applied sciences

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Characterisation and Antibiotic Resistance of Selected Bacterial Pathogens Recovered from Dairy Cattle Manure during Anaerobic Mono-Digestion in a Balloon-Type Digester

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Received: 31 July 2018; Accepted: 21 September 2018; Published: 29 October 2018

Abstract: Dairy cattle manure serves as a potential source of contamination and infection of animals, humans and the environment. Manure samples withdrawn from a balloon-type digester during anaerobic digestion were evaluated for the presence of antibiotic-resistant bacterial pathogens. The bacterial load of the samples was determined via a viable plate count method and the recovered isolates were subjected to characterisation and identification. These isolates were employed in antibiotic susceptibility testing using a disc diffusion method against a suite of 10 conventional antibiotics. The multiple antibiotic resistance (MAR) index was calculated and MAR phenotypes were generated. Although all the bacterial pathogens showed a certain degree of resistance to the studied antibiotics, a marked resistance was demonstrated by *Campylobacter* sp. to co-trimoxazole (87.5%) and nalidixic acid (81.5%). Remarkably, a high resistance (82.42%) was demonstrated against the antibiotic class, macrolide, followed by beta-lactams (40.44%), suggesting that bacterial resistance depended on the chemical structure of the antibiotics. However, individual bacterial isolates varied in resistance to particular antibiotics. Of the 83 bacterial isolates, 40(48.19%) observed MAR > 0.2 and, thus, were described as multidrug-resistant isolates. A total of 28 MAR phenotypes were revealed with the highest frequency of MAR phenotypes (37.5%) expressed against 3 antibiotics. Results indicated a high risk of exposure to various antibiotics and wide diversity of antibiotic resistance.

Keywords: zoonotic pathogens; cattle manure; antibiotic resistance; multiple antibiotic resistance; bio-digester; South Africa

1. Introduction

Naturally, animals harbour significant food- water-borne pathogens in their gastrointestinal tract which they shed in feces, and thus the animals serve as reservoirs and as potential sources of contamination [1]. Contamination of the environment, crops and humans may occur via unplanned release of these wastes by runoffs from wastes and housing facilities or from manure applied as fertilizer on farms or leachate infiltrating through soils and polluting underground springs and wells. Consequently, human health is exposed to threats of numerous gastrointestinal tract infections

associated with these zoonotic pathogens [2]. Some of the bacterial pathogens so far identified and characterized from animal manure included *E. coli*, *Salmonella* and *Campylobacter* species which are of relevance to public health at local, national and global dimensions [3]. Furthermore, Cole et al. [4] reported that the type and concentration of pathogenic microbes present in animal manure varies from farm to farm and is dependent on the species and age of the animal, the ration of diet and the physicochemical composition of the manure. Also, the geographical location, local conditions, population size and seasonal variation may influence the level of these microbes reported in manure [5].

Exposure of human, farm produce and animals to *E. coli*, *Salmonella*, *Shigella* and *Campylobacter* species is a plausible route of transmission and subsequent infection; therefore, they are regarded as important causes of enteric infections globally [6,7]. Enteric infections are prime issues in the public health sectors across the globe as they are associated with significant economic impacts [8]. It is therefore of relevance to monitor the occurrence of these microbes on farms and implement strategies to reduce, control or eliminate them before employing manure as a fertilizer for soil amendment [9]. Anaerobic digestion of animal manure in an airtight chamber (bio-digester) has been viewed as a plausible method for the control of bacterial pathogens resulting in the reduction of the microbes to threshold levels that depict human safety [10]. However, the rate of decimation of the microorganisms depends on the temperature of the process, the particular bacterium, the retention time and the metabolites generated during the process [11,12].

Antimicrobials are quite essential in the chemotherapy of infections caused by bacteria, combating the illness by shortening of its lifespan, reducing its symptoms and chances of transmission in the community [13,14]. However, there has been growing increase in the resistance of some microorganisms to the potentially available antibiotics. More especially, some antibiotics that are vital for treatment of bacterial infections of humans are currently used in farm practices for therapeutic and growth-promotion purposes [15,16]. Consequently, antibiotic-resistant bacteria may emerge which may negatively influence or compromise the outcome of treatment regimens issued to patients resulting in increased morbidity, mortality and economic burdens [17,18]. More elaborately, antibiotic-resistant bacteria are widespread and their numbers are growing; this represents a significant global health problem attributed to the use and misuse of antibiotics [19]. As a consequence, in the last decade, environmental bacteria have been regarded as reservoirs of antibiotic resistance determinants and prospective agents of novel resistance genes that could actually contribute to resistance in clinical settings via transfer of these genes to clinical pathogens. Accordingly, knowledge on the origin of these antibiotic resistance genes, their distribution in the environment and factors that favour their spread is crucial in a bid to establish strategies to circumvent antibiotic resistance [19].

In this light, the monitoring of the prevalence of antibiotic-resistant bacteria are of significance regardless of the source (clinical or environmental) as antibiotic susceptibility profiles of microorganisms vary widely from country to country and from one geographical location to the other [20]. Furthermore, organisms are vulnerable to developing unpredictable resistance patterns, thus making it necessary for constant surveillance of the antibiogram profiles in order to be current with the trend [18]. However, there is a dearth of information on the presence of the aforementioned enteropathogens in animal manure procured from dairy farms and treated in anaerobic digesters, in the Eastern Cape Province, South Africa, as well as their antibiotic resistance patterns.

It is well known that in developing countries, especially in rural areas, there is a high possibility of close contact of humans with food animals which in turn offers great chances for exposure to zoonotic bacteria. In particular, individuals living in both rural and urban areas in South Africa own and keep cattle for several reasons including generating income, providing food, producing manure which can be used as fertiliser, enhancing social status and presenting them as financial aids [21]. However, Johnson and colleagues [22] have noted the high prevalence of human immunodeficiency virus (HIV) in South Africa. In addition, it has been highlighted that HIV-positively diagnosed individuals are more vulnerable to develop enteric fever with frequent relapses [23]. Against this background, our

study investigated antibiotic resistance in zoonotic bacteria, which is vital as the population in South Africa constitutes of a great proportion of immune-compromised individuals.

Apparently, this study is first of its kind to assess the level of *Escherichia coli*, *Salmonella* and *Campylobacter* species as well as other Gram-negative bacteria (*Shigella* species) in dairy cattle manure obtained from the Fort Hare Dairy Trust, Alice, Eastern Cape Province, South Africa, which was fed into a balloon-type digester for anaerobic digestion. In addition, the antibiotic resistance profiles, taking into consideration the multiple antibiotic resistance (MAR) index and predominant MAR phenotypes, were determined.

2. Materials and Methods

2.1. Study Site and Sample Collection

Approximately 1700 kg of dairy cow manure required for charging a balloon-type digester was obtained from the Fort Hare Dairy Trust in Alice, Eastern Cape Province, South Africa. The manure collection involved three samplings (with multiple samples that were pooled together as a representative sample for each sampling) conducted over three successive days during which portions were transferred into sterilized screw-capped bottles. The slurry was prepared after each day's collection and fed into the digester. The digester was operated under batch mode and samples were collected every seven- or 14-day interval over a period of six (6) months at different positions of the digester for bacterial count and subsequent isolation and identification of the enteropathogens of interest [24]. Each sample was withdrawn and introduced into the tryptone soy broth medium (Liofilchem Diagnostic, Roseto degli Abruzzi, Italy) and transported on ice [25] to the Applied and Environmental Microbiology Research Laboratory, University of Fort Hare, Alice, and processed within 24 h upon arrival at the laboratory.

2.2. Bacterial Counts

Total viable counts of *E. coli*, *Salmonella*, and *Campylobacter* species were estimated by plate counts as per the method of Poudel et al. [26]. A total of 24 pooled samples were cultured. Briefly, 1g of each wet sample (slurry) was 10-fold serially diluted in 9 mL of sterile physiological saline. One hundred microlitres of each diluted sample (from 10^{-1} to 10^{-5}) was spread on different selective microbiological media: Chromogenic *E. coli* agar (Conda, Spain), *Salmonella/Shigella* agar (Conda, Spain), and modified *Campylobacter* cefoperazone desoxycholate agar (mCCDA; Conda, Spain) for the isolation of *E. coli*, *Salmonella/Shigella* and *Campylobacter* species, respectively. All inoculated plates were aerobically incubated at 37 °C for 18–24 h except the mCCDA plates for the growth of *Campylobacter* species that were incubated at 42 °C in a microaerophilic environment provided by a gas generating kit (BR0038, Oxoid, UK) for 48–72 h. After incubation, the number of colonies on each plate was counted and expressed as the means of three replicates [27].

2.3. Isolation and Identification of Bacterial Isolates

Following bacterial counts, distinct and well isolated colonies were picked from the different media plates and subcultured on their respective freshly prepared plates to ensure purity and for further identification and characterisation. Presumptive identification of these bacteria was based on their growth on selective media, cultural and morphological characteristics according to Cheesbrough [28]. Confirmation of the different bacteria isolates was based on biochemical tests, including catalase, oxidase, indole test, hippurate hydrolysis, hydrogen sulphide production and sugar fermentation on the triple sugar iron test, microaerobic growth at 37 °C and 42 °C and susceptibility to nalidixic acid [28]. Confirmed isolates were restreaked several times to ensure purity of the isolates, and were subsequently stored at −80 °C in tryptone soy broth in 20% glycerol [29].

2.4. Antibiotic Susceptibility Testing of Bacterial Isolates

Stock cultures of confirmed isolates were revived on Nutrient Agar (Merck, South Africa) and Mueller Hinton Agar (Conda, Spain) depending on the particular bacterial species. The Kirby–Bauer disc method was employed to determine the sensitivity of the isolates to a host of commercially available antibiotics (Mast Diagnostics, UK), and procedures were performed in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI) [30]. A standardized inoculum size, containing 10^8 cfu (which corresponded to a 0.5 Mc Farland standard) of each bacterial isolate was prepared and spread on solidified agar plates with sterile cotton swab sticks to produce a lawn of bacterial growth. Inoculated plates were allowed to dry for some minutes and the commercial discs were then aseptically placed at equidistance from each other and from the edge of the plates to prevent overlapping of inhibition zones [11]. The antimicrobials used included; co-trimoxazole (25 µg), erythromycin (15 µg), ciprofloxacin (5 µg), amoxicillin (10 µg), nalidixic (30 µg), tetracycline (25 µg), augmentin (30 µg), ampicillin (25 µg), gentamicin (10 µg) and chloramphenicol (30 µg). Plates with *Campylobacter* isolates were incubated under microaerophilic conditions for 48 h while for *Enterobacteriaceae*, the plates were incubated aerobically within 18–24 h. After the incubation period, plates were examined for zones of inhibition around each disc. The diameters of the zones of inhibition were measured and recorded in millimeters; each value represented the mean of triplicate assays. Results were interpreted as susceptible or resistant based on the breakpoint criteria of CLSI [24].

2.5. Determination of Multiple Antibiotic Resistance Index and Resistance Phenotypes

The multiple antibiotic resistance (MAR) index was calculated in order to determine the level of antibiotic resistance of individual bacterial isolate according to Resende et al. [31] by dividing the number of antibiotics to which the isolate was resistant to by the total number of antibiotics to which the isolate was exposed to in the study. A MAR value >0.2 was indicative of multiple antibiotic resistant bacteria. In simplified terms, multiple antibiotic resistance to more than 2 antibiotics resulted in greater than 20% resistance in this study, where 10 antibiotics were utilised. MAR phenotypes of isolates that showed resistance to three or more antibiotics were generated [32].

2.6. Data Analysis

Data were recorded on Excel spreadsheet and analysed using Matlab software (R2015a). The output of the analysis was represented on frequency tables.

3. Results

- Total viable counts of the target bacterial species in the influent manure are presented in Table 1 below. Presumptive *E. coli* counts was in the order of $2.0 \times 10^6 \pm 8.7 \times 10^5$ cfu/g, while presumptive *Salmonella* was in the order $2.7 \times 10^4 \pm 2.5 \times 10^4$ cfu/g and *Campylobacter* species counts was in the order of $3.4 \times 10^4 \pm 4.2 \times 10^4$ cfu/g.
- Overall, following the morphological and biochemical characterisation, a total of 83 bacterial isolates were obtained comprising of 16 *Campylobacter* species, 30 *E. coli*, 33 *Salmonella* and 4 *Shigella* species that were then subjected to an antibiotic sensitivity study. Of all the *Campylobacter* isolates, 4 isolates were confirmed as *C. jejuni* following the hydrolysis of sodium hippurate. The percentages of sensitive and resistant bacteria isolate to the antibiotics used are shown in Table 2 and the values varied with the type of bacteria.
- All the bacterial isolates belonging to the family *Enterobacteriaceae* exhibited a striking sensitivity to ciprofloxacin but varying levels of resistance to the other antibiotics. Nevertheless, the *Campylobacter* sp. belonging to the family *Campylobacteriaceae* demonstrated marked sensitivity of 87.5% to both tetracycline and chloramphenicol and a resistance of same magnitude (87.5%) to co-trimoxazole.

- The MAR index is presented in Table 3. Multiple antibiotic resistance is considered as resistance of a bacterial isolate to three or more antibiotics. A total of 48.19% (40/83) of the bacterial isolates were characterised as multi-resistant strains (MAR > 0.2) as they exhibited resistance to ≥ 3 antibiotics. The MAR index varied with the bacterial species, but ranged from 0.3 to 1. 75% of *Shigella* species displayed MAR > 0.2 while 23.33% of *E. coli* species exhibited MAR > 0.2. In addition, the MAR phenotypes among the 40 multidrug resistant isolates varied as represented in Table 4. Of all the bacterial species isolated, 37.5% (15) showed multidrug resistance to 3 antibiotics. Only a small percentage of 2.12% (1) showed a 10 MAR pattern which indicated resistance to all the antibiotics employed in this study, a situation that merits attention due to the possibility of horizontal gene transfer.
- Based on the bacterial pathogen, the predominant phenotypes were AP-A-GM-E-AUG, A-E-AP and A-E-TS at 15.79% for *Salmonella* sp.; TS-E-NA-CIP-GM and TS-NA-AUG-A at 18.18% for *Campylobacter* sp.; C-T-E at 42.86 for *E. coli* and C-AUG-T-A-E at 66.57% for *Shigella* sp.

Table 1. Characteristic properties of influent manure fed into the bio-digester.

Parameter	Values
Escherichia coli	$2.0 \times 10^6 \pm 8.7 \times 10^5$ cfu/g
Salmonella sp.	$2.7 \times 10^4 \pm 2.5 \times 10^4$ cfu/g
Campylobacter sp.	$3.4 \times 10^4 \pm 4.2 \times 10^4$ cfu/g
Shigella sp.	ND
% moisture content	88.8%
% total solids	11.2%
% total volatile solids	61.5%
% ash content	32.5%
pH	6.6

Table 2. Antibiogram of the confirmed bacterial isolates recovered from dairy manure in the Eastern Cape Province of South Africa.

Antibiotics	*E. coli* (n = 30)		*Salmonella* sp. (n = 33)		*Campylobacter* sp. (n = 16)		*Shigella* sp. (n = 4)	
	% S	% R	% S	% R	% S	% R	% S	% R
Ampicillin	96.7	3.3	60.6	39.4	81.3	18.8	75	25
Augmentin	96.7	3.3	69.7	30.3	62.5	37.5	25	75
Amoxicillin	80	20	36.4	63.6	62.5	37.5	25	75
Nalidixic acid	90	10	81.8	18.2	18.8	81.3	100	0
Tetracycline	76.7	23.3	87.9	12.1	87.5	12.5	25	75
Gentamicin	100	0	81.8	18.2	68.8	31.3	100	0
Ciprofloxacin	100	0	100	0	68.8	31.3	100	0
Erythromycin	6.7	93.3	0	100	81.3	18.8	0	100
Co-trimoxazole	96.7	3.3	81.8	18.2	12.5	87.5	100	0
Chloramphenicol	70	30	90.9	9.1	87.5	12.5	25	75

n, number of isolates for each bacteria; S, susceptible; R, resistant.

Table 3. Multiple antibiotic resistance (MAR) index of different bacteria isolated from balloon-type anaerobic digester samples.

Bacterial Isolates	MAR (Frequency of Determination %)		Number of Isolates
	≤0.2	>0.2	
Salmonella sp.	42.42	57.58	33
E. coli	76.67	23.33	30
Campylobacter sp.	31.25	68.75	16
Shigella sp.	25.00	75.00	4

Table 4. Multiple antibiotic resistant (MAR) phenotypes identified in bacterial isolates recovered from cattle manure during anaerobic digestion to 10 antibiotics.

***Salmonella* Species (n = 19)**			
MAR Phenotypes	**No. of Antibiotics**	**No. of Observed Isolates**	**Percentage**
C-AUG-T-A-E-AP-NA	7	2	10.53
AP-A-E-NA-AUG-TS	6	1	5.26
T-A-GM-E-NA-TS	6	1	5.26
AP-A-E-NA-AUG	5	2	10.53
AP-A-GM-E-AUG	5	3	15.79
AP-A-E-AUG-TS	5	1	5.26
C-A-E-AP	4	1	5.26
A-E-AP	3	3	15.79
A-E-TS	3	3	15.79
A-GM-E	3	1	5.26
A-E-AUG	3	1	5.26
Escherichia coli (n = 7)			
MAR phenotypes	No. of antibiotics	No. of observed isolates	Percentage
C-T-E-A-AUG	5	1	14.29
C-T-E-A	4	1	14.29
C-T-E	3	3	42.86
C-E-A	3	1	14.29
T-E-TS	3	1	14.29
Campylobacter species (n = 11)			
MAR phenotypes	No. of antibiotics	No. of observed isolates	Percentage
TS-C-E-NA-AUG-A-CIP-T-GM-AP	10	1	9.09
TS-NA-AUG-A-CIP-AP	6	1	9.09
TS-C-NA-CIP-GM	5	1	9.09
TS-E-NA-CIP-GM	5	2	18.18
TS-NA-AUG-A	4	2	18.18
NA-AUG-A-AP	4	1	9.09
TS-NA-T-GM	4	1	9.09
TS-NA-A	3	1	9.09
TS-NA-AUG	3	1	9.09
Shigella species (n = 3)			
MAR phenotypes	No. of antibiotics	No. of observed isolates	Percentage
C-AUG-T-A-E-AP	6	1	33.33
C-AUG-T-A-E	5	2	66.67

A, amoxicillin; AP, ampicillin; AUG, augmentin; C, chloramphenicol; CIP, ciprofloxacin; E, erythromycin; GM, gentamicin; NA, nalidixic; T, tetracycline; TS, Co-trimoxazole.

4. Discussion

In South Africa, Meissner et al. [33] reported a dramatic increase in the number of middle-class population due to improved income resulting in an equivalent rise in the demand of livestock products. Thus, intensive farming embracing huge quantities of antibiotics becomes crucial to meet demand [34]. According to Eagar et al. [35] antibiotics are mostly consumed as in-feed medications to the food animals in the country. However, the volume and pattern of the drugs consumed are not known due to lack of information. Naturally, the use of antibiotics is associated with the phenomenon of antibiotic resistance. Bacteria in food-producing animals are released into the environment and the food chain via manure, wastewater and animal-derived products, and in turn are transmitted to humans causing infections. Isolation, identification and characterisation of any particular disease-causing agent in a geographical location or country are important ingredients for the prevention and control of the disease that it causes [36]. The isolation, characterisation and antibiotic susceptibility and resistance

profiles of *E. coli*, *Salmonella*, *Campylobacter* and *Shigella* species obtained from a balloon-type digester, anaerobically degrading dairy cattle manure procured from a dairy farm in the Eastern Cape Province of South Africa, have being investigated and reported in this study.

In this study, we identified *E. coli*, *Salmonella*, *Shigella* and *Campylobacter* bacteria in the influent manure which is an indication that dairy manure is a potential source of water, food and soil pollution if this waste is not properly managed and contained. The levels of these bacteria as depicted by their counts are not surprising as Brown [37] in his study reported that the gastrointestinal tract of humans and animals could serve as major reservoirs of *Salmonella*, *Shigella* and *E. coli* whereas the rumen of animals have been identified as major sources of *Campylobacter* species that infect humans. Undoubtedly, these animals may shed these pathogens in their feces, which could act as a vehicle of transmission [26]. However, this poses a threat to public health since the infectious agents are viable. Apparently, the infective dose of *Salmonella* sp. can be as low as in the range of 15–100 bacterial cells per millilitres [38] similar to a low infective dose of *E. coli* attributed to as few as 10 cells [3]. Hence, modes of transmission become feasible and infections are bound to occur [39].

In detail, the counts (in numbers) obtained for *Salmonella* species and *E. coli* in this study were approximately 200 times and 20,000 times greater than the infective dose attributed to *Salmonella* and *E. coli*, respectively.

It has been reported that the antimicrobial resistant strains of *E. coli*, *Salmonella*, *Campylobacter* and *Enterococcus* bacteria obtained from agricultural sources can affect humans [15], thus the antibiogram profile of these bacteria, isolated and identified from our sample, becomes crucial. In addition, the continuous growth in antimicrobial resistance necessitates the need for continuous surveillance and new approaches in order to decrease the emergence and rise in antibiotic–resistant bacteria as well as to prevent dissemination of drug resistance [25].

Accordingly, in this study the different bacterial isolates exhibited varying levels of resistance to the antibiotics employed.

Resistance to peculiar antibiotic of specific bacterial isolates were observed including 87.5% and 81.8% resistance of *Campylobacter* isolates to co-trimoxazole and nalidixic acid, respectively; 75% resistance of *Shigella* species to augmentin, amoxicillin, tetracycline and chloramphenicol; 63% resistance of *Salmonella* species to amoxicillin and a resistance at 30% of *E. coli* isolates to chloramphenicol. Thus, the frequency of resistance varied with bacterial isolates and the antibiotics and the percentage of the resistant isolates is often considered as an indication of the level of antimicrobial resistance. The findings are in agreement with other studies which investigated the antibiotic resistance of enteropathogens from environmental sources, elsewhere [40,41].

Furthermore, Abo-State et al. [41] noted 100% resistance to eight antibiotics including tetracycline, of bacterial isolates belonging to the *Enterobacteriaceae* family which were recovered from water samples collected at Rosetta branch of the River Nile, Egypt. Similarly, Simango [40] reported a resistance of 82% to co-trimoxazole of *Campylobacter* species recovered from chicken faeces obtained from chicken farms in Harare, Zimbabwe. Owing to the possibility of antibiotic-resistant bacteria endowed with resistance genes to be disseminated via air, water, food, and rainfall; these bacteria might eventually end up in the environment [42]. It is clear that humans do interact with their environment. Therefore, the findings in this study are very vital in clinical settings due to the likelihood of the transfer of resistance genes via lateral gene transfer to human pathogens. This then results in increase in resistance in a clinical setting which causes a rise in costs due to the morbidity and mortality of infected individuals, human therapies associated with severe and persistent infections and long hospital stays, laboratory workloads, the discovery and production of new antibacterial agents against drug-resistant bacteria, as well as the increase in resources for suitable infection control programs [43]. In addition, the clinical pathogen might receive antibiotic-resistance genes alongside virulent genes thereby causing an increase in virulence and pathogenicity of the said bacterium [44]. Furthermore, all the enteric zoonotic bacterial isolates revealed the presence of multidrug resistance which has both ecological and public health implications. Apparently, these antibiotic-resistant bacteria can cause zoonotic infections in

humans with more severe and unfavourable outcomes in individuals who are immuno-compromised, the elderly and young children; leading to the greater use of broad spectrum and newer antibacterial agents in a bid to exclude the expected higher morbidity and mortality that would have resulted when an unsuitable agent is initially employed [45].

The data obtained showed that dairy manure contained pathogenic bacteria that were shown to be resistant to some antibiotics, indicating a potential public health hazard. Interestingly, the results emphasised the necessity of the application of hygiene practices and proactive measures to reduce the risk of infections. Clearly, the noted level of antimicrobial resistance is significant to public health in that the antimicrobial-resistant strains of these bacteria may play an important role in the introduction, development and persistence of disease. Furthermore, the observed resistance is a threat, especially to the immuno-compromised population whose immunity relies on the use of antimicrobials [15], as well as the menace of accumulation of antibiotic-resistance determinants in the environment.

Furthermore, most of the bacterial isolates demonstrated profound resistance to the antibiotic class, a macrolide (erythromycin) followed by beta lactams, phenicols, as opposed to the other antibiotics tested. Therefore, this revealed that bacterial resistance in the present study was dependent on the chemical structure of the antibiotics [46] as well as the presence of resistance genes, although not investigated in this study. Consequently, our result may indicate the possible use of some of these antibiotics on the dairy farm as reported by several authors elsewhere for disease prevention and treatment [16,47]. Hence, erythromycin cannot be efficient in the control of these pathogens [41].

On the other hand, all the *Salmonella*, *E. coli* and *Shigella* species (100%) were highly sensitive to ciprofloxacin but only 68.8% of *Campylobacter* sp. were susceptible to ciprofloxacin. According to European Committee on Antimicrobial Susceptibility Testing [48] EUCAST antibiotic sensitivity testing involving ciprofloxacin (5 µg) often results in low detectable levels of resistance of *Salmonella* sp. to the drug. In this light, another antibiotic disc, pefloxacin (5 µg) should be used; therefore, the susceptibility or resistance of *Salmonella* sp. can be inferred from pefloxacin disc sensitivity testing. This is because low levels of resistance in *Salmonella* species can only be detected with pefloxacin disc since ciprofloxacin cannot detect them; also, using ciprofloxacin, there is no certainty with which the *Salmonella* isolates are described as sensitive. Apparently, for the detection of high-level resistance, ciprofloxacin can be used but not for low level as it will result in false sensitive *Salmonella* isolates.

Also, all the *E. coli* and *Shigella* species (100%) as well as 81.8% *Salmonella*, and 68.8% *Campylobacter* species were sensitive to gentamicin. Moreover, the least resistance was demonstrated against aminoglycosides (gentamicin) and Christabel et al. [49] mentioned that the abuse to gentamicin is uncommon owing to its peculiar mode of administration which happens to be mostly intramuscular. Ciprofloxacin is the most expensive and first line drug approved for the treatment of resistant and non-resistant cases of typhoid in developing countries owing to the multidrug resistance of *Salmonella* isolates to all the three first line inexpensive drugs, chloramphenicol, co-trimoxazole and amoxicillin which constitute the mainstay therapy for the infection [50]. In addition, the price or the cost of an antibiotic determines its availability for use or its employment in a prescription [51]. More elaborately, Briyne et al. [52] in their study reported that the cost or price of an antibiotic is amongst other factors considered by veterinarians in decision making during prescription with antibiotics. Hence, the costlier or expensive antibiotics are rarely used injudiciously as Grace [53] noted that commonly used antibiotics are inexpensive.

Therefore, we concluded that ciprofloxacin and gentamicin were the most effective antibiotics in the control of the bacteria in this study. Consequently, a general or pre-knowledge of the anticipated susceptibility profile of these bacteria to a range of available antibiotics is a prerequisite to the commencement of treatment with an appropriate antibiotic [54].

Magiorakos et al. [55] termed a bacterial strain presenting with a resistant phenotype in ≥ 3 antibiotics as multidrug resistant. Also, the MAR index has been employed extensively, in several environments to classify multidrug-resistant organisms. Of the 83 isolates recovered, 40 (48.19%) bacterial isolates demonstrated resistance to three and more antibiotics (MAR > 0.2); thus,

these were described as multidrug-resistant bacterial enteropathogens. Only one bacterial isolate (*Campylobacter* sp.) demonstrated resistance to all the 10 antibiotics. The said isolate is a host to many antibiotic-resistance genes and, thus, can serve as a reservoir to disseminate resistance genes via lateral gene transfer to other pathogenic, environmental species and commensal bacteria thus causing the spread and rise of antibiotic resistance [56] which in turn presents with ecological and public health implications. Besides, Florea [57] highlighted that a calculated value of MAR index >0.25 poses a high risk contamination and the isolate recorded a MAR index of 1; hence, it is a threat as it can cause cross resistance to other antibiotics in circumstances where the antibiotics employed share the same mode of actions as well as it might cause difficult to treat infections [58].

Approximately 48% frequency of MAR > 0.2 among the bacterial isolates recovered from the digesting substrate (dairy cattle manure) in an anaerobic digester highlighted that the discharged effluent from the bio-digester may serve as a plausible environmental reservoir of antibiotic-resistance genes [31]. This imposes the implementation of other treatment methods for the post-treatment of the anaerobically treated effluent to ensure microbiological safety.

Moreover, from Table 3, the high frequency of MAR > 0.2 observed in, *Shigella* sp. (75%), *Campylobacter* sp. (68.75%) and *Salmonella* sp. (57.78%) suggested that these microorganisms have been exposed to erythromycin, amoxicillin, tetracycline and co-trimoxazole which are antibiotics employed as feed additives, prophylactics or therapeutics in cattle farms; hence, this reflected a microbial adaptive response [59]. In the present study, 28 antibiotic-resistance phenotypes were represented as shown in Table 4. This indicated a wide diversity of resistance which might persist in the environment and proliferate, thus creating great chances for the spread of resistance genes via horizontal or lateral gene transfer causing negative influences on human and animal antibiotic chemotherapy [60]. The findings corroborate those of Hinthong et al. [61] where 21 antibiotic-resistance patterns were elucidated. However, the overall predominant phenotypes were AP-A-GM-E-AUG, A-E-AP and A-E-TS added to C-T-E resistance patterns observed in *Salmonella* and *E. coli* species, respectively.

In summary, the aforementioned bacterial enteropathogens are responsible for gastrointestinal diseases in humans. Diarrhoea is attributed to inadequate sanitation and hygiene, a situation very common in Sub-Saharan African regions and the second-highest cause of death among children less than five years of age. Therefore, in developing countries e.g., South Africa, where sanitation is poor and the rate of diarrhoeal diseases is at its optimum, antimicrobial resistance in enteropathogens (enteric pathogens) is vital. More especially, the continuous rise in antimicrobial resistance among these enteropathogens in the aforesaid countries is becoming a critical area of concern.

5. Conclusions

Manure samples withdrawn from a balloon-type bio-digester during anaerobic digestion are endowed with the potential to cause contamination to the environment and subsequent infections in humans and animals due to the presence of antibiotic-resistant strains of *Salmonella*, *Shigella*, *Campylobacter* species and *E. coli*. Finally, data from this study serve as a baseline for future works on the identification of the virulence genes responsible for pathogenicity in these bacteria as well as determining the antibiotic-resistance genes.

Author Contributions: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Writing-Original Draft Preparation, Writing-review and editing, Funding acquisition, C.M-L.; Supervision, S.M.and E.M.; Supervision, Writing-Review and Editing, A.O.

Funding: This research was funded by [Claude Leon and National Research Foundations, the Department of Science and Technology, South Africa, grant number [98031]", and the University of Fort Hare for financial support [C114 and C702] and "The APC was funded by [Govan Mbeki Research and Development Centre, University of Fort Hare, Alice Campus, South Africa]".

Acknowledgments: We are grateful to the South Africa Medical Research Council for financial support.

Conflicts of Interest: "The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".

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