offers a unique opportunity to utilize ammonia to inactivate pathogens and produce a
221 hygienic safe fertilizer with reduced storage time allowing the farmers to apply the
222 manure when needed in the field. Ammonia (NH_3) is a small, uncharged molecule that
223 can cross the membrane of bacteria and damage the cell either by causing rapid
224 alkalization of the cytoplasm or through a decrease in intracellular K^+ concentration
225 (Kadam and Boone 1996; Park and Diez- Gonzalez 2003). A negative effect on Gram-
226 negative bacteria has been reported at ammonia concentrations > 30 mM (Park and
227 Diez- Gonzalez 2003). A higher pH will result in higher concentrations of uncharged
228 ammonia. Thus, a higher pathogen die-off from urea treatment can be achieved by a
229 combined effect of ammonia and carbonate by the addition Na_2CO_3 or lime stone
230 (CaCO_3) at pH 9.5. However, it should be noted that in the current experiments, mean
231 pH values were only 8.4 and 8.5 in manure heaps added rice straw/lime stone and rice

232 straw/urea, respectively (Table 1). Since ammonia concentrations were not measured in
233 the heaps, we do not know if the reported ammonia threshold value of 30 mM ammonia
234 needed to produce a hygienic safe fertilizer were obtained. In addition to reducing
235 bacterial faecal indicators and pathogens, ammonia is also known to cause similar
236 reductions of viruses and helminth parasites (Nordin et al. 2009; Vinnerås et al. 2008).
237 Further studies, including cost-benefit analyses, should be done to evaluate whether the
238 mixing of different concentrations of urea with pig manure are applicable to common
239 operational practices of rural Vietnamese farmers as well as meeting the soil
240 requirements for nitrogen. In particular, it is of interest to determine the level of
241 ammonia gas emission in manure heaps covered by clay as is the normal practice of
242 Vietnamese farmers. It could be that the clay cover together with the practice to apply
243 manure by hand reduces ammonia emission to acceptable low levels at which the
244 fertilizer value of the added nitrogen is maintained. Further, the addition of urea may
245 enable the farmers to obtain a more rapid hygienization of the stored manure that will
246 allow them to apply safe manure fertilizers when nutrients are demanded by crops.

247

248 **4. Conclusion**

249 Further studies should determine under which conditions the common practice of
250 Vietnamese farmers to cover their manure heaps with clay can be utilized to obtain a
251 safe and high-value fertilizer by adding urea and utilize the bactericidal effect of the
252 ammonia generated. Documentation should be generated on the percentage of urea,
253 amount of straw material, pH and other conditions needed to obtain ammonia
254 concentrations sufficient to inactivate both bacterial, viral and parasite pathogens.
255 Considerations should be given to cost-effectiveness and applicability to common
256 operational practices of rural Vietnamese farmers.

257

258 **Acknowledgements**

259 This study was a part of PhD project, funded by the Danish International Development
260 Assistance (Danida) (SUSANE- research project No. J.nr.104. Dan.8.L.722). We thank
261 Tien Minh Tran for collaboration in pilot studies and guidance in establishing the
262 manure heaps and conducting the analyses of physico-chemical parameters. Sang
263 Nguyen Anh provided excellent support managing the manure heaps and during sample
264 collection. Ms. Huong from the Hanoi Agricultural University is thanked for help with
265 the bacterial enumerations. Lars Stoumann Jensen is thanked for critical comments to
266 the manuscript.

267

268 **References**

269

References

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271 Guan, T.Y. & Holley, R.A. 2003. Pathogen survival in swine manure environments and
272 transmission of human enteric illness: A review. *J.Environ.Qual.*, 32, 383-392

273 Huynh, T.T.T., Aarnink, A.J.A., Drucker, A., & Verstegen, M.W.A. 2007. Pig
274 Production in Cambodia, Laos, Philippines, and Vietnam: A Review. *Asian*
275 *J.Agric.Dev.*, 4, 70-90

276 Kadam, P.C. & Boone, D.R. 1996. Influence of pH on ammonia accumulation and
277 toxicity in halophilic, methylotrophic methanogens. *Appl.Environ.Microbiol.*, 62, 4486-
278 4492

279 Lapar, M.L., Vu, T.B., & Ehui, S. 2003. Identifying barriers to entry to livestock input
280 and output markets in Southeast Asia: The case of Vietnam. *Socioecon.Policy Res.*, 56,
281 1-50

282 Nicholson, F.A., Groves, S.J., & Chambers, B.J. 2005. Pathogen survival during
283 livestock manure storage and following land application. *Bioresour.Technol.*, 96, 135-
284 143

285 Nordin, A., Nyberg, K., & Vinnerås, B. 2009. Inactivation of *Ascaris* eggs in
286 source-separated urine and feces by ammonia at ambient temperatures.
287 *Appl.Environ.Microbiol.*, 75, 662-667

288 Ottoson, J., Nordin, A., von Rosen, D., & Vinnerås, B. 2008. Salmonella reduction in
289 manure by the addition of urea and ammonia. *Bioresour.Technol.*, 99, 1610-1615

290 Park, G.W. & Diez- Gonzalez, F. 2003. Utilization of carbonate and ammonia-based
291 treatments to eliminate *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104
292 from cattle manure. *J.Appl.Microbiol.*, 94, 675-685

293 Petersen, A. & Dalsgaard, A. 2003. Species composition and antimicrobial resistance
294 genes of *Enterococcus* spp, isolated from integrated and traditional fish farms in
295 Thailand. *Environ.Microbiol.*, 5, 395-402

- 296 Unc, A. & Goss, M.J. 2004. Transport of bacteria from manure and protection of water
297 resources. *Appl.Soil Ecol.*, 25, 1-18
- 298 Vinnerås, B., Nordin, A., Niwagaba, C., & Nyberg, K. 2008. Inactivation of bacteria
299 and viruses in human urine depending on temperature and dilution rate. *Water Res.*, 42,
300 4067-4074
- 301 Vu, T.K.V., Tran, M.T., & Dang, T.T.S. 2007. A survey of manure management on pig
302 farms in Northern Vietnam. *Livest.Sci.*, 112, 288-297
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306 Table 1: Characteristics and composition of heaps with pig manure.

307

308 Figure 1. Temporal reduction of *E. coli* in heaps with stored pig manure following
309 different treatment methods.

310

311 Figure. 2. Temporal reduction of total coliforms in stored pig manure.

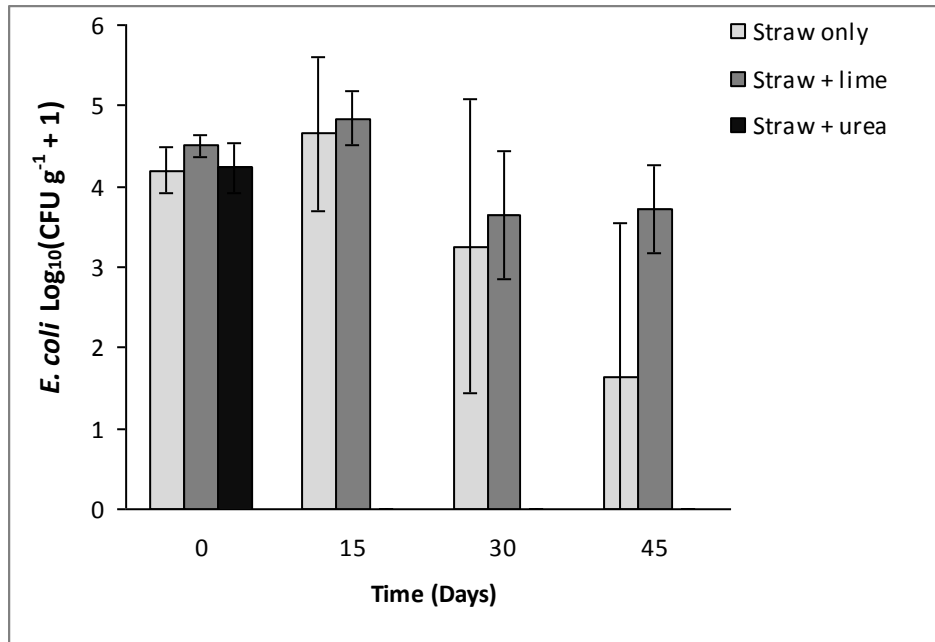
312

313 Figure. 3. Temporal reduction of *Enterococcus* spp. in stored pig manure.

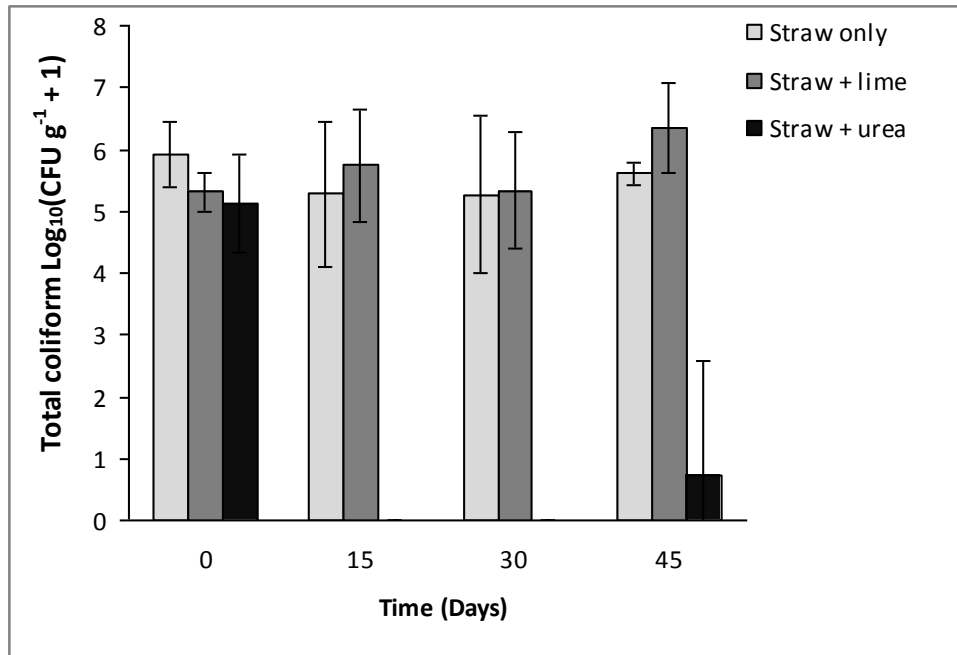
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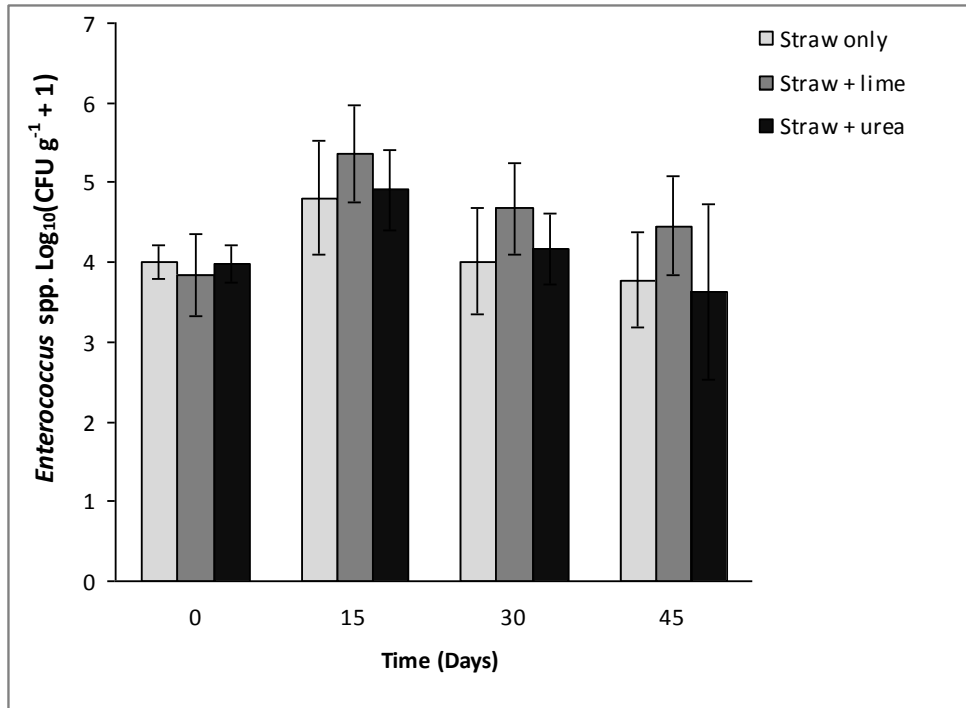
Table 1: Characteristics and composition of heaps with pig manure.

Characteristics/ Composition of manure heap	500 kg manure, 50 kg rice straw	500 kg manure, 50 kg rice straw, 2% lime stone (CaCO ₃)	500 kg manure, 50 kg rice straw, 2% w/w urea
Duration of storage (days)	45	45	45
Moisture (%)	67.9 ± 4.6	67.6 ± 3.8	67.7 ± 5.7
pH	7.5 ± 1.2	8.4 ± 0.6	8.5 ± 0.3
Temperature (°C)	34.3 ± 7.7	23.9 ± 2.2	26.4 ± 2.5
Maximum temperature (°C)	47	29	33
Time with highest measured temperature	Days 2, 3, 4	Days 10-18	Days 6, 7, 8



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