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ORIGINAL ARTICLE

Microbial load in bio-slurry from different biogas plants in Bangladesh

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ABSTRACT

Objective: The study was aimed to isolate, identify, and characterize common indicator bacteria, including *Escherichia coli*, *Salmonella* spp., and *Staphylococcus* spp. in manure and bio-slurry samples of different livestock farms and biogas plants of Bangladesh.

Materials and Methods: A total of 114 samples of manure and bio-slurry were collected from different livestock farms and biogas plants in Bangladesh. The total viable count (TVC), *E. coli, Salmonella* spp., and *Staphylococcus* spp. counts were determined by the spread plate technique method. Isolation and identification were performed by colony characteristics, staining, biochemical tests, and, finally, by using PCR. Antibiotic susceptibility test of the isolated bacteria was tested against commonly used antibiotics by using the disk diffusion method.

Results: The mean TVC, *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. counts were ranged from 8.19–10.75, 5.2–6.96, 5.81–6.87, 5.68–7.68 in manure samples and 7.26–8.65, 3.82–5.2, 4–5.54, 3.14–5.9 log cfu/gm in bio-slurry, respectively. In anaerobic digester after 30 days digestion, the presence of *E. coli, Salmonella* spp., and *Staphylococcus* spp. varied from 0–5.11, 0–4.84, and 0–5.59 log cfu/gm at 25°C, 27°C, 29°C, and 45°C temperature. Above-mentioned bacteria were absent in bio-slurry collected from anaerobic digester after 60 days digestion at environmental temperature. Bacterial counts were reduced significantly in both household slurry pits and experimental anaerobic digester. Antibiotic susceptibility results revealed that multidrug-resistant indicator bacteria were present in the bio-slurry samples.

Conclusion: Our findings conclude that the microbial load after treatment of animal manure via anaerobic digestion (Biogas plant) was grossly reduced and the reduction of bacterial pathogen depends on the duration and temperature of digestion.

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Manure; bio-slurry; anaerobic digester; indicator bacteria; antibiogram.

Introduction

Livestock manures are the undigested and discharged contents of farm animals which are used as fertilizer in soil land but this waste material has a significant effect on public health by contaminating the air, water, and soil. In Bangladesh, the livestock sector consists of 25.57 million large ruminants, 29.56 million small ruminants, and 337.99 million poultry where 70%–80% of them were raised by small household farms [1]. According to the Integrated Livestock Manure Management Policy [2], about 151.3 million tons of fresh manure are produced by farms animals and 4.52 million tons by poultry species per year [2].

Bio-slurry is an anaerobic processed natural material discharged as result from the biogas plant after generation of burnable methane gas for cooking, lighting, and running hardware [3]. It can also be widely used as fertilizer for crop production, containing higher nutrient than chemical fertilizer [4]. Untreated animal excreta like cow dung, poultry manure contain many diseases causing pathogenic microorganisms that might pose serious health problems to human being [5,6]. The bacterial pathogens, including *Salmonella*, *Listeria monocytogenes*, *Mycobacterium avium* subsp. *Paratuberculosis, Clostridium* spp., *Bacillus* spp., *Campylobacter, L. monocytogenes, Yersinia enterocolitica,*

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Staphylococcus, and *Streptococci* are isolated from the manure and bio-slurry by previous authors and interestingly most of the isolated bacteria have a significant relation to human health [7,8]. An important pathogenic *Escherichia coli* that are frequently recovered from livestock manures and most of them are pathogenic *E. coli* such as EHEC strains producing cytotoxins (*stx*1 and *stx*2) [9]. Discharging the biogas effluent in lands may lead to serious damage in both human and grazing animals as pathogens remain survive long time in soil, air, water, and even in underground water [10].

Livestock manure treated with anaerobic digestion system reduced the number of bacteria drastically [11]. Some studies noted that pathogen can even survive after anaerobic digestion [12] and also demonstrated that survived bacteria can grow in soil land after application [13]. Bacteria like *E. coli, Salmonella* completely eliminate from bio-slurry after 60 days of anaerobic digestion at 37°C, but *Listeria* spp. can remain [14]. Another study was carried out in Italy [15], which found *L. monocytogenes* in very low amount in bovine manure after treatment where *E. coli, Yersina*, and *Salmonella* spp. were completely reduced. A minimum storage period of at least 30 days was required to reduce the risk of pathogens and maintain at least 60 days interval between application of bio-slurry and planting [16]. In Bangladesh, biogas technology is getting popular day by day to meet the energy crisis. The residue produced from biogas plant has been used as the alternative fertilizer, nearly 31,000 biogas plants have been installed by the year of 2013 [17]. The huge amount of bio-slurry produced from the biogas plant is not being disposed of properly in soli lands; hence, it causes environmental pollution and also spreads zoonotic pathogens [4]. However, most of the research works conducted on bio-slurry in Bangladesh is related to fertilizer- and energy-based not based on bacterial pathogens. Against this background, this study sought (i) to determine the total viable bacteria and indicator bacterial load in manure and bio-slurry collected from different livestock farms and biogas plants of Bangladesh (ii) to isolate, identify, and study antibiogram profile of isolated *E. coli, Salmonella* spp., and *Staphylococcus* spp.

Materials and Methods

Sample collection

A total of 114 samples of manure and bio-slurry were collected from different livestock farms and biogas plants of different district of Bangladesh during the period from January to November 2017. The samples comprised of 24 manure samples from eight different livestock farms and 90 bio-slurry samples where 48 samples from 16 biogas plants, 12 from 4 experimental anaerobic digester after 30 days of digestion at different temperatures (25°C, 27°C, 29°C, and 45°C), and 30 from 10 different experimental anaerobic digester after 60 days of digestion at environmental temperature. From each farm, biogas plant, and anaerobic digester, samples were collected at three different time points. For microbial analysis, 1 gm of the sample was homogenized with 9 ml of phosphate-buffered saline solution. After mixing, serial dilution was made from 10^{-1} to 10^{-8} for culturing in different types of bacteriological media.

Microbial analysis

Spread plate technique was used to enumerate the total viable bacteria, *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. [18]. All the media were prepared according to manufactures instructions. For enumeration of total viable count (TVC), nutrient agar media (NA) were used. From each dilution, 0.1 ml was inoculated on the center of the respective agar media by sterile pipette and spread by a sterile glass rod. After that, the plates were incubated at 37°C for 24 h. Following incubation, colonies appeared on NA were counted and calculated by multiplying average number of colonies in particular dilution with dilution factors and recorded as colony-forming unit per gram of samples. *Escherichia coli*, *Salmonella* spp., and *Staphylococcus* spp. were enumerated the same way using eosin methylene blue (EMB), Salmonella-shigella (SS), and mannitol salt (MS) agar, respectively. Colonies shown in metallic sheen in EMB, black color in SS, and yellow color in MS were counted as *E. coli, Salmonella* spp., and *Staphylococcus* spp.

Isolation and identification of bacteria

To isolate pure colony, bacteria that grow on different media were subcultured on respective agar plates. All the isolates of respective bacteria were identified based on cultural characteristics, morphological characteristics, biochemical test including sugar fermentation, Methyl red, Voges–Proskauer, indole, coagulase tests [19] and finally confirmed by molecular characterization. Previously published genus-specific primers were used to identify the microorganisms [20–22] (Table 1).

Table 1. List of primers used.

Primer	Sequence	Size (bp)	References
E. coli (F)	5′-AATTGAAGAGTTTGATCATG-3′	704	[20]
E. coli (R)	5′-CTCTACGCATTTCACCGCTAC-3′		
Salmonella common (F)	5′-ACTGGCGTTATCCCTTTCTCTGGTG-3′	496	[21]
Salmonella common (R)	5′-ATGTTGTCCTGCCCCTGGTAAGAGA-3′		
Staphylococcus spp.16S (F)	5′-GGAGGAAGGTGGGG ATGACG-3′	241	[22]
Staphylococcus spp. 16S (R)	5′-ATGGTGTGACGGGC GGTGTG-3′		

Antibiotic susceptibility test

All the isolated bacteria were subjected to antimicrobial susceptibility test by using disk diffusion or Kirby-Bauer method [23]. In the current study, 14 commonly available antibiotics include amoxicillin (30 µg), ampicillin (25 µg), azithromycin (30 µg), ciprofloxacin (5 µg), neomycin (30 µg), oxacillin (1µg), norfloxacin (10 µg), gentamycin (10 µg), erythromycin (5 µg), penicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), streptomycin (10 µg), and nalidixic acid (30 µg) of HiMedia, India, were used. The zone inhibition produced by the respective bacteria was compared with the standards of the Clinical and Laboratory Standards Institute [24].

Results and Discussion

Bacterial load in manure and bio-slurry

In manure sample TVC, *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. were varied from log 8.19–10.75, 5.2–6.96, 5.81–6.87, and 5.68–7.68 cfu/gm (Table 2). Average TVC, *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. were recorded as 9.77, 6.11, 6.23, and 6.81 log cfu/gm, respectively. Microbial counts in manure sample in this study are in agreement with previously conducted research studies [15,25,26]. In bio-slurry samples of natural bio-slurry pits, TVC ranged from 7.26 to 8.65 log cfu/gm with the highest number of *E coli* was found at Singair-01, Manikgonj 5.20 log cfu/gm and lowest at Kapashia-1, Gazipur 3.82 log cfu/gm, *Salmonella* spp. was found highest at Buffalo farm BLRI, Savar 5.54 log cfu/gm and lowest at Phoenix-04, Gazipur 4.00 log cfu/gm samples and *Staphylococcus* spp. highest at Phoenix-03, Gazipur 5.90 log cfu/gm and lowest at Kapashia-2, Gazipur 3.14 log cfu/gm. Indicator bacteria (*E. coli, Salmonella* spp., and *Staphylococcus* spp.) were always present in all the samples from natural bio-slurry pits (Table 3). Huong et al. [27] enumerated the *Salmonella* and other indicator bacteria in pig bio-slurry samples in Vietnam and recovered a huge number of bacteria. In the current study, no significant difference was found between the manure of livestock farms and bio-slurry samples of natural bio-slurry pits of different farms.

Table 2. Bacterial load in manure samples.

Sample name/ Collection place (*n* = 24)	Bacterial load (log cfu/gm ± SD) in manure			
	TVC	*E. coli*	*Salmonella* spp.	*Staphylococcus* spp.
Dairy farm, BAU	10.66 ± 0.21	5.92 ± 0.01	6.34 ± 0.65	7.07 ± 0.72
Ambagan, BAU	9.42 ± 0.12	6.96 ± 0.04	6.02 ± 0.02	7.68 ± 0.37
Dairy farm, BLRI, Savar	10.19 ± 0.09	6.47 ± 0.23	6.4 ± 0.31	7.24 ± 0.08
Singair, Manikgonj	9.11 ± 0.31	5.89 ± 0.09	6.08 ± 0.48	6.66 ± 0.12
Kapashia, Gazipur	8.19 ± 0.20	6.02 ± 0.80	6.87 ± 0.12	6.82 ± 1.1
Phoenix Hatchery-01, Gazipur	9.95 ± 0.12	6.35 ± 1.2	5.98 ± 0.07	5.68 ± 0.45
Phoenix Hatchery-02, Gazipur	10.75 ± 0.17	5.2 ± 0.93	6.34 ± 0.10	6.34 ± 0.05
Fosiler More, BAU	9.88±0.05	6.05 ± 0.03	5.81 ± 0.03	6.97 ± 0.76

BAU = Bangladesh Agricultural University, BLRI = Bangladesh Livestock Research Institute TVC = Total Viable Count, SD = Standard deviation, cfu = Colony Forming Unit.

Bacterial load in experimental anaerobic digester after 30 and 60 days of digestion

After 30 days of digestion in an anaerobic digester, the highest number of *E coli* was obtained from the digester operated at 25°C at GEKH (Green Energy Knowledge Hub, Bangladesh Agricultural University (5.11 log cfu/gm), *Salmonella* spp. and *Staphylococcus* spp. were observed highest in number when the digester was operated at 27°C at GEKH, BAU (4.84 and 5.59 log cfu/gm). No common indicator bacteria were found when digester operated at 45°C at GEKH, BAU (Table 4). Among the 30 bio-slurry samples from anaerobic digester after 60 days at environmental temperature, TVC was found from 2.29 to 3.96 log cfu/gm. Common indicator bacteria *E. coli, Salmonella* spp., and *Staphylococcus* spp. were not found in any samples (Table 5). Similar results were also reported by several researchers in other parts of the world**.** Costa et al. [28] found the lower number of bacterial load including coliforms, lactobacillus, and streptococci after anaerobic digestion in bio-slurry samples. Philipp and Holzle [29] reported that *E coli* were absent when the digester was operated at 55°C. Wagner et al. [30] were also observed that *Salmonella* spp. and *E. coli* were reduced below the detection limit when the anaerobic digestion was performed at 50°C. Both of these results support current findings of the absence of common indicator bacteria when the anaerobic digester was operated at 45°C. The increased temperature has a great influence on the reduction of microbes during anaerobic digestion.

Average log reduction of bacterial load between manure and bio-slurry samples

The log reduction of TVC, *E. coli, Salmonella* spp., and *Staphylococcus* spp. were 1.89, 1.45, 1.64, and 1.37 from manure sample to natural bio-slurry pits and the reduction was statistically significant ($p < 0.05$). In every condition of an experimental anaerobic digester at GEKH,

BAU at different temperatures found the huge reduction of the bacterial population after 30 days digestion and reduction rate was statistically significant. From manure to 60 days digested bio-slurry at an environmental temperature in an experimental anaerobic digester, the log reduction of TVC was 6.64 and *E. coli, Salmonella* spp., and *Staphylococcus* spp. were fully reduced and this reduction was also statistically significant ($p < 0.05$) (Table 6). The results of the present study are in agreement with the findings of other studies on the bacterial reduction in biogas plants [31–33]. In this study, we found common indicator bacteria were reduced from manure to bio-slurry but not eliminated because the elimination of bacteria depends on several factors, pH, temperature, availability of nutrients, and also on their initial amount in the waste.

Isolation and identification of bacteria

A total of 60 isolates of *E. coli, Salmonella* spp., and *Staphylococcus* spp. were isolated from the samples where each organism was 20. Isolation was done based on their cultural characteristics in respective agar media, *E. coli* showed greenish-black color in EMB, *Salmonella* spp. black center color in SS, and *Staphylococcus* spp. yellowish color colonies in MS agar media, respectively. The findings of the current study support the results of previous studies [34–36]. In Gram's staining, *E. coli* appeared as pink color single or paired rod-shaped, *Salmonella* spp. as pink color rod-shaped, and *Staphylococcus* spp. as violet color cocci shaped arranged in grapes like a cluster. All the isolates of three organisms were found positive in their respective biochemical test. Finally, confirmation was done by PCR using genus-specific primers (Figs. 1–3). PCR results of this study were similar to the results of previous findings [20,37,38].

Antimicrobial susceptibility test

Antibiotic resistance is a global problem nowadays. Antibiotics used in the veterinary sector for animal production and subsequent application of their effluent in the soil environment increased antibiotic resistance. Antibiotic susceptibility testing revealed that all the isolates of *Staphylococcus* spp. were found 100% resistant to ampicillin, amoxicillin, and penicillin where less resistant rate was observed against azithromycin (0%), gentamycin (10%), norfloxacin (10%), nalidixic acid (10%), ciprofloxacin (10%), neomycin (15%), erythromycin (15%), and streptomycin (20%). *Salmonella* spp. was found resistant to ampicillin, amoxicillin (100%), and nalidixic acid (80%). *Escherichia coli* was susceptible against gentamycin, chloramphenicol, azithromycin, norfloxacin, erythromycin, and tetracycline (Table 7). Duriez and Topp [39] found *E. coli*

Table 3. Bacterial load in bio-slurry samples of natural bio-slurry pits.

Sample name/Collection place (*n* = 48)	Bacterial load (log cfu/gm ± SD) in bio-slurry			
	Total viable count	*E. coli*	*Salmonella* spp.	*Staphylococcus* spp.
Phoenix Hatchery-01, Gazipur	8.47 ± 0.02	4.92 ± 0.14	4.41 ± 0.08	5.65 ± 0.09
Phoenix Hatchery-02, Gazipur	7.74 ± 0.08	3.87 ± 0.22	4.64 ± 0.13	5.68 ± 0.12
Phoenix Hatchery-03, Gazipur	8.15 ± 0.04	3.95 ± 0.02	4.53 ± 0.11	5.90 ± 0.10
Phoenix Hatchery 04, Gazipur	8.65 ± 0.05	4.54 ± 0.15	4.00 ± 0.10	5.85 ± 0.09
Kapashia -1, Gazipur	8.05 ± 0.04	3.82 ± 0.02	4.30 ± 0.001	5.28 ± 0.13
Kapashia -2, Gazipur	7.26 ± 0.03	4.46 ± 0.18	4.18 ± 0.12	3.14 ± 0.06
Kapashia -3,Gazipur	7.29 ± 0.03	5.00 ± 0.12	4.90 ± 0.11	5.00 ± 0.07
Dairy Farm BLRI, Savar	8.20 ± 0.11	4.96 ± 0.08	4.20 ± 0.14	5.44 ± 0.13
Buffalo Farm BLRI, Savar	7.67 ± 0.01	4.75 ± 0.14	5.54 ± 0.09	5.62 ± 0.9
Singair-01,Manikgonj	7.69 ± 0.12	5.20 ± 0.03	4.76 ± 0.12	5.68 ± 0.12
Singair-02, Manikgonj	7.76 ± 0.13	4.48 ± 0.08	4.92 ± 0.03	5.74 ± 0.14
Vaccine project, BAU	8.24 ± 0.07	4.88 ± 0.13	4.85 ± 0.15	5.41 ± 0.08
Ambagan-1 ,BAU	7.77 ± 0.02	4.97 ± 0.07	4.20 ± 0.01	5.75 ± 0.12
Ambagan-2, BAU	7.77 ± 0.01	4.90 ± 0.01	4.78 ± 0.20	5.59 ± 0.12
Dairy Farm, BAU	7.73 ± 0.12	4.85 ± 0.01	4.58 ± 0.04	5.65 ± 0.02
Fosiler More, BAU	7.75 ± 0.09	5.04 ± 0.11	4.68 ± 0.90	5.73 ± 0.03

BAU = Bangladesh Agricultural University, BLRI = Bangladesh Livestock Research Institute, TVC = Total Viable Count, SD = Standard deviation, cfu = Colony Forming Unit.

Table 4. Bacterial load of the bio-slurry sample from anaerobic digester after digestion of 30 days.

Sample name/ Collection place (*n* = 12)	Bacterial load (log cfu/gm ± SD) in bio-slurry			
	TVC	*E. coli*	*Salmonella* spp.	*Staphylococcus* spp.
GEKH, BAU (45°C)	2.85 ± 0.02*	0	0	0
GEKH, BAU (29°C)	6.96 ± 0.03	4.41 ± 0.07	4.66 ± 0.11	5.02 ± 0.09
GEKH, BAU (27°C)	7.01 ± 0.12	4.53 ± 0.03	4.84 ± 0.09	5.59 ± 0.11
GEKH, BAU (25°C)	7.29 ± 0.02	5.11 ± 0.01	4.50 ± 0.06	5.23 ± 0.10

BAU = Bangladesh Agricultural University, GEKH = Green Energy Knowledge Hub, TVC = Total Viable Count, SD = Standard deviation, cfu = Colony Forming Unit.

Table 5. Bacterial load in bio-slurry samples of experimental anaerobic digester after digestion of 60 days.

Sample name (*n* = 30)	Bacterial load (log cfu/g ± SD) in bio-slurry			
	Total viable count	*E. coli*	*Salmonella* spp.	*Staphylococcus* spp.
D-1	2.85 ± 0.07	0	0	0
D-2	3.53 ± 0.03	0	0	0
D-3	2.85 ± 0.02	0	0	0
D-4	3.96 ± 0.13*	0	0	0
D-5	3.01 ± 0.12	0	0	0
D-6	2.29 ± 0.19*	0	0	0
D-7	3.85 ± 0.05	0	0	0
D-8	2.93 ± 0.03	0	0	0
D-9	2.81 ± 0.12	0	0	0
D-10	3.29 ± 0.03	0	0	0

D = Digester, SD = Standard deviation, cfu = Colony Forming Unit.

and other indicator bacteria resistance to ampicillin, chloramphenicol, streptomycin, nalidixic acid, cephalothin, tetracycline, sulfamethoxazole, kanamycin, and trimethoprim in manure samples.

Escherichia coli can cause bloody diarrhea, abdominal cramps, fever, vomiting and nausea, and sometimes can cause severe anemia or kidney failure that leads to death in individual, especially young children [9]. *Salmonella* spp. is commonly found in manure and may survive in the environment up to 1 year if get favorable conditions and can cause serious infection in both human and animals [40]. Another indicator bacteria abundantly found in bio-slurry is *Staphylococcus* spp., zoonotic pathogens, which can transmit to human easily through the food chain [41].

Anaerobic digestion in the bio-slurry pit is an effective system for managing the manure and converts it to bio-slurry in the livestock farms. However, present results documented that certain indicator bacteria can survive in the natural bio-slurry pit even in the experimental anaerobic digester at a lower temperature. Although bacterial pathogens reduced significantly from manure to bio-slurry samples but not fully eliminated. The indicator bacteria present in bio-slurry used as effluent in soil land can easily transmit to fertilized crops. Some vegetables consumed as raw forms such as salad, carrots, tomato, and cucumber can easily contaminate with pathogenic microorganisms and likely to transfer human body. In addition, this effluent sometimes discharges in an external environment like channels, river that may pollute the aquatic environment as well as underground water that may enter into household wells. That way antibiotic-resistant bacteria can transmit from environmental setting to human and animal bodies. Present research did not correlate common indicator bacterial count with the other factors, such as pH, availability of nutrients, and the initial

Table 6. Average log reduction of bacterial load between manure to bio-slurry samples.

Bacterial load	Manure	Log reduction from manure to bio-slurry samples in different condition					
		Bio-slurry pits	Anaerobic digestion after 30 days				Anaerobic digestion after 60 days
			25°C	27°C	29°C	45°C	
Total viable count	9.77	7.88 (1.89)	7.29 (2.48)	7.01 (2.76)	6.96 (2.81)	2.85 (6.92)	3.13 (6.64)
E. coli	6.11	4.66 (1.45)	5.11 (1)	4.53 (1.58)	4.41 (1.7)	0 (6.11)	0 (6.11)
Salmonella spp.	6.23	4.59 (1.64)	4.5 (1.73)	4.84 (1.39)	4.66 (1.57)	0 (6.23)	0 (6.23)
Staphylococcus spp.	6.81	5.44 (1.37)	5.23 (1.58)	5.59 (1.22)	5.02 (1.79)	0 (6.81)	0 (6.81)

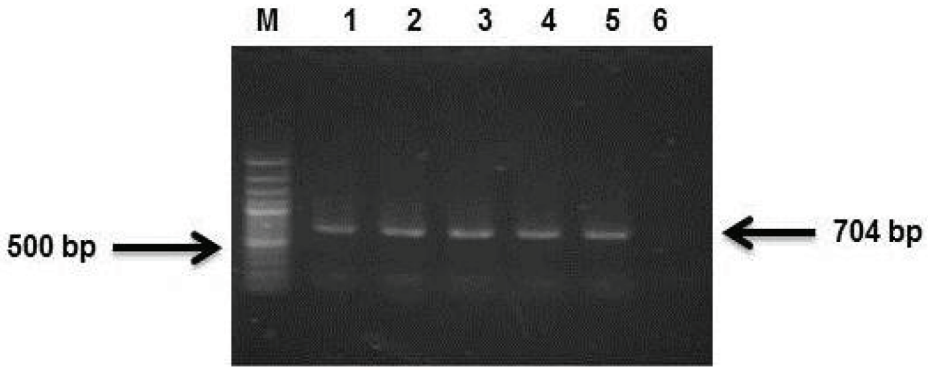

Figure 1. PCR for amplification of partial 16S rRNA gene of *E. coli*, Lane M: DNA marker, Lanes 1–4: tested isolates, Lane 5: positive control, and Lane 6: Negative control.

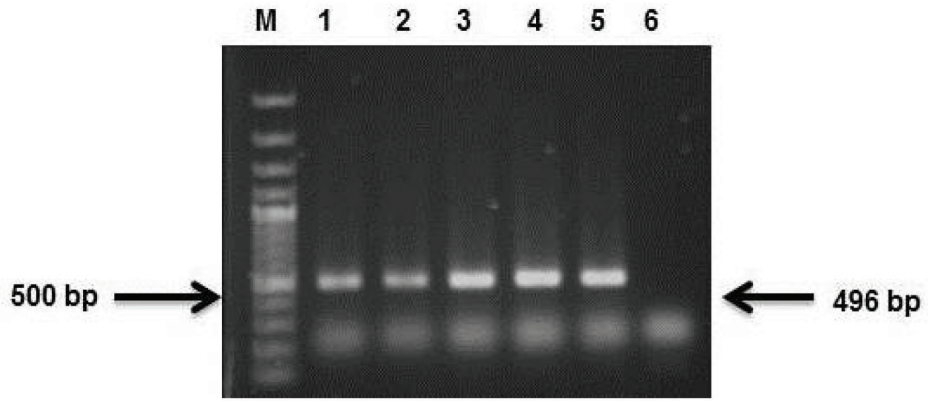

Figure 2. PCR for amplification of *Salmonella* genus, Lane M: DNA marker, Lanes 1–4: tested isolates, lane 5: positive control, and lane 6: negative control.

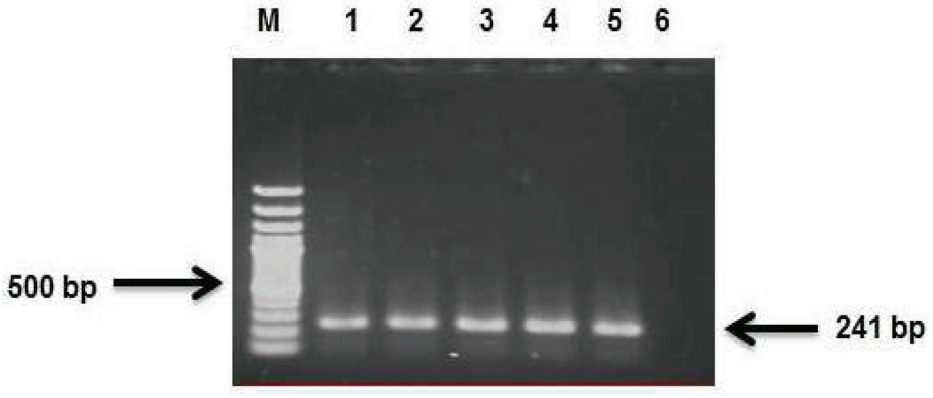

Figure 3. PCR for amplification of *Staphylococcus* genus, Lane M: DNA marker, Lanes 1–4: tested isolates, lane 5: positive control, and lane 6: negative control.

amount of manure. Here, isolated bacteria were identified up to genus level not to species. A more complete characterization of the bacteria is warranted, particularly in the context of understanding the zoonotic importance of pathogens. Hence, these limit us for a better conclusion of results.

Table 7. Antimicrobial susceptibility test of isolated bacteria.

Organisms	No (%) of resistance isolates												
	AMP	AMX	Gen	E	NX	AZM	CIP	N	P	S	C	TE	NA
Staphylococcus spp. (*n* = 20)	20 (100)	20(100)	2 (10)	3 (15)	2(10)	0(0)	2(10)	3(15)	20(100)	4(20)	-	-	-
Salmonella spp. (*n* = 20)	20(100)	20(100)	4(20)	8(40)	5(25)	2(10)	3(15)	-	-	-	2(10)	4(20)	16(80)
E. coli (*n* = 20)	20(100)	18(90)	3(15)	7(35)	6(30)	5(25)	8(40)	-	-	-	3(15)	7(35)	15(75)

AMP =Ampicillin, AMX = Amoxicillin, GEN = Gentamicin, E = Erythromycin, NX = Norfloxacin, AZM = Azithromycin, CIP = Ciprofloxacin, N = Neomycin, P = Penicillin, S = Streptomycin, C = Chloramphenicol, TE = Tetracycline, NA = Nalidixic acid.

Conclusion

Common indicator bacteria were present in all bio-slurry samples collected from natural bio-slurry pits. Significantly reduced numbers of indicator bacteria were present in bio-slurry samples after 30 days digestion in the experimental anaerobic digester at different temperatures (25°C, 27°C, and 29°C) and absence of common indicator bacteria at 45°C operated anaerobic digester. No indicator bacteria were present in bio-slurry after 60 days digestion in the experimental anaerobic digester at environmental temperature. To the best of our knowledge, this is the first report on the microbial status of manure and bio-slurry samples in Bangladesh.

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Conflict of interest

The authors declare that there is no conflict of interest toward the publication of this article.

Authors' contribution

MAI, PB, ZFH, and AAMS carried out the experiments, analyzed the data, and wrote the initial draft of the manuscript. CKS and MMA supplied the experimental anaerobic digested bio-slurry samples. SS and MTR designed and supervised research work, rewrite, and finalized the manuscript. All authors read and approved the manuscript before submission.

References

[1] District Livestock Services (DLS). Annual report 2017–2018. Department of Livestock Services, Bangladesh. Available via www.dls.gov.bd

[2] Integrated Livestock Manure Management Policy (ILMMP). Government of the People's Republic of Bangladesh, Ministry of Fisheries and Livestock, pp 1-2, 2015. Available via https://mofl.portal.gov.bd/sites/default/files/files/mofl.portal.gov.bd/page/221b5a19_4052_4486_ae71_18f1ff6863c1/ILMM%20Policy.pdf

[3] Islam MS. Use of bioslurry as organic fertilizer in Bangladesh agriculture. In Prepared for the presentation at the International Workshop on the Use of Bioslurry Domestic Biogas Programme. Bangkok, Thailand, 2006 Sep 27.

[4] Shaheb MR, Nazrul MI, Khan AM. Agro economic performance of Bio-slurry on boro rice cultivation in some sites of Moulvibazar district. Bangladesh J Agric Res 2017; 42(2):363–71; https://doi.org/10.3329/bjar.v42i2.32821

[5] US Environmental Protection Agency (USEPA). National pollutant discharge elimination system permit regulation and effluent limitations guidelines and standards for concentrated animal feeding operations: final rule. Fed Regist 2003; 68(29):7175–274.

[6] Albihn A, Vinnerås B. Biosecurity and arable use of manure and biowaste-Treatment alternatives. Livest Sci 2007; 112(3):232–9; https://doi.org/10.1016/j.livsci.2007.09.015

[7] Bagge E, Sahlström L, Albihn A. The effect of hygienic treatment on the microbial flora of biowaste at biogas plants. Water Res 2005; 39(20):4879–86; https://doi.org/10.1016/j.watres.2005.03.016

[8] Massé D, Gilbert Y, Topp E. Pathogen removal in farm-scale psychrophilic anaerobic digesters processing swine manure. Bioresour Technol 2011; 102(2):641–6; https://doi.org/10.1016/j.biortech.2010.08.020

[9] European Food Safety Authority (EFSA). Urgent advice on the public health risk of Shiga-toxin producing *Escherichia coli* in fresh vegetables. EFSA J 2011; 9(6):2274; https://doi.org/10.2903/j.efsa.2011.2274

[10] Nicholson FA, Groves SJ, Chambers BJ. Pathogen survival during livestock manure storage and following land application. Bioresour Technol 2005; 96(2):135–43; https://doi.org/10.1016/j.biortech.2004.02.030

[11] Aitken MD, Sobsey MD, Van Abel NA, Blauth KE, Singleton DR, Crunk PL, et al. Inactivation of *Escherichia coli* O157: H7 during thermophilic anaerobic digestion of manure from dairy cattle. Water Res 2007; 41(8):1659–66; https://doi.org/10.1016/j.watres.2007.01.034

[12] Sidhu JP, Toze SG. Human pathogens and their indicators in biosolids: a literature review. Environ Int 2009; 35(1):187–201; https://doi.org/10.1016/j.envint.2008.07.006

[13] Johansson M, Emmoth E, Salomonsson AC, Albihn A. Potential risks when spreading anaerobic digestion residues on grass silage crops-survival of bacteria, moulds and viruses. Grass Forage Sci 2005; 60(2):175–85; https://doi.org/10.1111/j.1365-2494.2005.00466.x

[14] Goberna M, Podmirseg SM, Waldhuber S, Knapp BA, García C, Insam H. Pathogenic bacteria and mineral N in soils following the land spreading of biogas digestates and fresh manure. Appl Soil Ecol 2011; 49:18–25; https://doi.org/10.1016/j.apsoil.2011.07.007

[15] Bonetta S, Ferretti E, Bonetta S, Fezia G, Carraro E. Microbiological contamination of digested products from anaerobic co-digestion of bovine manure and agricultural by-products. Lett Appl Microbiol 2011; 53(5):552–7; https://doi.org/10.1111/j.1472-765X.2011.03148.x
[16] Franz E, Semenov AV, Van Bruggen AH. Modelling the contamination of lettuce with *Escherichia coli* O157: H7 from manure-amended soil and the effect of intervention strategies. J Appl Microbiol 2008; 105(5):1569–84; https://doi.org/10.1111/j.1365-2672.2008.03915.x
[17] Haque MA, Jahiruddin M, Rahman MM, Saleque MA. Nitrogen mineralization of bioslurry and other manures in soil. Res Agric Livest Fish 2015; 2(2):221–8; https://doi.org/10.3329/ralf.v2i2.25002
[18] International Standards Organization (ISO-6579). Microbiology of food and animal feeding stuffs—horizontal method for detection of *Salmonella* spp. 4th edition, International Organization for Standardization, Geneva, Switzerland, pp 1–27, 2002.
[19] Cheesbrough M. Medical laboratory manual for tropical countries. 1st edition, Microbiology, English Language Book Society, London, UK, pp 400–80, 1985.
[20] Guan S, Xu R, Chen S, Odumeru J, Gyles C. Development of a procedure for discriminating among Escherichia coli isolates from animal and human sources. Appl Environ Microbiol 2002; 68(6):2690–8; https://doi.org/10.1128/aem.68.6.2690-2698.2002
[21] Cohen ND, Neibergs HL, McGruder ED, Whitford HW, Behle RW, Ray PM, et al. Genus-specific detection of salmonellae using the polymerase chain reaction (PCR). J Vet Diagn Invest 1993; 5(3):368–71; https://doi.org/10.1177/104063879300500311
[22] Stuhlmeier R, Stuhlmeier KM. Fast, simultaneous, and sensitive detection of staphylococci. J Clin Pathol 2003; 56(10):782–5; https://doi.org/10.1136/jcp.56.10.782
[23] Bauer A, Kirby W, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 1966; 45:493–6; https://doi.org/10.1093/ajcp/45.4_ts.493
[24] Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. 26th edition, CLSI supplement M100s. Clinical and Laboratory Standards Institute, Wayne, PA, 2016.
[25] Soupir ML, Mostaghimi S, Yagow ER, Hagedorn C, Vaughan DH. Transport of fecal bacteria from poultry litter and cattle manures applied to pastureland. Water Air Soil Pollut 2006; 169(1–4):125–36; https://doi.org/10.1007/s11270-006-1808-x
[26] Watcharasukarn M, Kaparaju P, Steyer JP, Krogfelt KA, Angelidaki I. Screening *Escherichia coli, Enterococcus faecalis,* and *Clostridium perfringens* as indicator organisms in evaluating pathogen-reducing capacity in biogas plants. Microb Ecol 2009; 58(2):221–30; https://doi.org/10.1007/s00248-009-9497-9
[27] Huong LQ, Forslund A, Madsen H, Dalsgaard A. Survival of Salmonella spp. and fecal indicator bacteria in Vietnamese biogas digesters receiving pig slurry. Int J Hyg Environ Health 2014; 217(7):785–95; https://doi.org/10.1016/j.ijheh.2014.04.004
[28] Costa A, Gusmara C, Gardoni D, Zaninelli M, Tambone F, Sala V, et al. The effect of anaerobic digestion and storage on indicator microorganisms in swine and dairy manure. Environ Sci Pollut Res 2017; 24(31):24135–46; https://doi.org/10.1007/s11356-017-0011-5
[29] Philipp W, Holzle LE. Germs in digestates from biogas plants? Gefahrstoffe Reinhaltung der Luft 2012; 72(5):216–20.
[30] Wagner AO, Gstraunthaler G, Illmer P. Survival of bacterial pathogens during the thermophilic anaerobic digestion of biowaste: laboratory experiments and in situ validation. Anaerobe 2008; 14(3):181–3; https://doi.org/10.1016/j.anaerobe.2008.03.004
[31] Pourcher AM, Marti R, Thorigné A, Jégou B, Dabert P. Effect of anaerobic storage and aerobic digestion on micro-organisms in pig manure: cultural and molecular approaches. In Proceedings XIII International Congress in Animal Hygiene ISAH Vol. 1, Estonian University of Life Sciences Tartu, Estonia, June 17–21, 2007, Tartu 2007.
[32] Son CK, Dung TT, Dung HT, Luong LM, Hau NY, Hung NV. Assess quality of bioslurry under biogas program for animal husbandry sector of Vietnam, Hanoi, Vietnam 2008.
[33] Poudel RC, Joshi DR, Dhakal NR, Karki AB. Evaluation of hygienic treatment of biowastes by anaerobic digestion in biogas plants. Nepal J Sci Technol 2009; 10:183–8; https://doi.org/10.3126/njst.v10i0.2958
[34] Nesa MK, Khan MS, Alam M. Isolation, identification and characterization of salmonella serovars from diarrhoeic stool samples of human. Bangl J Vet Med 2011; 9(1):85–93; https://doi.org/10.3329/bjvm.v9i1.11218
[35] Konuku S, Rajan MM, Muruhan S. Morphological and biochemical characteristics and antibiotic resistance pattern of *Staphylococcus aureus* isolated from grapes. Int J Nutr Pharmacol Neurol Dis 2012; 2(1):70–3; https://doi.org/10.4103/2231-0738.93135
[36] El-Aziz NKA, Eldesoky IE, Ammar AM, Eissa SI, Mohamed YH. Molecular studies on *M. gallisepticum* and avian pathogenic *E. coli* induced infections in broilers. Eur J Vet Med 2014; 2014:1–11.
[37] Ali R, Al-Achkar K, Al-Mariri A, Safi M. Role of Polymerase Chain Reaction (PCR) in the detection of antibiotic-resistant Staphylococcus aureus. Egyptian J Med Hum Genet 2014; 15(3):293–8; http://dx.doi.org/10.1016/j.ejmhg.2014.05.003
[38] Smith SI, Fowora MA, Atiba A, Anejo-Okopi J, Fingesi T, Mary EhiAdamu ME, Omonigbehin EA, Ugo-Ijeh MI, Bamidele M, Odeigah P. Molecular detection of some virulence genes in *Salmonella* spp. isolated from food samples in Lagos, Nigeria. Anim Vete Sci 2015; 3 (1):22–7; https://doi.org/10.11648/j.avs.20150301.15
[39] Duriez P, Topp E. Temporal dynamics and impact of manure storage on antibiotic resistance patterns and population structure of *Escherichia coli* isolates from a commercial swine farm. Appl Environ Microbiol 2007; 73(17):5486–93; https://doi.org/10.1128/aem.00218-07
[40] Groot LD, Bogdanski A. Bioslurry= brown gold? A review of scientific literature on the co-product of biogas production. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, 2013.
[41] Rao RT, Jayakumar K, Kumar P. Bovine origin *Staphylococcus aureus*: a new zoonotic agent? Vet World 2017; 10(10):1275–80; https://doi.org/10.14202/vetworld.2017.1275-1280