The Effect of Thermophilic Anaerobic Digestion on Ceftiofur and Antibiotic Resistant Gene Concentrations in Dairy Manure

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ABSTRACT

The prevalence of antibiotics on farms for therapeutic and prophylactic use in animals can cause negative effects on biomethane production during anaerobic digestion. Previous literature has found decreased biomethane production rates from a variety of antibiotics, but biogas inhibition differs between studies of continuous and batch reactors and the type of antibiotic studied. Cephalosporin drugs are the most common antibiotic class used to treat mastitis in dairy cows and can retain most of their bioactivity after excretion. Ceftiofur is a commonly used cephalosporin drug but no previous study investigating the effect of Ceftiofur on biomethane during continuous anaerobic digestion has been performed.

The aim of this study was to examine the effect on biomethane production when manure from cows treated with Ceftiofur was anaerobically digested. Laboratory sized anaerobic digesters (AD) were run at thermophilic (55°C) temperatures and a 10 day hydraulic retention time. Manure from cows treated with Ceftiofur were fed to the antibiotic treatment reactors for 50 days. The reactor performance was measured by i) biomethane production, ii) waste stabilization in terms of solids and chemical oxygen demand, iii) change in mass of Ceftiofur and iv) change in concentration of antibiotic resistant genes, specifically cfx(A), mef(A), and tet(Q). There was statistically significant decrease in cumulative gas production due to the addition of Ceftiofur into the reactors, but no significant difference between treatments in waste stabilization in terms of percent volatile solids (VS) and total chemical oxygen demand (TCOD) reduction. Anaerobic digestion decreased the amount of Ceftiofur in manure, and the amount of Ceftiofur in the reactors reduced over the time of the experiment. Change in antibiotic resistant genes (ARGs) were gene dependent over time. Concentrations of tet(Q) reduced significantly between feed and effluent of both treatments, and cfx(A) reduced significantly for the control treatment but not the Ceftiofur treatment. Concentrations of mef(A) increased over time in both treatments. Overall,
the addition of Ceftiofur in continuously operated anaerobic digesters negatively affected biomethane production, a value-added product responsible for on-farm renewable energy. However, anaerobic digestion does decrease the mass of Ceftiofur within manure, thereby reducing the environmental loading from run-off from farms.
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GENERAL AUDIENCE ABSTRACT

Anaerobic digestion is a biological treatment technology used on farms to treat manure. It can be used to reduce potential environmental damage from contaminants and manure, homogenize manure for fertilizer, and produce methane gas for renewable energy. An emerging challenge in manure management is the presence of antibiotics such as ceftiofur used in animal production to prevent and treat illnesses. When antibiotics are used on livestock, they are excreted from the animal in manure. When the manure is added to the digester, the antibiotic molecules within the manure can kill the bacteria responsible for manure homogenization and gas production. Ceftiofur is a type of cephalosporin antibiotic used to treat dairy cows for mastitis, a bacterial infection of the udder. When the cows are treated with Ceftiofur, it can remain in the excreted manure and enter the digester. The use of antibiotics on farms is also leading to a global phenomenon known as antibiotic resistance. The bacteria that are exposed to antibiotics can develop mutations to become immune to the antibiotic, and can spread the mutations through antibiotic resistant genes (ARGs). ARGs can spread to bacteria which have never been exposed to antibiotics, making them resistant. This causes a significant concern in regards to disease treatment across the world as the efficacy of antibiotics is threatened. Understanding how ARGs move and how they can be eliminated is crucial to preventing global antibiotic resistance.

The purpose of this study was to assess the effect of anaerobic digestion on Ceftiofur and ARGs. Four continuous lab-scale anaerobic digesters, two using control manure and two using manure from cows treated with Ceftiofur, were run at 55°C for a period of 50 days. Over time, the reactor with manure from cows treated with the Ceftiofur antibiotic produced less gas as compared to the control digesters. The amount of Ceftiofur within the digesters decreased over time, demonstrating anaerobic digestion’s ability to degrade the antibiotic molecule. The effect of anaerobic digestion on the ARG concentration was gene specific. The concentration of the
tet(\textit{Q}) gene, a gene responsible for resistance against the very common antibiotic tetracycline, was reduced by anaerobic digestion. These results demonstrate that anaerobic digestion is a technology which can reduce the environmental impact of manure from Ceftiofur-treated cows. This shows that manure treatment can be a first step in combating antibiotic resistance across the globe.
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1 INTRODUCTION

1.1 The US Dairy Industry

Agriculture is at the intersection of food security, renewable energy, and environmental protection. Beyond providing food, fiber, and fuel for the growing population, the agricultural industry also provides employment to one third of the world’s population (FAO, 2014). Food security relates to sufficient access, availability, and use of food necessary to provide health and wellness to all people at all times (FAO, 2014). Renewable energy produces energy from sources that can be constantly replenished without environmental damage (NREL, 2016). The production of renewable energy is a key attribute to energy security, which requires sources of electricity and transportation fuels to be accessible, reliable, and affordable (IEA, 2016). Natural disasters, volatile markets, and political and social disputes impact energy availability around the world, and renewable energy seeks to improve the United States’ energy security, environmental quality, and energy economy (DOE, 2016). Agriculture inhabits the natural environment, and has direct impact on soil, air, and water quality. Building a framework for sustainable agriculture impacts key environmental aspects such as land use, water quality, air pollution, climate change, biodiversity, and future productivity of natural resources (FAO, 2014). The need for sustainable agriculture practices for production of animal based proteins is evident in order to meet demand of the growing population without sacrificing the natural resources necessary for a balanced ecosystem.

The growth of wealth and human population around the globe has caused significant increase in the demand for animal proteins, including milk, meat, and eggs (FAO, 1996). In order to meet the increasing demand for milk and milk products against a volatile market, dairy farms in the United States have increased in size and reduced in number. In 1970 there were 648,000 dairy farms in the US, but only 75,000 farms in 2006 (ERS, 2016). The decrease in the number of farms has in turn increased the average farm size from 19 cows per farm in 1970 to 120 cows per farm in 2006 (ERS, 2016). Despite having fewer cows, milk production increased due to improved cow genetics. About 9,000 pounds of milk were produced per cow per year in 1970 and an average of 19,951 pounds per milk per cow per year in 2006 (ERS, 2016). But with the increase in milk production comes an increase in manure. The average cow produces 2.7 kg of
Manure contains organic matter, nutrients, bacteria, and emerging pollutants such as hormones and antibiotics, which, depending on management, can cause damage to soil, air, and water quality and pose potential health hazard to humans (NRCS, 1992). If not managed properly, the organic matter and nutrients in the manure deplete oxygen, and nitrogen and phosphorus cause algal blooms phosphorus in waterways (NRCS, 1992). If the bacteria in manure reaches surface and ground water and accumulates beyond the allowable concentration limits, the waters may become polluted, making them unsuitable for drinking, fishing, or recreation (NRCS, 1992). The bacteria in animal manures are a potential source of pathogens of concern such as \textit{E. coli}, campylobacter, salmonella, and giardia (Humenik, 2001). Dairy manure management is a key part of farm operations with a focus on ways to minimize treatment costs without sacrificing environmental safety and farm profits. Proper manure management should aim to: reduce the strength of organic matter and nutrients that can cause damage to waterways, remove odors and noxious air pollutants, inactivate or decrease levels of pathogenic bacteria, and produce value-added products as an additional revenue source on the farm.

1.2 Dairy Manure Management

Good manure management begins with knowing the quantity and characteristics of the manure produced on a farm. This knowledge helps in determining the treatment approach to use. Manure quantity defines the mass or volume of waste produced and cumulated over a given time period. The size of treatment and storage systems are designed based on the quantity of manure produced and the desired storage time. The mass of manure remaining after water is removed is defined as total solids, which includes both organic and inorganic matter or volatile (VS) and fixed (FS) solids (APHA, 1995). The VS is the fraction of TS that burns off when the solids are ignited at 550°C (APHA, 1995). In general, dairy manure about 87% water and 13% TS when
excreted (PNNL, 2003), and VS is typically 80% of TS. Knowledge of the amount of solids in manure is important in determining which treatment is most suitable given the composition of the manure and its strength. Waste strength is characterized generally as oxygen demand, expressed as biological oxygen demand (BOD) or chemical oxygen demand (COD) (Merkel, 1981). BOD determines the molecular oxygen used during the biochemical degradation of the organic materials and oxidation of the inorganic materials, while COD quantifies the oxygen equivalent of the organic matter by analyzing the manure components which are susceptible to oxidation by a chemical oxidant (APHA, 1995). The BOD and COD have a direct linear relationship with manure’s organic matter. The manure organic matter can be estimated by VS, and is the amount of manure which can be easily converted into secondary products.

In general, manure is usually considered a low volume, high strength waste stream compared to municipal wastewater (Merkel, 1981). While the same principles and types of treatments are employed for both types of wastes, the challenges in conducting the treatments differ due to their characteristics. Thus, some experiences in municipal wastewater treatment are included in this literature review to provide perspectives of physical, chemical, and biological processes.

1.2.1 Value Added Products from Manure

The ultimate objective of manure management is to reduce the potential negative impacts on the environment without loss of farm revenue (Merkel, 1981). Animal manures are a resource on the farm but will incur some cost to be disposed. Thus, deriving value added products with the goal of minimizing or reducing the disposal costs and/or providing an additional source of revenue on a farm is desirable. Value added products are goods which are transformed from a lesser value feedstock such as manure, into a final product with higher economic value. Manure can be used as a soil additive (e.g. fertilizer and source of organic matter (Cao et al., 2009)) and feedstock for: manufacture of rubber, printing ink, tiles, and charcoal briquettes (Merkel, 1981), polyhydroxyalkoanate (PHA) production (Coats et al., 2007, 2010) producing precursors for transportation fuels (Huber, 2006), and biomethane as renewable energy (ASERTTI, 2011). Several treatment techniques have been developed, and the selection of what to use on a farm is governed by local laws, economics, ultimate end use, and the size of the farm operation.
1.2.2 Treatment Processes

The main processes used in manure treatment include physical, thermal, chemical, and biological, or combination (DeBusk et al., 2008). These process are described below.

*Physical*

Physical processes use gravity or mechanical forces to treat manure. Sedimentation is a low-cost separation technique, which uses gravity to separate solids from liquids. Mechanical equipment, such as screens and belt presses, speed up separation processes by using a mechanical device to apply pressure to the manure and a physical barrier to trap the solids (Ogejo, 2009). Separation of suspended solids reduces the oxygen demand of the manure, reducing the environmental impact after release and overall cost of treatment (Merkel, 1981). Separation techniques reduce the mass and volume of solids, to prevent clogging in pipes, concentrating the solids for reuse, and reducing the volume of material for transport and storage (Ogejo, 2009).

*Thermal*

Thermal treatment processes use heat and include incineration, gasification, combustion, torrefaction, and pyrolysis. The common theme of these processes is the volatilization of organic matter in the feedstock (manure). The advantages of thermal processes are: short processing time, high temperature pathogen elimination, efficient phosphorus recovery, and small footprint for the technology (Cantrell et al., 2008). Disadvantages include: cost of fuel for heat processing, release of environmentally damaging gases such as nitrogen and greenhouse gases, and the cost of equipment. During incineration, gasification, and combustion, the volatile the organic matter in the manure is burned off, leaving behind ash. The ash can be used as light weight aggregates for bricks and cement, as activated carbon, and as a fertilizer (Merkel, 1981). Gasification, pyrolysis, and torrefaction can change manure into single or combinations of different gas, liquid, and solid products, depending on the operating conditions. The resulting products can be used for energy (Tyagi et al., 2009; Bakri et al., 2016).
**Incineration**

Incineration burns manure at temperatures ranging from 400 to 900 °C in the presence of oxygen (Huang et al., 2011). The organic matter and some phosphorus and nitrogen volatilize into a gas, and approximately 30-35% of the manure remains as ash (Huang et al., 2011). The volatilization of VS reduces the amount of manure to handle, and the heat can sterilize any components that may be hazardous (Merkel, 1981).

**Gasification**

Gasification bakes manure in a low oxygen environment to turn the volatile solids into a mixture of carbon monoxide and hydrogen to create syngas, and the non-volatile solids remain as ash or char (Buckley & Schwarz, 2003). Dry gasification requires a low moisture content and a TS upwards of 90% by weight (Cantrell et al., 2008). Wet gasification however can have a much higher moisture content and a TS as low as 8% (Cantrell et al., 2008). Dry gasification of manure is performed at atmospheric pressure and at around 850°C (Cantrell et al., 2008). Wet gasification uses higher pressures of 21 MPa and lower temperatures of around 350°C (Cantrell et al., 2008). The advantage of dry gasification is faster processing times compared to wet gasification, but wet gasification does not require an additional step to concentration the solids to a high TS.

**Pyrolysis**

Pyrolysis decomposes the biomass in the absence of oxygen at temperatures up to 1000°C to produce pyrolysis oil, syngas, and char (Fernandez-Lopez et al., 2015; Cantrell et al., 2008). The pyrolysis oil can be used as a liquid fuel and the char can be converted into black carbon or fertilizer, or used as a feedstock to manufacture printing ink, rubber, tiles, or charcoal briquettes (Merkel, 1981).
**Torrefaction**

Torrefaction heats the biomass in the absence of oxygen at temperatures 200 to 300°C to condense the carbon into a solid product which is free of pathogens, easily transported and stored, and has a high energy value (Bakri et al., 2016). The final solid product is often used as a coal substitute due to its energy density, hydrophobicity, and resistance to microbial degradation (Bakri et al., 2016).

**Chemical**

In general, chemical processes use oxidation, ion exchange, coagulation, flocculation and/or precipitation to remove nutrients such as nitrogen (N), phosphorus (P), heavy metals, organic compounds, and odor causing compounds. The disadvantage of chemical processing is the cost of chemical additives, but many are working to combine chemical treatment technologies to minimize overall cost (Zhang & Lei, 1998).

**Oxidation**

Oxidation uses electrolysis to minimize odors and kill odor causing bacteria by O₂ toxicity (Bejan et al., 2007) or oxidants to react with odor causing agents such as hydrogen sulfide (Xue & Chen, 1999). Potassium permanganate and hydrogen peroxide are chemical oxidants which react with hydrogen sulfide, mercaptans, and sulfides to form non-odorous products (Xue and Chen, 1999). Hydrogen peroxide has been reported to react with hydrogen sulfide into sulfur and water in under 90 minutes, providing a fast and economical treatment of odorous gases (Xue and Chen, 1999).

**Ion Exchange**

Ion exchange resins can be used to bind and remove nitrogen and phosphorus from manure (Milan et al., 1997). Zeolite resins in a packed column can be used to remove ammonium from manure (Guo et al., 2006). Ion exchange columns allow for fast processing times around 30 minutes for ammonium removal and can be used multiple times if the columns are regenerated.
properly (Guo et al., 2006). However, using ion exchange for large quantities of manure treatment can be more expensive and laborious as compared to other treatment methods.

*Coagulation, Flocculation, Precipitation*

Coagulation, flocculation, and precipitation are used to remove particles of organic matter, nitrogen, and phosphorus from manure. Coagulation uses electrolytes or organic polymers to cause aggregation of suspended particles, which then settle out due to gravity (Zhang & Lei 1998). This is often used to remove solid particles which remain suspended in water after solid-liquid separation (PNNL, 2003). Coagulation and flocculation aggregate particles through coagulants such as aluminum sulfate, aluminum chloride, ferric chloride, ferric sulfate, lime or polymers (DeBuck et al., 2008). These aggregations of particles known as flocs can then be separated or precipitated out of solution. Precipitation processes can be used to recover nitrogen (N) and phosphorus (P) from manure e.g. as struvite (Rico et al, 2010; Uludag-Demirer & Othman, 2009). Phosphorus removal is often achieved through the addition of coagulants to cause precipitation and then settle out the P (DeBusk et al., 2008).

*Biological*

In biological processes, microorganisms such as bacteria transform organic matter into a more stabilized product. Organic matter is comprised of proteins, lipids, and carbohydrates which are broken down into amino acids, volatile fatty acids, and sugars and alcohols respectively (Merkel, 1981). Using microorganisms instead of energy intensive processes that require, for example, heat and pressure, makes biological conversion processes much cheaper than thermal conversion, but the processing time is much longer (Candell et al., 2008). Biological processes also produce nutrient rich end products, which are highly suitable as fertilizers (Candell et al., 2008). The breakdown of animal wastes by biological mechanisms is seen in Figure 1.

**1.2.3 Description of Treatment Technologies**

Manure treatment technologies take advantage of any or a combination of the physical, chemical, and biological processes discussed. Manure treatments technologies that have been used on dairy
farms include solid-liquid separation, aerobic digestion, lagoons, composting, and anaerobic digestion (Humenik, 2001, eXtension 2013).

**Solid-liquid separation**

Separation processes include sedimentation, filtration, centrifugation, screening, chemical precipitation, and particle coagulation and flocculation (Zhang & Westerman, 1997). Screening and sedimentation are the most commonly used due to ease of use and low cost (Zhang & Westerman, 1997). Filtration is used mostly as a secondary separation technique, to the remove the finer particles such as N and P, which are precursors to odor causing agents (Zhang & Westerman, 1997). Centrifugation cause separation via centrifugal forces and separate particles based on density (Zhang & Westerman, 1997). Chemical precipitation uses metal based coagulants of iron (Fe$^{2+}$, Fe$^{3+}$), aluminum (Al$^{3+}$), and calcium (Ca$^{2+}$) to chemically react with nutrients, forming an insoluble precipitate (Zhang & Westerman, 1997). The solids are removed during these processes to reduce the organic loading in other treatments, and can be used as bedding in CAFOs or composted separately (eXtension, 2013).

**Aerobic Digestion**

Aerobic digestion uses biological processes in the presence of oxygen to oxidize organic carbon and nitrogen compounds, thereby minimizing the production and release of odors, ammonia, and greenhouse gases (eXtension, 2013). Microbial growth consume and degrade suspended and soluble organics in the manure (Merkel, 1981). The advantages of aerobic digestion include odor reduction and high BOD and settleable solid reduction ($\geq 90\%$) (Merkel, 1981). Aerobic digestion is able to handle wastes with different organic matter content, thereby minimizing pretreatment requirements of wastes that may be high in organic matter content. Two major disadvantages of aerobic systems are (1) the cost of energy and equipment needed and (2) required skill to manage and maintain biological growths (Merkel, 1981). Resources needed to manage aerobic systems are not common on farms, so aerobic digestion is used mostly in municipal wastewater treatment centers.
Figure 1: Components of Animal Waste (Merkel 1981)


Lagoons

Lagoons provide both manure treatment and storage on farms. They can be anaerobic or aerobic. Aerobic lagoons work with the same principles as aerobic digestion described above. In general, anaerobic lagoons degrade organic matter through microbial activity in the absence of oxygen (Grady et al., 2011). Some lagoons have facultative bacteria which use some oxygen during metabolism to convert the complex organic molecules (proteins, lipids, carbohydrates) into simpler compounds (Grady et al., 2011). The strictly anaerobic methanogenic bacteria then convert the simpler molecules into carbon dioxide and methane, which leave the system, reducing the amount of carbon in the lagoon (Grady et al., 2011). These lagoons are able to treat dilute and concentrated organic wastes well with high loading rates and can minimize the amount of sludge that accumulates. Anaerobic lagoons do not require mechanical agitation or aeration, both minimizing the amount of land necessary for construction and the cost of energy (Grady et al., 2011). They also provide a central locale for manure collection. The main disadvantage is the production of odorous gases, which can be mitigated by covering the lagoon.

Composting

Composting is an aerobic biological process in which microorganisms convert organic matter in manure to form a soil humus material - compost (Merkel, 1981). During the composting process, microbial activity on the waste generates heat, increasing the internal temperature of the compost pile (Merkel, 1981). Composting temperatures spans mesophilic to thermophilic, and remains at thermophilic temperatures for periods long enough to slow down or inactivate microbial activity. Usually falling temperature of the compost after sustaining the thermophilic temperatures is an indication of completion of the breakdown of organic matter. The final product is odorless and granular and used as a soil amendment or erosion control material while being free of pathogens and seeds (eXtension, 2013; Merkel, 1981). Compared to raw manure, composted manure possess better quality and characteristics as a soil amendment, is easy to handle and store, and has minimal odor, pathogens, and seeds (Hao et al., 2001). Composting also reduces the volume of manure. The reduced volume and mass allow for the compost to be transported further at a lower cost (Hao et al., 2001). The disadvantage to composting is the carbon and nitrogen losses
as greenhouse gases, creating a potential to cause environmental damage and reducing or losing some of the fertilizer value of the compost (Hao et al., 2001). Composting also demands a high solids content compared to lagoons and anaerobic digestion, requiring a liquid-solids separation processing step before composting begins.

**Anaerobic Digestion**

Anaerobic digestion (AD), is a biological process that decomposes organic matter into a stable product while producing biogas in the absence of oxygen. AD reduces odor, pathogen count, and organic matter in the manure while creating fertilizer and biogas, which can be sold as value-added products (Zieminski & Frac, 2012). The technology has been used for centuries, first in India in 1859, and multiple configurations have been made since then to take advantages of processing parameters to make significant improvements in performance (Abdelgadir et al., 2014). The advantages of anaerobic digestion include reduction of greenhouse gas emissions by conversion and capture of carbon to methane, a higher efficiency of biomass degradation and waste stabilization, a smaller space requirement, and a lower input demand as compared to lagoons (Abdelgadir et al., 2014). Generally, the cost of anaerobic digestion is lower than aerobic digestion as anaerobic digestion forgoes aeration (Humenik, 2001). Additionally, abstracting value-added products such as biomethane and fertilizer provide an extra source of revenue to the farm and will offset the cost of treatment.

The major factors that impact AD performance are temperature, hydraulic retention time (HRT), organic loading rate (OLR), and pH (Abdelgadir et al., 2014). Reactor design may also affect costs and influence what value-added products are attainable. During the AD process, the conversion of biomass consists of four major steps: hydrolysis, fermentation, acetogenesis, and methanogenesis. The process parameters are usually adjusted to optimize the synergy between all steps. The factors that affect anaerobic digestion are briefly described below.

*Temperature*
Temperatures affect gas production and the rate of organic matter degradation. Commonly, on-farm AD are operated at mesophilic (30-38°C) with a few at thermophilic (50-60°C) temperatures. In general, ADs operated at mesophilic temperatures are preferred due to lower energy input required to maintain the digester temperature thereby minimizing the cost of heating while still stabilizing manure and producing biogas. Thermophilic temperatures, while requiring higher operating costs, use shorter treatment times due to the higher metabolic rates (Abdelgadir et al., 2014). Thermophilic digesters are more efficient, require smaller tank sizes, a lower capital cost, and also require a shorter hydraulic retention time (Abdelgadir et al., 2014).

**Hydraulic Retention Time**

Hydraulic retention time (HRT) is defined as the time that a soluble component remains within the reactor and is important to ensure proper biomass degradation (Abdelgadir et al., 2014). It is calculated as the volume of the reactor divided by the influent flow rate (Equation 1) (Abdelgadir et al., 2014).

\[
HRT \ (\text{days}) = \frac{\text{Volume of the reactor (L)}}{\text{Influent flow rate (L/day)}} \quad (1)
\]

The standard HRT for a completely mix digester at mesophilic conditions is 17 days (NRCS, 2009).

**Organic Loading Rate**

The organic loading rate (OLR) refers to the amount of organic matter entering the reactor per day, and is equal to the mass of the volatile solids in the influent divided by the volume of the reactor (Equation 2).

\[
OLR \ \left( \frac{\text{g}}{\text{L}} \right) = \frac{\text{Weight of VS (g)}}{\text{Volume of reactor (L)}} \quad (2)
\]
High OLR can lead to the accumulation of undigested materials and cause operational problems (Abdelgadir et al., 2014). Thus, it is important to balance the OLR and HRT to achieve sufficient biomass degradation.

**pH**

pH is critical for the microbial communities within the reactor. Methanogens prefer a neutral pH, creating a narrow optimum range of 7.0 to 7.2, with acceptable ranges being from 6.6 to 7.6, but the pH can drop to lower than 6.0 due to an increased acid production (Abdelgadir et al., 2014). Balance of the microbial communities of each processing step is crucial for effective digestion and biogas production.

**Digester Design**

Anaerobic digesters are designed to meet varying criteria such as gas production rates, operating costs, and feeding rates. Four different types are commonly used: batch, mixed, plug flow, or two-stage (Parsons, 1984).

Batch digesters are filled completely with feedstock, e.g., manure and held for one HRT before emptying (Parsons, 1984). Batch systems require little attention during digestion, but gas production rates are not consistent, with a slow initial gas production and decline after peak (Parsons, 1984). Batch digesters are not commonly used on farms due to the variable gas production rates but are useful in a lab setting to determine the methane potential of a feedstock. Continuous feeding of reactors is more desirable on farms to provide a constant and reliable gas production rate. Mixed digesters use a mixing mechanism such as paddles or an agitator to integrate fresh slurry from the influent into the rest of the digester, allowing for the better distribution of the bacteria, and therefore improved gas production and slurry treatment (Parsons, 1984). The digesters can be continuously or periodically mixed, but the periodically mixed reactor is more cost effective due to intermittent power demand. Plug flow digesters are less expensive compared to their mixed digester counterparts, both for construction and operation, and are mechanically simpler to install and fix (Parsons, 1984). In plug flow digesters the
influent slurry is fed at one end and pushes the oldest slurry out of the other end in a horizontal manner (Parsons, 1984). Two-stage digesters work like mixed digesters with the exception of having two reactors in series for series to separate the acidogenic and methanogenic steps. Two stage digesters offer several advantages: creating separate optimums for the two major groups of bacteria for each phase (described below); preventing toxic material build up and overload thereby increasing stability; preventing pH shock of methanogens from low pH fermentative bacteria; shortening the hydraulic retention time and allowing for higher organic loading rate, ultimately making a more cost efficient operation (Demirer & Chen, 2005). The design of a reactor must allow for synergy between the four steps (described below) of anaerobic digestion to promote biodegradation and methane production.

Hydrolysis

The hydrolysis process, represented by Eq. 3, is the first stage of organic matter decomposition. The process uses a variety of enzymes such as amylases, proteases, and lipases to break down water-insoluble carbohydrates, proteins, and fats into sugar and monosaccharides, amino acids, and fatty acids, respectively (Shah et al., 2014). Hydrolytic enzymes break down only 50% of the organic matter fed to a digester, leaving complex polymers such as cellulose undigested (Shah et al., 2014). Depending on the complexity of the organic matter, particle size, pH, and rates of diffusion and adsorption of enzymes, hydrolysis can be the rate limiting step for biogas production (Shah et al., 2014).

\[ C_{24}H_{40}O_{20} + 3H_2O \rightarrow 4C_6H_{12}O_6 \]  \hspace{1cm} (3)

Hydrolysis is performed facultative bacteria, such as *Streptococcus* and *Enterobacterium* (Shah et al., 2014). These facultative bacteria consume oxygen to create a completely anaerobic environment necessary for the other microbial populations, such as fermentative bacteria.

Fermentation
Fermentation, also known as acidogenesis, is the conversion of water soluble organic matter into a variety of small molecules (Angelidaki et al., 2011). Hydrolysis products, monosaccharides, and amino acids are degraded into short-chain volatile fatty acids (VFAs) such as acetic, propionic, butyric, and valeric acids, aldehydes, carbon dioxide, and hydrogen gas (Angelidaki et al., 2011; Shah et al., 2014). Fermentation occurs in two stages: hydrogenation and dehydrogenation. In the first step, hydrogenation, products such as acetates, carbon dioxide, and hydrogen gas are formed (Shah et al., 2014). The acetates are used directly by methanogens for methanogenesis, and the concentration of hydrogen gas increases within the digester (Shah et al., 2014). In the second step, dehydrogenation, bacteria begin producing volatile fatty acids such as propionic and butyric acids (Shah et al., 2014). These VFAs are used by a separate group of bacteria that oxidizes them into acetate for substrates for the methanogens (Shah et al., 2014).

The generalized reactions for fermentation are presented in equations 4, 5 and 6.

\[
C_6H_{12}O_6 \rightarrow 2CH_3CH_2 + 2CO_2 \quad (4)
\]

\[
C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (5)
\]

\[
C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (6)
\]

Fermentation takes place by obligatory anaerobic bacteria of the genera Psuedomonas, Bacillus, Clostridium, Micrococcus, and Flavobacterium (Shah et al., 2014).

**Acetogenesis**

The generalized reactions for acetogenesis are presented in equations 7, 8, and 9.

\[
CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2 \quad (7)
\]

\[
C_6H_{12}O_6 + 2H_2O \rightarrow CH_3COOH + 2CO_2 + 4H_2 \quad (8)
\]

\[
CH_3CH_2OH + 2H_2O \rightarrow CH_3COO^- + 2H_2 + H^+ \quad (9)
\]
As the quantity of fermentation acids (formic, acetic, propionic, butyric, and valeric) increase, the pH in the digester decreases, enhancing the production of acetate by acetogenic bacteria such as *Syntrophomonas* and *Syntrophobacter* (Shah et al., 2014). The VFAs produced during fermentation are converted into acetate and hydrogen during acetogenesis. The acetogenic step accounts for 25% of all the acetates and 11% of the hydrogen produced (Shah et al., 2014). Hydrogen gas is toxic to the acetogenic bacteria, forcing a symbiosis with the autotrophic methane bacteria, which consume H₂, in order for the digester to function (Shah et al., 2014). The acetate then become the main substrate for methanogens.

*Methanogenesis*

Methanogenesis is the final step of anaerobic digestion. Methanogens convert acetic acid/acetate, hydrogen, formate, methanol, methylamine, and dimethyl sulfide, into the final products of carbon dioxide and methane, known as biogas (Shah et al., 2014). The gas leaves the system as the final removal of carbon to reduce the strength of the effluent, and is collected as biogas (Abdelgadir et al., 2014). This biogas is either burned for heat or energy, or scrubbed, stored and transported (Abdelgadir et al., 2014). Methanogens breakdown and modify several substrates including hydrogen, acetate, formate, methanol, carbon monoxide, trimethyl-, dimethyl- and methyl- lamines, methyl mercaptans, and metals, making them excellent waste processors, as presented in equations 10 to 21, respectively (Shah et al., 2014).

\[
CH_3COOH \rightarrow CH_4 + CO_2 \tag{10}
\]

\[
CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \tag{11}
\]

\[
4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \tag{12}
\]

\[
CH_3COOH \rightarrow CH_4 + CO_2 \tag{13}
\]

\[
4HCOOH \rightarrow CH_4 + CO_2 + 2H_2O \tag{14}
\]
Methanogenesis is performed by archaea, unlike the previous, bacterial-mediated steps. The methanogenic archaea are chemolithotrophic and strictly anaerobic (Shah et al., 2014). Two types of methanogens, acetotrophic and hydrogenotrophic, form a symbiotic relationship with the diverse bacterial community in a digester to create biogas. Acetotrophic methanogens metabolize the methyl groups of acetate to CO₂ and CH₄ gases (Shah et al., 2014). This process consumes VFAs and acetate produced during fermentation and acetogenesis, thereby, maintaining the pH at a neutral balance. Hydrogenotrophic methanogens use hydrogen and carbon dioxide gas as their substrates for methane production (Shah et al., 2014). The consumption of hydrogen gas maintains a stable partial pressure of hydrogen to prevent toxicity to the acetogenic bacteria in the previous step (Shah et al., 2014). The methanogens are responsible for methane production, and the temperature of digestion can affect the most prominent species involved in digestion (Demirel & Scherer, 2008). *Methanosaeta* is more dominant in mesophilic conditions, while *Methanosarcina* dominates at thermophilic conditions (Shah et al., 2014).

The average rate of biogas production from single substrate (manure) is variable. Many operators are adopting a co-digestion approach, where food and other agricultural wastes mixed with manure as feedstock to increase the quantity and quality biogas produced (Macias-Corral et al.,

\[
4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (15)
\]

\[
4CO + 2H_2O \rightarrow CH_4 + 3H_2CO_3 \quad (16)
\]

\[
4(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3 \quad (17)
\]

\[
2(CH_3)_2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3 \quad (18)
\]

\[
4(CH_3)NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3 \quad (19)
\]

\[
2(CH_3)_2S + 3H_2O \rightarrow 3CH_4 + CO_2 + H_2S \quad (20)
\]

\[
4Me^0 + 8H^+ + CO_2 \rightarrow 4Me^{++} + CH_4 + 2H_2O \quad (21)
\]
Typical biogas production rates that have been reported for manure range from 0.17 to 0.319 L CH₄ g⁻¹ VS⁻¹ for mesophilic reactors (Stowe et al., 2015; El-Mashad & Zhang, 2007; Solli et al., 2014), and 0.180 to 0.246 L CH₄ g⁻¹ VS⁻¹ (Kaparaju et al., 2008; Moset et al., 2015) for thermophilic reactors.

The balance of specific microbial communities is of paramount importance to the stability and operation of anaerobic digesters. The prevalence use of antibiotics on farms presents a potential for unused and potent antibiotics excreted in manure to inhibit microbial growth in digesters, affecting both manure stabilization and gas production.

1.3 Antibiotics and Bacterial Targets

Antimicrobials are drugs which kill or inhibit growth of microorganisms and antibiotics are targeted to kill and inhibit bacteria (CDC, 2015). In food-producing animals such as cattle and hogs, antimicrobials are used for three reasons: therapeutically (to treat animals with illness), prophylactically (to prevent infections), or as growth promotors (as a feed supplement) (Halbert et al., 2006). In general, antibiotics kill and/or inhibit bacterial growth by attacking specific parts of cells of interest. Antibiotics are classified based on their target organism, activity, spectrum, and chemical structure.

1.3.1 The Structure of Bacteria

Bacteria are prokaryotic microorganisms with a cellular envelope composed of a cell wall and plasma membrane (Anderson et al., 2012). The cell wall protects the bacteria from osmotic pressure changes while the plasma membrane holds the cytoplasm (Anderson et al., 2012). There are two classes of bacteria, gram negative and gram positive, differentiated by their cellular envelopes. Gram-positive bacteria have an inner membrane made up of phospholipids and a layer of peptidoglycan bonded with teichoic acids (Clark & Pazdernik, 2013). On the other hand, gram-negative bacteria have three layers: the inner membrane with phospholipids; a single layer wall made of peptidoglycan and periplasmic space; and a final outer membrane which is connected through the periplasmic space to the inner membrane by lipoproteins (Clark & Pazdernik, 2013). The cellular envelope structures are used to differentiate antibiotic modes of
attack. Bacteria have DNA in a nucleoid or plasmid structure in the cytoplasm, and also synthesize many of their own enzymatic co-factors, such as folic acid (Anderson et al., 2012). The differences in bacterial biochemistry are used to design antibiotics to selectively attack species or genera of bacteria. Antibiotics target one of four areas to kill bacteria or prevent new growth: DNA replication, metabolism, protein synthesis, or cell-wall synthesis. A generalized chart demonstrating the specific target areas in a bacteria cell is shown in Figure 2.

1.3.2 Antibiotic Targets in Bacteria

DNA Replication

Three classes of antibiotics, quinolones, rifamycin, and nitroimidazole target DNA replication with the goal of preventing the growth of new bacterial cells. The process of forming a new bacterium requires duplication of DNA for binary fission. Typically, DNA replication occurs by separating the double helix into two strands via a DNA helicase. Then a DNA primase synthesizes and binds to a primer DNA strand to begin DNA synthesis (Anderson et al., 2012). A DNA polymerase performs DNA replication and new strand synthesizes while a DNA endonuclease proof-reads the fidelity of the copied strand (Anderson et al., 2012). Antibiotics target specific areas of this replication process to prevent new cells from being formed. Quinolones act by binding to the topoisomerase enzymes responsible for relaxing the DNA chain from the tightly coiled double helix (Anderson et al., 2012). Without a relaxed DNA chain, the DNA cannot be replicated. Rifamycin antibiotics bind to the RNAP holoenzyme binding site, which normally attaches to the promoter DNA to initiate transcription (Anderson et al., 2012). By preventing binding to the RNAP holoenzyme, transcription is hindered (Anderson et al., 2012). Nitroimidazole antibacterial agents release radicals within the cytoplasm to attack the DNA molecule itself (Anderson et al., 2012).
Figure 2: Targets of antibiotic within bacteria cell (modified from Wikipedia, 2007)
Metabolism

Sulfonamide antibiotics damage cells by targeting bacterial metabolism. For example, a key metabolic process in prokaryotes is folic acid synthesis, an important enzymatic co-factor for DNA synthesis (Anderson et al., 2012). Sulfonamide antibiotics interfere with the folic acid synthesis process by mimicking para-aminobenzoic acid (PABA) and binding to the active site dihydroperoate synthase (Anderson et al., 2012). This prevents folic acid synthesis and DNA replication (Anderson et al., 2012) and subsequently, blocks cell repair and the formation of new bacterial cells.

Protein Synthesis

The antibiotics aminoglycoside, macrolide, tetracycline, and chloramphenicol target protein synthesis to prevent the growth of new and the repair of existing bacterial cells. Protein synthesis occurs by DNA replication, transcription of RNA via mRNA, and translation to form the protein using a ribosome. The ribosome is made of two subunits, 50S and 30S, which come together to translate the protein into its final form. Aminoglycoside antibiotics bind to the 30S subunit of the ribosome, causing misreading of the mRNA to prevent protein synthesis (Anderson et al., 2012). Macrolide antibiotics bind to a high-affinity site within the nascent protein tunnel in the 23S subunit of the 50S subunit of the ribosome for inhibition (Anderson et al., 2012). Tetracycline antibiotics inhibit bacterial growth by binding to the 30S subunit of the ribosome via a high-affinity binding site (Anderson et al., 2012). Cloramphenicol binds to the 50S subunit of the ribosome and prevents binding of tRNA for translation by binding to the peptidyl transferase center A site (Anderson et al., 2012).

Cell-Wall Synthesis

Beta (β)-lactam antibiotics prevent cell wall synthesis to kill both existing and growing cells (Anderson et al., 2012). Beta lactam antibiotics inhibit the penicillin-binding protein (PBP) or transpeptidase enzyme, which is responsible for crosslinking peptidoglycan strands within the cell wall (Anderson et al., 2012). Because peptidoglycan is present in both gram positive and
negative bacteria types but not eukaryotic cells, it can provide a broad spectrum of activity with no effect on human or animal host cells (Anderson et al., 2012). Beta lactam antibiotics include penicillins, cephalosporins, monobactams, and carbapenems (Anderson et al., 2012).

1.3.3 Antibiotics and the Dairy Industry

The use of antimicrobials is prevalent in livestock production, with over 13 million kg of antimicrobials sold in the USA for use in food-producing animals in 2010 (FDA, 2014). Different types of antimicrobials are used to treat different diseases in livestock, and cephalosporin drugs are the most common antibiotic used to treat mastitis. Mastitis is an infection of the udder of dairy cows, affecting milk production and impacting income from milk. It is common within the dairy industry, with 16.4% of all dairy cows afflicted with the disease in 2007 (APHIS, 2008). Over 53% of cows treated for mastitis were treated with cephalosporins (such as ceftiofur, cephapirin, and cefazolin) (APHIS, 2008) and more than 24,000 kg of cephalosporin drugs were sold in the USA in 2010 (FDA, 2014).

Ceftiofur

Ceftiofur is a third generation cephalosporin, and a broad spectrum bactericidal antibiotic under the broader classification of beta-lactams. There are many generations of antimicrobials, often distinguished by their spectrum of activity. Cephalosporins are classified into four generations: 1, 2, 3, 4, with narrow, expanded, broad, and extended spectrums, respectively (Hornish & Kotarski, 2002). Ceftiofur is a veterinary specific, broad spectrum, third generation cephalosporin (Hornish & Kotarski, 2002). All four generations are semi-synthetic antibiotics, originating from cephalosporin C, a compound produced by the Cephalosporium acremonium fungus (Hornish & Kotarski, 2002). The defining characteristic of cephalosporins is the 7-aminocephalosporanic acid nucleus which is made of six-membered dihydrothiazine ring connected with four-membered beta-lactam ring (Hornish & Kotarski, 2002). This beta-lactam ring disrupts the penicillin-binding proteins inside the cell walls of bacteria (Hornish & Kotarski, 2002). The chemical structure of Ceftiofur, presented in Figure 3, has an oxyiminoaminothiazolyl group attached to the 7-amino cephalosporin nucleus instead of an amino-acyl at position 7- beta, as found in other cephalosporins (Hornish & Kotarski, 2002). As
a third generation cephalosporin, Ceftiofur also includes a furoic acid thioester at position 3 (Hornish & Kotarski, 2002).

The degradation of Ceftiofur results in two main metabolites, desfuroyl-ceftiofur (DFC) and defuroyl-ceftiofur dimer (DFC-dimer). These metabolites are formed by the cleavage of the furoic acid by hydrolysis of the thioester bond, and condensing of the DFC molecule to retain the beta-lactam ring, respectively (Hornish & Kotarski, 2002). Both metabolites keep their beta-lactam ring and therefore their antimicrobial activity (Hornish & Kotarski, 2002). Ceftiofur is mostly metabolized, either as a sodium salt or hydrochloride salt, with a half-life of less than 10 minutes (Hornish & Kotarski, 2002). Approximately 57-63% of Ceftiofur metabolites excreted by cattle is in the urine and the rest is contained in the feces (Hornish & Kotarski, 2002). Any antimicrobial activity left in the excreted material is expected to degrade quickly by the hydrolytic, photolytic, and biological degradation mechanisms found in the environment (Hornish & Kotarski, 2002).
Figure 3: Chemical structure of Ceftiofur (Wikipedia, 2008)
1.3.4 Antibiotics in Anaerobic Digestion

Antibiotics are designed to kill and inhibit the growth of disease causing bacteria in animals. However, some of the antibiotics are excreted in manure and may impact the bacteria community in anaerobic digestion systems. Many studies have investigated the effect of antibiotics on anaerobic digestion of manure and wastewater sludge, including: antibiotics which inhibit protein synthesis (aminoglycosides, tetracyclines, amphenicols, MLS), antibiotics that affect cell wall synthesis (beta-lactams), and antibiotics which inhibit DNA replication (rifampicin, sulfonamides, quinolones). Findings about the effect of antibiotics on biogas production have been varied and dependent on the antibiotic type. Typically, results are reported in terms of biogas inhibition, defined as the percent difference in biogas yield between control and antibiotic treatments. The results of the studies with a focused on aminoglycosides, tetracyclines, amphenicols, MLS, beta-lactams, rifampicin, sulfonamides, and quinolones are presented below.

Aminoglycosides

One of the pioneer studies on the impact of aminoglycoside antibiotics on anaerobic digestion was conducted by Sanz et al. (1996). In their study they added a synthetic volatile fatty acid stock to wastewater sludge in a batch biomethane potential (BMP) test at mesophilic temperatures (30 °C) to determine antibiotic concentrations that inhibited biogas production. Biogas was inhibited 20% by all aminoglycoside antibiotics (streptomycin, spectinomycin, neomycin, hygromycin B, kanamycin) with a minimum dosage (Sanz et al., 1996). Streptomycin inhibited biogas production 20% at a 18 mg L⁻¹ dosage, spectinomycin and neomycin at 20 mg L⁻¹, and gentamicin at a 35 mg L⁻¹ dosage (Sanz et al., 1996). Hygromycin B inhibited gas production 20% at 64 mg L⁻¹ dosage and kanamycin at 100 mg L⁻¹ dosage (Sanz et al., 1996). Lins et al. (2015) confirmed Sanz’s inhibition results, reporting a 27% biogas inhibition at 100 mg L⁻¹ of kanamycin sulfate, and Sara et al. (2013) found 22% biogas inhibition at 37 mg L⁻¹ of spectinomycin combined with 18.5 mg L⁻¹ lincomycin in a mesophilic batch reactor with pig manure. Higher inhibitions were found for many of the aminoglycosides. Lins et al. (2015) reported an 81% biogas inhibition at 100 mg L⁻¹ of gentamicin, an 89% inhibition at 100 mg L⁻¹ of neomycin sulfate, and Sanz et al. (1996) reported a 50% biogas inhibition at 210 mg L⁻¹ of hygromycin B.
Tetracyclines

Tetracyclines have been vastly studied because of their prevalent use in livestock production. Approximately 42% of all antimicrobials used in livestock are tetracyclines (FDA, 2014). A summary of some of the work showing antibiotic concentrations and related biogas inhibitions reported in literature are presented in Figure 4. Of note, Bauer et al. (2014) conducted a continuous mesophilic (37.5 °C) reactor of pig manure, and found that biogas production was inhibited at lower antibiotic concentrations as compared with the other studies. All studies with the exception of Lins et al. (2015) reported biogas inhibition by tetracycline at concentrations as low as 1 mg L⁻¹. These results suggest potential inhibition of biogas production during anaerobic digestion by tetracycline at low concentrations.

Amphenicols

Results on biogas inhibition from amphenicols are varied. There are reports that amphenicols can inhibit biogas production a dosages as low as 11 mg L⁻¹ (Sanz et al., 1996; Mitchel et al., 2013; Lins et al., 2015). Sanz et al. (1996) reported a 20% inhibition at 11 mg L⁻¹ of chloramphenicol in the same experiment as described above. However, Lins et al. (2015) described negligible biogas inhibition at 100 mg L⁻¹ of chloramphenicol. In florfenicol experiments, Mitchell et al. (2013) reported a 40% inhibition at 36 mg L⁻¹. At higher dosages, Sanz et al. (1996) reported an 80% inhibition at 41 mg L⁻¹ and Mitchell et al. (2013) recorded 80% inhibition at dosages between 180 and 360 mg L⁻¹. In summary, these studies present mixed results.

Macrolide-Lincosamide-Streptogramines (MLS)

Erythromycin, spiramycin, and tylosin are three macrolides that have been reported in literature. Lincomycin, a lincosamide, has also been studied for effect on biogas production during anaerobic digestion. No studies on streptogramines were found. Biogas inhibition relative to the concentration of MLS antibiotics is presented in Figure 5. Aydin et al. (2015) and Sara et al. (2013) investigated inhibitory effects of MLS antibiotics in combination with other antibiotics.
Aydin et al. (2015) studied the effect of combinations of erythromycin (E), sulfamethoxazole (S), and tetracycline (T) on biogas production on a batch reactor using synthetic volatile fatty acids as feedstock. They reported inhibition at 10 mg L$^{-1}$ of erythromycin in all combination studies (ET, ES, ETS). Sara et al. (2013) performed a batch anaerobic digestion experiment of pig manure and lincomycin and spectinomycin at 37°C. They reported 18% inhibition when 4.6 mg L$^{-1}$ lincomycin and 9.2 mg L$^{-1}$ spectinomycin. Mitchell et al. (2013) found no total biogas inhibition during tylosin loading at concentrations of 0.92, 9.2, and 92 mg L$^{-1}$. Sanz et al. (1996) however reported a 20% inhibition at 15 mg L$^{-1}$ of tylosin in the same experiment as described above. The results of these experiments are mixed and further investigation on how antibiotics inhibit biogas production need to be performed.
**Figure 4:** Reported biogas inhibition relative to tetracycline concentration (for ETS, antibiotic concentration refers to antibiotic concentration of each individual component)

**Figure 5:** Reported biogas inhibition relative to macrolide and lincosamide concentration. Lincomycin+spectinomycin reports antibiotic concentration of lincomycin only.
**Beta-Lactams**

Penicillins and cephalosporin beta-lactam drugs have been investigated for their effect on biogas production. Variable results have been reported on the influence of penicillins in anaerobic digestion. Sanz et al. (1996) reported a 20% biogas inhibition at 10 mg L\(^{-1}\) of ampicillin, while Lins et al. (2015) and Mitchell et al. (2013) describe negligible inhibition for concentrations up to 100 mg L\(^{-1}\) of ampicillin. Penicillin G had the same varied results. Sanz et al. (1996) reported 20% inhibition at 10 mg L\(^{-1}\) of penicillin, and Lins et al. (2015) reported negligible inhibition at 100 mg L\(^{-1}\) of penicillin.

Lu et al. (2014), Beneragama et al. (2013), and Sara et al. (2013) tested the effect of cephalosporins on batch reactors. Lu et al. (2014) studied the effect of cephalexin, a first generation narrow spectrum cephalosporin, on mesophilic (35°C) waste activated sludge. There was no biogas inhibition with antibiotic concentration of 2,000 mg L\(^{-1}\) (Lu et al., 2014). Beneragama et al. (2013) tested cefazolin, another first generation narrow spectrum cephalosporin, on biogas production from cow manure at thermophilic (55°C) temperatures and also reported no biogas inhibition. Sara et al. (2013) tested ceftiofur, a third generation cephalosporin, on biogas inhibition of anaerobic digestion of pig manure mesophilic (37°C) temperatures. There was no effect on biogas production at 1.7 mg L\(^{-1}\) (Sara et al., 2013). In summary, batch anaerobic digestion of cephalosporins report no biogas inhibition.

**Rifampicin**

Rifampicin was investigated in batch trials by Lins et al. (2015) and Sanz et al. (1996). Both found an inhibition to biogas production at different concentrations. Sanz et al. (1996) reported a 20% inhibition at 100 mg L\(^{-1}\) dosage of rifampicin and 50% inhibition at dosages greater than 250 mg L\(^{-1}\), but Lins et al. (2015) described a 72% inhibition at 50 mg L\(^{-1}\). The difference may partly be attributed to the differences in temperatures, as Sanz used mesophilic (30°C) conditions and Lins performed digestion at thermophilic (52°C) conditions.

**Sulfonamides**
Aydin et al. (2015) investigated the impact of sulfamethoxazole combined with tetracycline at the conditions described above. They reported a 26% inhibition at 1 mg L\(^{-1}\) concentration of each antibiotic, and a maximum reported inhibition of 61% for 250 mg L\(^{-1}\) concentration of each antibiotic. Mitchell et al. (2013) reported no inhibition effects of sulfamethazine on biogas production for concentrations between 0.001 mg L\(^{-1}\) and 1.0 mg L\(^{-1}\).

Quinolones

Danofloxacin, enrofloxacin, and novobiocin were investigated by Sara et al. (2013), Bauer et al. (2014), and Sanz et al. (1996) respectively. Sara et al. (2013) described a 10% biogas inhibition at 1.1 mg L\(^{-1}\), 16% inhibition at 4.3 mg L\(^{-1}\), and 17% inhibition at 8.5 mg L\(^{-1}\) of danofloxacin. Bauer et al. (2014) reported a 26% inhibition at 1.85 mg L\(^{-1}\) of enrofloxacin for a continuous experiment, and Sanz et al. (1996) published a 20% inhibition at 10 mg L\(^{-1}\) of novobiocin.

1.4 Antibiotic Resistance

The increased use of antibiotics in both human and animal medicine is leading to the emergence of resistant strains of bacteria. The increase in bacteria strains resistant to antibiotics is becoming one of the largest threats to global health, generating questions about the future of medicine and disease treatment (WHO, 2015). Several studies (Hofacre et al., 2000; Smith et al., 2002; Jordan et al., 2005; Kumar & Schweizer, 2005; Sato et al., 2005; Halbert et al., 2006; Walczak & Xu, 2011) have reported the presence of antibiotic resistant bacteria (ARB) in animal manure, raising questions and concerns about the fate and transport of antibiotic resistant on farms, and potential transfer of antibiotics from farm animals to humans. Sapkota et al. (2007) reported presence of enterococci resistant to erythromycin and tetracyclines in surface waters and enterococci resistant to clindamycin and tetracyclines in groundwater near a swine CAFO. Anderson and Sobsey (2006) quantified movement of antibiotic resistant \(E.\ coli\) from swine farms into ground water in North Carolina. They reported almost 70% of \(E.\ coli\) were resistant to 1 to 6 antimicrobials.
1.4.1 Antibiotic Resistant Genes

ARB can use different mechanisms to become resistant to antibiotics. Enzymatic and target modification, antibiotic inactivation and degradation, target bypass, and efflux pump are some of the mechanisms reported in literature associated with transfer during anaerobic digestion (Ghosh et al., 2009; Miller et al., 2013; Zhang et al., 2015). Briefly, with enzymatic modification, bacteria use a newly produced enzyme to destroy or inactivate the antibiotic molecule (Kaufman, 2011). Target modification entails changing the antibiotic receptor in the bacterium to prevent binding (Kaufman, 2011). Target modification of the bacterial cell wall also prevents the antibiotic from acting on the cell wall (Kaufman, 2011). Efflux pump refers to a resistance mechanism where a protein structure embedded in the cell wall pumps out the antibiotic from inside the cell, preventing the concentration of antibiotic from reaching critical efficacy levels (Kaufman, 2011). Efflux pump has been reported as the most common resistance mechanism in wastewater sludge because it presents an efficient way for bacteria to fight against multiple environmental stresses including antibiotics, heavy metals, and toxins (Zhang et al., 2015).

Antibiotic resistance is transferred between bacteria via antibiotic resistant genes (ARGs). These genes encode the mode of action to the bacteria to prevent attack by antibiotics. Antibiotic gene resistance transfer between bacterium can be horizontal or vertical. Horizontal gene transfer involves gene movements between bacteria of the same generation, and not passage through generations of evolution. Passing gene resistance through generations is known as vertical gene transfer (Tenover, 2006). Horizontal gene transfer allows adaptations from one species to pass to another. The transfer of genes is critical in antibiotic resistance, where resistance mechanisms are passed between species, allowing bacteria that have never been exposed to antibiotics to acquire immunity. One condition for horizontal gene transfer is the use of a mobile genetic element. Mobile genetic elements are the elements which change gene expression within a bacterium, allowing for them to become resistant. Mobile genetic elements (MGE) include any DNA which move between and within genomes (Siefert, 2009). These MGE components can move in one of three ways: transformation, conjugation, transduction.
Transformation occurs when bacteria incorporate DNA segments from the environment that have been previously released by other cells into their genetic code (Tenover, 2006). This route allows DNA to pass from lysed cells to be passed to living bacterium. Conjugation requires direct interaction between two bacterium through a pilus (Tenover, 2006). The pilus transfers a plasmid containing ARGs from one bacterium to another. Transduction transfers DNA from one bacterium to another using a bacteriophage, a bacterial virus (Tenover, 2006). While ARG transfer can occur through any of these mechanisms, conjugation is the most common (Barlow, 2009).

**Mobile genetic elements (MGE)**

As noted above, MGEs are good for transferring resistant genes horizontally between bacteria. MGEs are categorized into four groups: transposons, plasmids and bacteriophages, and integrons (Siefert, 2009).

**Transposons**

Transposons move from one location to another within a cell genome and alter gene expression by modifying the genomic dynamics of a cell (McDermott et al., 2003; Siefert, 2009). These elements serve as promoters, silencers, enhancers, inverters, translocators, and as alternative splicing sites, all which can change the expression of gene conferring resistance ((Le Rouzic & Capy, 2009; Siefert, 2009). Three classes of transposons have been identified based on their movement mechanism: retrotransposons, DNA transposons, and insertion sequences (IS).

**Retrotransposons**

Retrotransposons are the “copy and paste” transposons. They incorporate themselves into the genome of a target bacterium by first being transcribed into RNA and then DNA using a reverse transcriptase (Siefert, 2009). This transcriptase is encoded by the transposon itself, allowing it to place multiple copies of genes into the target genome (Siefert, 2009).
DNA Transposons

A DNA transposon also “cuts and pastes” itself into the genome, but does not include an RNA intermediate (Siefert, 2009). Instead it move directly from one position to another within the genome, allowing it to be very specific (Siefert, 2009). A transposase enzyme creates “sticky ends” at the targeted DNA site, and the transposon is cut by the enzyme and pastes itself at the targeted DNA site, causing duplication (Siefert, 2009).

Insertion Sequences

Insertion sequences are the simplest form of transposons, as they only code for transposition (McDermott et al., 2003). Insertion sequences are short DNA sequences of 700-2500 bp length which only code for proteins which the transposon uses, such as transposases to catalyze reactions and regulatory proteins to stimulate or inhibit activity (Siefert, 2009). ISs are often involved in composite or complex transposons, where they frame accessory genes, genes which are not present in all members of a species, such as antibiotic-resistant genes (Siefert, 2009; Ozer et al., 2014). Because of this, they often move neighboring genes to effect antibiotic resistance (Siefert, 2009).

Plasmids and bacteriophages

Plasmids and bacteriophages are very commonly used in biotechnology for gene transfer. Plasmids are chromosomal DNA molecules which are able to replicate themselves outside of the main chromosomal DNA of a cell (Siefert, 2009). They are circular or double stranded, and can code for multiple resistances by harboring multiple genes simultaneously (McDermott et al., 2003). Bacteriophages are bacteria infecting viruses, and can transfer genetic material as single stranded or double stranded RNA or DNA (Siefert, 2009).
**Integrons**

The last type of MGE is the integron. Integrons are the genetic elements which can modify and acquire open reading frames (ORFs) from gene cassettes and convert them into functional genes for expression in the genome (Cambray et al., 2010). A key integron gene, *intI1*, encodes a site-specific tyrosine recombinase to specifically integrate or cut specific gene cassettes (Cambray et al., 2010). “Gene cassettes are discrete genetic elements that may exist as free, circular, non-replicating DNA molecules when moving from one genetic site to another, but which are normally found as linear sequences that constitute part of a larger DNA molecule” (Bennett, 1999). Gene cassettes are normally promoterless and therefore not expressed, and integrons can convert these into expressive genes, which is especially important as it can take dormant resistance genes and express them. Class 1 integrons reduce the “genetic cost” of incorporating a resistant gene into a bacterial genome, and is a key signal to resistance (Ghosh et al., 2009).

**ARGs Related to Beta-Lactam Resistance**

Different ARGs have been identified as indicators for resistance to specific antibiotics. There are many genes which impart resistance to antibiotics, but not all genes are found in every resistant bacterium. Cows treated with Ceftiofur provided a higher abundance of beta-lactam and multiple resistance genes compared to cows without antibiotic treatment (Chambers et al., 2015). This study investigated the change in *cfx(A)*, *mef(A)*, and *tet(Q)* in response to Ceftiofur treatment, which were the genes identified by a previous study (Chambers et al., 2015).

**1.4.2 Fate of ARGs in Anaerobic Digestion**

Waste treatment has the potential to minimize transportation and proliferation of ARGs to mitigate the transfer of antibiotic resistance within the environment. ARGs have been found in both human and animal wastes, after the host was treated with antibiotics. Thus, waste treatment and disposal can be a route through which ARGs are released into the environment. Anaerobic digestion is an old and reliable technology that is used in both wastewater treatment plants and on farms to treat wastes. Several studies reported the effect of anaerobic digestion on antibiotic resistance genes, but there no consensus result has been agreed upon. Studies on mesophilic
digestion of wastewater sludge shows varying degree of change in concentrations of ARGs with mixed or inconclusive results on the effectiveness on ARGs reductions.

Although various genes have been studied to determine the fate of ARGs during anaerobic digestion, there are still no conclusive results about how or why ARGs change during anaerobic digestion. Ghosh et al. (2009) investigated the concentrations of tet(A), tet(O), tet(X), and intl1 genes before and after anaerobic digestion in a full scale two stage, thermophilic (50-60°C) – mesophilic (35-37°C) wastewater digester. They reported no change in concentrations of tet(A), tet(O), tet(X), and intl1 genes before and after the mesophilic stage, but significant reduction was found for all genes after the thermophilic stage (Ghosh et al., 2009). Genes tet(A) and tet(O) were reduced 50 to 80% in the effluent as compared to the influent, intl1 was reduced 80-95%, and tet(X) was almost entirely eliminated, with reduction between 85 and 99% (Ghosh et al., 2009). Diehl & LaPara (2010) anaerobically digested wastewater sludge in bench-scale reactors at varying temperatures (22°C, 37°C, 46°C, 55°C). They reported no change in the tet(A) and tet(L) genes at 22°C, but found an increase in the concentration of tet(O) genes at 37°C. Genes tet(W), tet(X), and intl1 reduced at all temperatures (22°C, 37°C, 46°C, 55°C) but most genes reduced only at the higher temperatures (Diehl & LaPara, 2010). Genes tet(A) and tet(L) were reduced at the higher temperatures of 46°C and 55°C, and tet(W) was reduced at 22°C, 46°C, and 55°C (Diehl & LaPara, 2010). Miller et al. (2013) investigated the effects of mesophilic (37°C) and thermophilic (55°C) anaerobic digestion on concentrations of ARGs in wastewater sludge at bench scale. They reported a reduction in the concentration of tet(O) and tet(W) genes at thermophilic conditions but no change at mesophilic conditions (Miller et al., 2013). A reduction in sulI, sulII, and intl1 genes was reported at both conditions (Miller et al., 2013). Zhang et al. (2015) operated bench scale anaerobic digesters at 35°C and 55°C digesting wastewater sludge to test the change of ARGs associated with resistance of specific antibiotic groups. Mixed results were described by Zhang et al. (2015). At mesophilic temperatures, most groups of ARGs were reduced, some more successfully than other. ARGs associated with quinolones and fosmidomycin were reduced 100% compared to the influent, and ARGs associated with beta-lactam and bacitracin antibiotics were reduced 96.8% and 82.6%, respectively (Zhang et al., 2015). Multidrug ARGs and acriflavine ARGs were reduced 71.6% and 67.4% respectively, while tetracycline ARGs and aminoglycoside ARGs were reduced 42.7% and 37.9% respectively.
(Zhang et al., 2015). The concentration of ARGs associated with antibiotic resistance for 3 classes of antibiotics were reported to increase. Sulfonamide ARGs increased 26.3%, MLS ARGs increased 61.3%, and chloramphenicol ARGs were reported to increase 138% (Zhang et al., 2015). At thermophilic temperatures, Zhang et al. (2015) reported reduction of genes between 3% and 90% for all genes except those associated with chloramphenicol resistance, which increased 614%. Resende et al. (2014) investigated the change in ARGs during anaerobic digestion of cow manure. A full scale anaerobic digester at mesophilic (24°C-34°C) and ambient (14°C-25°C) investigated the change in concentrations of *ermB, aphA2*, and *blaTEM-1* (Resende et al., 2014). They reported a reduction in all genes at both temperature ranges (Resende et al., 2014).

In summary, these studies demonstrate that anaerobic digestion has some effects on ARG concentration, specific to individual genes. The exact mechanism of the fate of ARGs is unknown, but a hypothesis suggests higher temperatures mitigate gene proliferation due to pathogen destruction (Diehl & LaPara, 2010; Sun et al., 2016).
1.5 Objectives

Based on the knowledge of how antibiotics work, there is an expectation that antibiotics should inhibit the AD process. However, there is no conclusive results on the impact of antibiotics on biogas production through anaerobic digestion (AD) in the literature. The cause of the diverse results is not clear, and may be due to due to differences in digester substrate, operating conditions (batch v. continuous, mesophilic v. thermophilic), and microbial community relating to the inoculum. Various studies on AD’s effect on antibiotic resistant genes (ARGs) also have no conclusive results, and few studies have looked at the difference in ARGs in the presence of antibiotics during anaerobic digestion. To my knowledge, work like this study of continuous anaerobic digestion of manure from cows treated with Ceftiofur has not been reported in the literature.

The purpose of this study was to assess the effect of thermophilic anaerobic digestion on Ceftiofur and ARGs in dairy manure. The findings of this study add knowledge to the scientific literature about antibiotics and AD on the environment, VAP production, and the development of antibiotic resistance.
2 MATERIALS AND METHODS

2.1 Manure Source, Collection, and Preparation

Manure used in this study was collected from dairy cows in a research study conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol 13-145-DASC). A group of six healthy, late lactation dairy cows were used. Three cows received subcutaneous administration of Ceftiofur (150 mg Ceftiofur per 45.4 kg body weight; 2 doses, 72 h apart) and the other three cows were used as negative controls with no antibiotic treatment. The cows used in the experiment were selected for homogeneity in terms of body weight and stage of lactation, and none had received antibiotic treatment in the current lactation period. All the cows were offered free choice water and ad libitum total mixed ration. The cows were housed in a metabolism barn throughout the study. On day 1 of the study, cows were fitted with urinary catheters to allow separate feces and urine collection. After 24 h of acclimation to the catheters, the three cows assigned to the Ceftiofur treatment received were treated accordingly. Total collection of feces and urine for 24 h was conducted on day 3 post treatment. Feces and urine from the 3 cows of each treatment were composited and mixed separately to achieve homogeneous dairy manure for the Control and Ceftiofur. The manure collected was placed in 53 L HDPE totes and stored in a freezer at -20 °C until use. Manure from control cows contained no antibiotics and served as control dairy manure. When needed, the manure was removed from the freezer and thawed. Once thawed, the feed for the digesters was prepared by mixing 1 kg of manure with 2.5 kg of DI water. The mixture was blended for 30 seconds, and then passed through a 2 mm sieve to attain the desired consistency and total solids. The prepared feed was stored at 4 °C until used.

2.2 Reactor Design and Operation

A total of four upright cylindrical completely mixed reactors were used in this study. Two reactors, designated R1 and R2, were used as the Control treatment (Control) and fed manure from cows not treated with Ceftiofur. Two different reactors, designated R4, and R5, were used as the Ceftiofur treatment (Ceftiofur) and fed manure from cows treated with Ceftiofur. The working and head space volumes, organic loading rates (OLR), and feeding rates of the reactors
are summarized in Table 1. All reactors were operated at a thermophilic temperature of 55 ± 2°C with 10 d HRT. The temperature of the digester content was maintained by heating blankets (Model SSHB121236012, SRFG 12/12/5, Omega Engineering, Stamford, CT) wrapped around the reactors. Temperature was controlled by Digital Temperature Controller Model WH7016F, Model CN79022 and CN1504-TC (Omega Engineering, Stamford, CT) in conjunction with solid state relays (Model SSRL240DC10, SSR330DC25, Omega Engineering, Stamford, CT). Heating was activated by thermocouples submerged in the reactors (Traceable Digital Temperature Controller, Fisher Scientific, Waltham, MA; Model TJ36-CASS-18U-12, Omega Engineering, Stamford, CT). The contents of the reactors were mixed by top mounted electric motor (Dayton Motor Model 4Z064A, Grainger, Lake Forest, IL) driven propellers. The mixers were ran for 1 min every 5 min at motor speeds of 47 rpm at 60 Hz. The mixing was managed by a program encoded in a Chrontrol (XT Table Top-AC Outlet, Chrontrol Corp, San Diego, CA). Each digester was fed once every 24 hours. The feeding and discharging was performed using a peristaltic pump (Masterflex Model 77200-62, Cole Parmer Instrument Co., Chicago, IL). Before feeding the reactors, the digester content in the volume equivalent to the feed volume was removed as effluent. Gas volume was measured via a wet-tip gas meter (Rebel wet-tip gas meter company, Nashville, TN).
Table 1. Operating characteristics of reactors

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Working Volume (L)</th>
<th>Headspace (L)</th>
<th>OLR (g L(^{-1}))</th>
<th>Feed Volume (L)</th>
<th>Effluent Volume (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>5.0</td>
<td>1.0</td>
<td>2.33</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>R2</td>
<td>14.0</td>
<td>2.0</td>
<td>2.33</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>R4</td>
<td>12.0</td>
<td>2.0</td>
<td>2.52</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>R5</td>
<td>12.0</td>
<td>2.0</td>
<td>2.52</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>
2.3 Reactor Start Up

The reactors were started using equal volumes (1:1) of seed (effluent from a sludge fed anaerobic digester) and dairy cow manure. The seed was obtained from the effluent of an active anaerobic digester at Christiansburg Wastewater Treatment Plant (Christiansburg, VA). Each reactor was filled to the working volume level with the startup mixture. The reactors were then operated for 1 HRT (10 days) before regular daily feeding began. After 1 HRT, the appropriate effluent volume was pumped out of each reactor and feed added every 24 h. The reactors were operated for 102 d as two thermophilic-mesophilic two stage systems and a single mesophilic reactor. Thereafter, the reactors were reconfigured to operate as single thermophilic reactors for approximately 2 HRTs before manure from cows treated with Ceftiofur was fed into R4 and R5. During the start-up phase the reactors were fed with manure from cows not treated with Ceftiofur.

2.4 Sampling

Biogas volume was recorded every day at the time the reactors were fed. Reactor feed and effluent were collected every 3 d for analysis. During each sampling event, first, the reactor contents were thoroughly mixed, and then a volume equivalent to daily feed for the reactor was pumped out. The effluent was allowed to run for a few minutes into a waste container to flush the tubing and afterwards samples were taken directly from the hose into the sample bottles. Influent samples were taken directly from the storage buckets at the time of use when feeding the reactors. Samples used for the determination of manure characteristics (solids, COD, pH, and total nitrogen and phosphorus) were collected into 250 mL screw top bottles. Samples for Ceftiofur and ARG analysis were collected into 100 mL sterile centrifuge tubes. Samples for manure characteristics and antibiotic/ARG analysis were stored at 4°C and -20°C, respectively, until analysis.

2.5 Analytical Methods

Total solids (TS), volatile solids (VS) were analyzed according to Standard Methods (APHA, 1995) and total and soluble COD were analyzed according to Hach Method 8000 (Hach, Loveland, CO). Briefly, for solids analysis, aliquots of 20 mL were drawn from well-mixed
sample and placed into a crucible and dried for 24 h in an oven set at 105°C. The weights of crucible with and without the samples was determined and TS calculated using Equation 22. The dried solids were then placed in a furnace at 550°C for 1 hour, and weighed for the determination of volatile solids. VS was calculated using Equation 23. To determine the TCOD, 20 mL of sample was mixed with 380 mL DI water for a 1:20 dilution, to bring the concentration within the range of measurement method used. The dilution was blended for 30 s and 20 mL were diluted with 180 mL of DI water to create a final 1:100 dilution. Two mL of the resulting mixture was placed in COD vials (Cat. 2125915, Hach, Loveland, CO), digested for 2 hours at 105°C (COD Reactor Model 45600, Hach, Loveland, CO), and analyzed on a spectrophotometer (DR2800, Hach, Loveland, CO). Soluble chemical oxygen demand was analyzed by mixing 20 mL of sample with 380 mL of DI water and mixing well. Forty-five mL of the well mixed samples were centrifuged (5804R, Eppendorf, Hauppauge, NY) at 14,000 rpm for 20 minutes. The centrate was then vacuum filtered through a 0.45 micron filter (Filter RW03, Millipore, Billerica, MA). Two mL of the filtrate was placed in COD vials (Cat. 2125915, Hach, Loveland, CO), digested for 2 hours at 105°C (COD Reactor Model 45600, Hach, Loveland, CO), and analyzed on a spectrophotometer (DR2800, Hach, Loveland, CO). pH was measured using a pH meter (WTW Multi 3420, Cole-Parmer, Vernon Hills, IL). Total phosphorus and total nitrogen were analyzed by the molybdovanadate with acid persulfate digestion method (Method 10127, HACH, Loveland, CO), and the persulfate digestion method (Method 10072, HACH, Loveland, CO), respectively. Total phosphorus and total nitrogen samples were analyzed on an HRT basis.

Five mL of samples from R1 and R2 from days 0, 3, 6, 9 were mixed to form a single sample for Control effluent for HRT 1. Samples from R4 and R5 from days 0, 3, 6, 9 were mixed for form a single sample for Ceftiofur effluent for HRT 1. Control feed, Ceftiofur feed, and R3 effluent were blended individually. Days 0, 3, 6, 9 formed the sample for HRT 1, days 21, 24, 27, and 30 formed the sample for HRT 3, and days 45 and 50 combined for HRT 5. Table 2 shows the reactor samples and days that were combined to form the TP and TN samples. After the samples were made, 2 mL of well mixed sample was diluted to a 1:20 dilution with 38 mL of DI water. The resulting mixture was well mixed and placed into the TP and TN vials (Cat. 2767245, Hach, Loveland, CO); digested for 2 hours (COD Reactor Model 45600, Hach, Loveland, CO), and analyzed on a spectrophotometer (DR2800, Hach, Loveland, CO).
Total Solids = \( \frac{\text{Dry Weight of Sample (g)}}{\text{Wet Weight of Sample (g)}} \) \hspace{1cm} (22)

Volatile Solids = \( \frac{\text{Dry Weight of Sample (g)} - \text{Ash Weight of Sample (g)}}{\text{Wet Weight of Sample (g)}} \) \hspace{1cm} (23)
Table 2: Composite Sampling Protocol for TN and TP Analysis

<table>
<thead>
<tr>
<th>HRT</th>
<th>Day</th>
<th>Samples</th>
<th>Composite Sample ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0, 3, 6, 9</td>
<td>Control Feed</td>
<td>Control Feed, HRT 1</td>
</tr>
<tr>
<td></td>
<td>0, 3, 6, 9</td>
<td>Ceftiofur Feed</td>
<td>Ceftiofur Feed, HRT 1</td>
</tr>
<tr>
<td></td>
<td>0, 3, 6, 9</td>
<td>R1, R2 effluent</td>
<td>Control Effluent, HRT 1</td>
</tr>
<tr>
<td></td>
<td>0, 3, 6, 9</td>
<td>R4, R5 effluent</td>
<td>Ceftiofur Effluent, HRT 1</td>
</tr>
<tr>
<td>3</td>
<td>21, 24, 27, 30</td>
<td>Control Feed</td>
<td>Control Feed, HRT 3</td>
</tr>
<tr>
<td></td>
<td>21, 24, 27, 30</td>
<td>Ceftiofur Feed</td>
<td>Ceftiofur Feed, HRT 3</td>
</tr>
<tr>
<td></td>
<td>21, 24, 27, 30</td>
<td>R1, R2 effluent</td>
<td>Control Effluent, HRT 3</td>
</tr>
<tr>
<td></td>
<td>21, 24, 27, 30</td>
<td>R4, R5 effluent</td>
<td>Ceftiofur Effluent, HRT 3</td>
</tr>
<tr>
<td>5</td>
<td>45, 50</td>
<td>Control Feed</td>
<td>Control Feed, HRT 5</td>
</tr>
<tr>
<td></td>
<td>45, 50</td>
<td>Ceftiofur Feed</td>
<td>Ceftiofur Feed, HRT 5</td>
</tr>
<tr>
<td></td>
<td>45, 50</td>
<td>R1, R2 effluent</td>
<td>Control Effluent, HRT 5</td>
</tr>
<tr>
<td></td>
<td>45, 50</td>
<td>R4, R5 effluent</td>
<td>Ceftiofur Effluent, HRT 5</td>
</tr>
</tbody>
</table>
Biogas samples were collected at least once a month in 1 L Tedlar bags and analyzed for quality, i.e. methane and carbon dioxide composition using a gas chromatograph (Model GC-14A, Shimadzu Scientific Instruments, Columbia, MD) equipped with a 1.8 m packed column with Haysept D packing and a thermal conductivity detector. Helium was used as the carrier gas. The quantity of biogas was normalized to the mass of VS fed to the digester.

### 2.6 Ceftiofur Concentration

Ceftiofur concentrations were determined via liquid chromatography mass spectrometry (LC MS). Briefly, frozen digester effluent samples were thawed at room temperature. After thawing, the sample was mixed thoroughly and 0.05 grams drawn and placed into a 30 mL centrifuge tube. Each sample tube received 10 mL of 0.2 M borate buffer and 0.5 mL of 25% ammonia solution. The sample tubes were then capped, vortexed, and placed into horizontal shaker (Model E6000, Eberbach Corp., Ann Arbor, MI) for 15 min at 14,777 rpm. The tubes were then placed in 60°C water bath and incubated for 20 hours. After 20 hours, the prepared samples were cleaned from the feces using Solid Phase Extraction. To perform the Solid Phase Extraction, OASIS HLB cartridges (CAT#WAT094226, Waters Corp, Milford, MA) were connected to a vacuum chamber and conditioned with 5 mL of methanol and then 5 mL of DI water. Prepared samples were added and flowed through cartridges at rate of 2-3 drops per second to elute a sample cleaned of the feces and buffer. Cartridges were then washed with 3 mL of water. Eluent was collected into 6 mL glass tubes with 5 mL of methanol:acetonitrile (50:50, v/v) mixture. The mixture was vortexed and 1 mL drawn and dried at 35°C in a N₂ evaporator. Standards were prepared in the same manner concurrently with the samples. 1 mL of the standards were mixed with 1 mL of the extracted Control matrix solution to create 2 mL volume dried. For samples, 1 mL of dried residue was re-dissolved using 1 mL of the mobile phase (70% water, 30% methanol, 0.1% formic acid). The samples were then filtered through PVDF filter of 0.2 μm (Cat# 28145-491, VRM, Appledoorn, The Netherlands) into amber glass vial and quantified on LCMS-MS.
Estimates of the total mass of Ceftiofur in the reactors were calculated before (MCB) and after (MCA) feeding (Equations 24 and 25). It was assumed that comparing MCA and MCB provides a relative measure for the treatment effect.

\[
\text{MCB (ng) = } c_{\text{out}} \times \rho_{\text{out}} \times V_{\text{out}}
\]

(24)

\[
MCA \text{ (ng) = (} c_{\text{in}} \times \rho_{\text{in}} \times V_{\text{in}}) + (c_{\text{out}} \times \rho_{\text{out}} \times (V_{\text{out}} - V_{\text{in}}))
\]

(25)

Calculations for MCB are represented in Equation 24 and based on the concentration of antibiotic in the effluent \( (c_{\text{out}}, \text{ng g}^{-1}) \), volume of effluent \( (V_{\text{out}}, \text{L}) \), and the density of the reactor effluent \( (\rho_{\text{out}}, \text{g L}^{-1}) \). Calculations for MCA, represented in equation 25, exhibit initial mass of antibiotic before digestion. MCA is based on the concentration in the influent \( (c_{\text{in}}, \text{ng g}^{-1}) \), volume of influent \( (V_{\text{in}}, \text{L}) \), density of the influent \( (\rho_{\text{in}}, \text{g L}^{-1}) \), and the remaining mass of Ceftiofur after sampling.

### 2.7 Quantification of Antibiotic Resistant Genes

The DNA from the samples was extracted using the QiaAmp Fast DNA Stool Mini Kit, according to manufacturer’s instructions. Extracted DNA was stored at -20°C until qPCR analysis was conducted for 16S rRNA, \( tet(Q) \), \( cfx(A) \), and \( mef(A) \) genes. Briefly, the qPCR procedure entailed, first, using test plates to determine appropriate dilution i.e., 1:10, 1:20, 1:50, 1:70, 1:80, and 1:100. Then, the dilution factor from test plates with the highest copies of DNA was selected and used to process all DNA samples. qPCR plate wells were filled with 1 µL of DNA, 2.4 µL molecular biology-grade water, 0.8 µL each of forward and reverse primer, and 5 µL SsoFast Evagreen Supermix (Bio-Rad, Hercules, CA). Six standards were prepared through serial dilutions to create points ranging from \( 10^2 \) copies of DNA per µL and \( 10^8 \) copies of DNA per µL. Standards, reagent blanks, and samples were run in triplicate. Results were accepted when the data and standards fit along a curve with high correlation \( (R^2>0.90) \). Ratio of \( cfx(A) \), \( mef(A) \), and \( tet(Q) \) genes were normalized to 16S gene concentration to account for differences in bacterial biomass between treatments (Eq 26).
Gene/16S ratio: $\frac{\text{Concentration of gene of interest (copies of DNA per } \mu\text{L)}}{\text{Concentration of 16S genes (copies of DNA per } \mu\text{L)}}$ (26)

2.8 Statistical Analysis

Statistical analyses were performed using JMP Pro 13® desktop software (SAS, Cary, NC). First, an analysis of variance (ANOVA) was conducted to determine a treatment effect. Then, differences in biogas production and ARG concentrations between treatments were determined via paired t-tests. A Wilcoxon Signed-Rank Test was performed in addition to the paired t-test to account for the non-normal distribution of the cumulative gas data. Further, a measure of difference, a part of the paired t-test in JMP was calculated by taking the absolute value of the difference between two values. Significant difference was determined at alpha values of 0.05. Best fit lines were calculated via linear regression in excel. A fit was considered acceptable with high correlation.
3 RESULTS AND DISCUSSION

3.1 Manure Characteristics and Reactor Performance

The manure characteristics of interest expressed as mean (±standard deviation) are presented in Table 3. The pH and TS in both the influent and effluent were similar for both treatments (p>0.05). The organic loading rates for the Control and Ceftiofur treatments were similar (p=0.39) at 2.33 g VS L⁻¹ and 2.52 g VS L⁻¹, respectively.

There was no statistically significant difference in waste stabilization (destruction of VS and TCOD) and nutrient (TN and TP) content, between treatments, averaged over the course of the experiment. On average, VS reductions were 42 and 44% for the Control and Ceftiofur treatments, respectively. The VS reductions obtained in this study are comparable, consistent, and within the range (41 to 59%) of what has been reported for thermophilic AD (Kobayashi et al., 2008). The observed TCOD reductions had similar trend and magnitude as VS, with the Control (43%) being slightly higher than Ceftiofur treatment (38%). These values are also comparable to previously reported values of an average COD removal between 39.7% and 50.3% for thermophilic AD (Kobayashi et al., 2008). Some of the loss of VS and TCOD is due to the consumption of the carbon to convert to methane gas and CO₂, and is a key factor of the waste stabilization aspect of AD process. By removing the carbon, the manure loses some of its strength and is thereby stabilized, as compared to raw manure. TP concentration in the effluent were lower than the influent by 49% and 30% for Control and Ceftiofur treatments respectively. We do not expect the TP and TN to change after AD. The differences in TP concentrations may have been caused by precipitation and settling of P in the reactor as described in Gungor and Karthikeyan (2008). They reported increased concentrations of insoluble P following anaerobic digestion. Some of the differences may also be attributed to imperfect mixing resulting in settled precipitated P at the bottom of the reactor below the sampling port. The lower values of TN concentration in the effluent compared to the feed i.e. 34% and 25% for the Control and the Ceftiofur treatment, respectively, was not a surprise either. Some of the N may have been lost as ammonia (2 to 6%) in the biogas as has been reported by Xue & Chen (1999). It is also possible that some N was in the settled material at the bottom of reactors explained above. The TN:TP
ratio is an important measure for the value of the manure as a fertilizer. The TN:TP ratio for the Control and Ceftiofur treatments after AD are 5:1 and 13:2 respectively. While the ratios of TN:TP vary depending on the crop, this ratio is nitrogen high as compared with recommended TN:TP ratios of 3:1 for rice, of 29:9 for tobacco, and of 24:25 for oats (Buchholz, 1983). Additional processing steps to optimize the TN:TP ratio would be effective to improve the value of the manure as a fertilizer.

While the average values of TCOD were not statistically different over the course of the experiment, there were different when broken down per HRT. During the first 10 days of the experiment, the average percent reduction were 45% and 53% for Control and Ceftiofur treatments respectively. During HRT 2, Control removed more organic matter (43%) compared to Ceftiofur (35%). By HRT 5, there was a large difference in TCOD reduction, as the control removed 51% of the organic matter while the Ceftiofur treatment removed 26%. VS reduction was also higher in the Control treatment for VS (51%), as compared to the 43% VS reduction in the Ceftiofur treatment. This difference in waste stabilization is seen especially when comparing sCOD reduction values. During the first HRT, sCOD reduction values for Control and Ceftiofur treatments were comparable (57% and 52% respectively). But from HRT 2 onward, Ceftiofur was much lower, with HRT 4 and 5 showing the greatest differences (HRT 4: Control: 61%, Ceftiofur: 19%; HRT 5: Control: 66%, Ceftiofur: 20%). This demonstrates that over time, Ceftiofur removes less carbon and is produces less stabilized waste, which is further shown through the biogas analysis.
Table 3: Mean (±standard deviation) characteristics of the manure fed and effluent from the reactors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Control Influent</th>
<th>Control Effluent</th>
<th>Ceftiofur Influent</th>
<th>Ceftiofur Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>--</td>
<td>6.71 (±0.47)</td>
<td>7.82 (±0.22)</td>
<td>6.95 (±0.51)</td>
<td>7.87 (±0.21)</td>
</tr>
<tr>
<td>TS</td>
<td>g L⁻¹</td>
<td>28.76 (±9.65)</td>
<td>17.38 (±4.46)</td>
<td>30.63 (±10.46)</td>
<td>18.45 (±6.19)</td>
</tr>
<tr>
<td>VS</td>
<td>g L⁻¹</td>
<td>23.31 (±8.18)</td>
<td>12.76 (±3.52)</td>
<td>25.23 (±9.57)</td>
<td>13.73 (±4.95)</td>
</tr>
<tr>
<td>TCOD</td>
<td>g L⁻¹</td>
<td>39.09 (±12.24)</td>
<td>21.09 (±5.41)</td>
<td>42.25 (±11.67)</td>
<td>25.87 (±8.41)</td>
</tr>
<tr>
<td>sCOD</td>
<td>g L⁻¹</td>
<td>12.17 (±3.47)</td>
<td>4.88 (±1.04)</td>
<td>14.81 (±2.94)</td>
<td>10.84 (±2.99)</td>
</tr>
<tr>
<td>TN</td>
<td>g L⁻¹</td>
<td>1.72 (±0.40)</td>
<td>1.14 (±0.53)</td>
<td>2.12 (±0.66)</td>
<td>1.60 (±0.40)</td>
</tr>
<tr>
<td>TP</td>
<td>g L⁻¹</td>
<td>0.37 (±0.11)</td>
<td>0.19 (±0.11)</td>
<td>0.33 (±0.15)</td>
<td>0.23 (±0.10)</td>
</tr>
</tbody>
</table>
3.2 Biogas and Methane Production

The cumulative biogas produced from both treatments expressed in terms of mass of VS fed to the reactors is presented in Figure 6. There was no statistically significant difference in cumulative biogas produced during the first HRT. Similar observations have also been made in previous batch studies using manure containing Ceftiofur (Sara et al., 2013) and cefazolin (Beneragama et al., 2013). Both report no statistically significant difference in gas production between the control and cephalosporin-treated reactors. In the first HRT, the Ceftiofur-treated reactors produced slightly higher amounts of biogas, despite expecting the Control to produce more. This type of result was previously reported in Miller et al. (2013). They reported a 30% increase in biogas production during the first few days of using manure with sulfamethoxazole in a continuous fed AD. After the first few days, the biogas production from the antibiotic-treated reactor dropped below the control, as observed in this study. After the first HRT, the average biogas yield from reactors Ceftiofur treatment were consistently lower than Control (Figure 6). Linear fit was performed on the data to compare with other models reported in the literature. The linear fit model demonstrated a constant rate of gas production. The difference in linear trends of biogas yields exhibits increasing divergence for Control and Ceftiofur treated reactors. Based on the linear trend-lines (Control, \( y = 9.21x \), \( R^2 = 0.99 \); Ceftiofur, \( y = 7.97x \), \( R^2 = 0.97 \)), there is an increasing difference in gas yield between the control and Ceftiofur treatments.

The biogas quality was similar (p=0.13) for both treatments. The biogas contained 59% and 52% methane for Control and Ceftiofur treatments, respectively. These values are similar to literature values, which report average methane values of 61-64% for thermophilic AD of manure without antibiotics (Kaparaju et al., 2008; Kobayashi et al., 2008). Based on these results, we conclude there is similar quality of biogas being produced but a difference in amount, as described below.

To ascertain the effect of Ceftiofur during AD, the biogas yield during each HRT i.e. every 10 d was plotted (Figure 7). The difference in cumulative gas yield gas yield increased with time of digestion with the Control consistently larger than the Ceftiofur treatment after HRT 1. The gas production rates of the Control correspond with the increased percent reduction in VS. In the third HRT, there was only a 7% difference in biogas production values, but by the fourth HRT,
the difference grew to 13%. By HRT 5, the difference in biogas production between the treatments was 15%, and the percent VS reduction was 46% and 41% for Control and Ceftiofur treatments respectively. This displays the increasing difference in gas production as time continues. The delay in difference of biogas production until 1 HRT suggests that at least one 1 HRT of antibiotic input is necessary to begin to affect the microbial community within the reactors. This effect can be explained due to the mechanism of action of Ceftiofur.

The divergence of cumulative biogas produced between the Control and Ceftiofur treatment suggests that Ceftiofur could be affecting biogas production over time, perhaps related to the mode of action of the antibiotic Ceftiofur. Ceftiofur is a broad spectrum bactericidal which affects both gram negative and gram positive bacteria. It is known to be more active against gram-positive bacteria, which would namely effect hydrolysis and acidogenesis. Based on the noted divergence and a time series analysis that predicted continual divergence of the biogas yields, it is possible the bacterial community in the Ceftiofur treatment could be very different from the Control due to the death of many gram negative and gram positive bacteria responsible for hydrolysis, acidogenesis, or acetogenesis. Methanogens are archaea and not expected to be affected by either antibiotics, leading to the hypothesis that the difference in biogas production could come mainly from an un-balancing of the acidogenesis and methanogenesis reactions. If the VFAs creation is limited because of Ceftiofur attacking the bacteria responsible for acidogenesis, then the methanogens are unable to grow to the necessary density because of the lack of substrate. This inhibits biogas production, limiting the ability for carbon to be removed from the reactor.

The action against these groups of bacteria responsible for acidogenesis and methanogenesis could be proven by metagenomic analysis of the microbial community, where we would expect to see bacteria involved in hydrolysis and acidogenesis in smaller proportions in the antibiotic reactors to those found in the Control. The bactericidal effects would continue to kill off the susceptible bacteria as long as the antibiotic is being fed to the system. The input of both new bacteria and Ceftiofur into the reactor could allow the system to produce biogas for some time, but we hypothesize after extended periods of exposure to Ceftiofur, the reactions would become un-balanced between substrate generation and consumption, and biogas production would be
extremely limited due to the lack of substrate being produced during acidogenesis or acetogenesis.
**Figure 6:** Cumulative biogas yield for Control and Ceftiofur reactors with linear fit lines

**Figure 7:** Cumulative biogas yield averaged over HRT (1 HRT=10 d)
Table 4: Comparison of average cumulative gas (mL g VS fed⁻¹) per HRT

| Period (HRT) | N  | Treatment       | Measure of difference | Prob > |t| | Prob>|S| |
|--------------|----|-----------------|-----------------------|--------|---|---------------|
|              |    | Control         | Ceftiofur             |        |   |               |
|              |    | Mean (+/- SD)   | Mean                  | |Δ|    |                |
| 1            | 10 | 45 (±28)        | 62 (±34)              | 16.4   | 0.0002 | 0.002 |
| 2            | 10 | 160 (±36)       | 130 (±11)             | 30.15  | 0.0047 | 0.0117 |
| 3            | 10 | 246 (±23)       | 228 (±25)             | 18.45  | <.0001 | 0.002 |
| 4            | 10 | 332 (± 25)      | 287 (±14)             | 44.65  | <.0001 | 0.002 |
| 5            | 10 | 404 (± 27)      | 342 (± 25)            | 61.35  | <.0001 | 0.002 |

N is the number of samples per calculation, the measure of difference is the difference between the average cumulative gas of Control and the average cumulative gas of Ceftiofur for the stated HRT. Prob > |t| is the statistical p-value for a paired t-test to compare the values. A value <0.05 demonstrates significant differences between the treatments. Prob > |S| is the statistical p-value for a Wilcoxon sign test to take into account for the non-normal distribution of the data. A value of <0.05 shows significant differences between treatments.
3.3 Mass of Ceftiofur in Reactors

The concentration of Ceftiofur in the manure fed to the reactors and the effluent from the reactors is presented in Figure 8. The concentration of Ceftiofur in the effluent after treatment was statistically different (p<0.0001) than the concentration of Ceftiofur in the influent. The average concentration in the feed was 10 ng Ceftiofur g⁻¹ influent⁻¹, while the average concentration in the effluent sample was 2.3 ng Ceftiofur g⁻¹ effluent⁻¹. This demonstrates that anaerobic digestion was responsible for reducing the concentration of Ceftiofur.

In order to include the 10 day retention time of the reactors in the Ceftiofur analysis, the mass of Ceftiofur in the manure fed and reactor effluents were plotted (Figure 9). In general, the mass of Ceftiofur in the digester effluent after treatment (MCB) increased during the first few days of feeding, reaching a maximum on day 12 before declining over time (Figure 9). Up to day 12, for the first HRT, the manure from Ceftiofur treated cows was replacing the non-treated manure used to prepare the reactors. At day 10, the manure in reactors Ceftiofur was entirely from Ceftiofur treated cows and the peak of mass of Ceftiofur in the reactors is seen in Figure 9. After day 15, the mass of Ceftiofur steadily decreased to the end of the test, day 65. Feeding Ceftiofur based manure was stopped on day 50 and samples from days 55, 60, and 65 were analyzed for the residual Ceftiofur. The percent reduction of Ceftiofur between “before feeding” and “after feeding” showed statistically significant differences in HRT 2, 3, and 4. The second HRT period (days 11 to 20) had an average mass of Ceftiofur before and after feeding of 40 and 48 μg, respectively, with an 18% loss of Ceftiofur mass. The third and fourth HRTs (days 21-30 and 31 to 40) had 24% and 23% reduction in antibiotic respectively, and by the fifth HRT (days 41 to 50), the percent reduction was negligible. The significant reduction over the entire experiment (p<0.05) signifies the reduction in the mass of Ceftiofur due to the AD process. This is also reiterated by the continual reduction in mass of Ceftiofur as digestion time increases. Reduction in Ceftiofur concentration during AD has been reported by Sara et al. (2013) during mesophilic batch AD of pig manure. They reported 70% degradation of Ceftiofur during their trials. The lower percent reduction from this study is likely due to the continuous nature of the reactor, where the continual input of Ceftiofur damages the microbial community, which may be
responsible for Ceftiofur degradation (Rafii et al., 2009). The potential disruption to the microbial community is also supported by the decreasing percent reduction as time continues.
Figure 8: Concentration of Ceftiofur in the influent and effluent during the course of the experiment.

Figure 9: Mass of Ceftiofur in the reactors over time, before and after feeding.
3.4 Antibiotic Resistant Gene Concentration in the Reactors

3.4.1 Effect of Anaerobic Digestion on ARG Concentration

The average concentrations of each ARG in the feed and effluent of the reactors over the course of the experiment are presented in Figure 10. In the Ceftiofur treatment, no statistically significant difference (p=0.14) was observed between feed and effluent concentration of 16S (p=0.19), cfx(A) (p=0.96), and mef(A). However, there was a statistically significant difference (p<0.0001) in tet(Q) concentrations, with the reduction of 78% of copies of tet(Q) per µL of sample. The Control treatment had statistically significant reductions of 16S (p=0.03), cfx(A) (p=0.02), and tet(Q) (p=0.0002), but not statistically significant reduction in mef(A) concentration.

Change in 16S Concentration

The average concentration of 16S genes decreased slightly but was not statistically significant during the Ceftiofur treatment. The average concentration of the influent was $1.95 \times 10^8$ copies of DNA per µL, and the effluent contained an average of $1.16 \times 10^8$ copies of DNA per µL. In contrast, the Control treatment had an average 76% reduction in 16S gene concentration between the influent and effluent (influent=$2.27 \times 10^8$ copies DNA per µL, effluent=$5.38 \times 10^7$ copies DNA per µL). 16S genes are a measure of bacterial count within the reactors, and can be used to provide rough comparisons of differences in bacterial biomass growth (Rosselli et al., 2016). It was to be expected for both treatments to have significant reduction in bacterial count when comparing feed and effluent due to the reduction in bacterial diversity from thermophilic anaerobic digestion (Kobayashi et al., 2008). However, in our study only the Control showed significant reductions in 16S genes. We speculate this may be due to the reduction in bacterial community to give way to a biogas-producing archael community, while the Ceftiofur reactors did not develop a high biomass archael community (Turker et al., 2016). Another possibility is the reduction in bacterial species richness due to the thermophilic environment, as found in previous metagenomics studies (Kobayashi et al., 2008; Sun et al., 2015). This would account for the difference in biogas production as discussed earlier. To account for the differences in 16S
concentration, the concentration of the other ARGs are normalized to 16S to give a ratio, as previously described in Miller et al. (2013) and Ghosh et al. (2009).

**Change in cfx(A) Concentration**

The was no statistically significant difference (p=0.7419) in the concentration of cfx(A) between the influent and effluent for the Ceftiofur treatment. The average influent and effluent concentrations were $2.80 \times 10^4$ and $3.25 \times 10^4$ copies DNA per $\mu$L respectively. When normalized to a 16S ratio, there was no statistically significant difference (p=0.1813) in the ratio of $cfx(A)/16S$ between the influent and effluent of the Ceftiofur treatment. It did however represent a 77% decrease between the influent and effluent ratios.

The concentration of $cfx(A)$ was statistically significantly lowered (p=0.0424) by the Control treatment, reducing the influent concentration 71% from $4.62 \times 10^4$ copies of DNA per $\mu$L to $1.34 \times 10^4$ copies of DNA per $\mu$L. Once normalized, the concentration of $cfx(A)/16S$ was not statistically significantly lower (p=0.2160). These results lead us to hypothesize that the $cfx(A)$ gene is transferred via bacteria. When the bacterial count is not taken into account, the Control has statistically significant reductions in $cfx(A)$ concentrations, but is no longer significant once normalized to the bacterial count of the reactors. Sun et al. (2016) report that the bacterial community is the key mechanism of ARG transfer within reactors, and the reduction of the bacterial community in the Control reactors is likely the cause of the reduction of the $cfx(A)$ gene concentrations. This hypothesis needs to be confirmed by further studies, but no previous work investigating the $cfx(A)$ gene could be found.

**Change in mef(A) Concentration**

The concentration of mef(A) genes statistically significantly increased in both treatments (Control, p=0.0350; Ceftiofur, p=0.0210). mef(A) increased 174% in the Control treatment, from $6.06 \times 10^4$ to $1.66 \times 10^5$ copies of DNA per $\mu$L, and almost 500% in the Ceftiofur treatment, from $1.07 \times 10^5$ to $6.37 \times 10^5$ copies of DNA per $\mu$L. Few previous studies have investigated macrolide resistance genes. Zhang et al. (2016) found concentrations of mef(A) to increase significantly
during batch mesophilic digestion, similar to this study. The effluent samples contain higher ratios of \textit{mef}(A) to 16S genes in both treatments (Fig 11), but were not statistically significant (Control, \( p=0.6458 \); Ceftiofur, \( p=0.1546 \)). Similarly, to \textit{cfx}(A), this likely signifies the transfer of ARGs through a specific bacterial species, which is removed during the thermophilic digestion process. There was a statistically significant difference in the ratios of \textit{mef}(A) and 16S when comparing the Control and Ceftiofur feeds (\( p=0.0450 \)), signifying the higher average concentration of normalized \textit{mef}(A) genes in the Ceftiofur treatment. This demonstrates that more of the bacteria coming from the Ceftiofur treated cows have the \textit{mef}(A) gene as compared to the Control cows.

\textit{Change in tet}(Q) \textit{Concentration}

The concentration of \textit{tet}(Q) genes was statistically significantly lowered by anaerobic digestion for both treatments (Control, \( p=0.0096 \); Ceftiofur, \( p=0.0009 \)). Concentration of \textit{tet}(Q) was reduced 78\% and 74\% for the Control treatment and Ceftiofur treatments respectively. Once normalized, \textit{tet}(Q) was still statistically significantly reduced (Control, \( p=0.0075 \); Ceftiofur, \( p=0.0008 \)), with the Control reducing 85\% and Ceftiofur reducing 91\% of the concentration of \textit{tetQ} as normalized to 16S gene concentrations (Fig 11). This is similar to previously reported values, both normalized and un-normalized.

Diehl & LaPara (2010) reported reduction in all \textit{tet} genes during thermophilic anaerobic digestion for a semi continuous operation of wastewater solids. They found reduction in concentration of \textit{tet}(A), \textit{tet}(L), \textit{tet}(O), \textit{tet}(W), \textit{tet}(X), and \textit{intI} genes at 55\°C. Miller et al. (2013) found a reduction in \textit{sulII}, \textit{sulII}, \textit{tet}(W), \textit{tet}(O), and \textit{intII} gene concentrations between the feed and both thermophilic Control reactors and reactors treated with sulfamethoxazole. Ghosh et al. (2009) found that concentrations of \textit{tet}(A), \textit{tet}(O), \textit{tet}(X), and \textit{intI} were significantly reduced between feed and thermophilic digestion for wastewater treatment samples, with \textit{tet}(X) decreasing 85-99\%. Sun et al. (2016) report that the thermophilic temperatures of anaerobic digestion of dairy manure is responsible for the elimination of transfer of \textit{tet} genes by reducing the number of bacteria which can transfer the genes. These reported values and the results of this
study demonstrate that thermophilic anaerobic digestion is a useful technology for reducing the amount of *tet* ARGs found in wastewater and manure.

The difference in ARG reduction is gene specific in this study. Many studies have investigated the effect of AD on *tet* genes (Sun et al., 2016; Diehl & LaPara, 2010; Miller et al., 2013), but fewer studies have investigated the overarching effect of ARG reduction and transfer across multiple genes of interest. Jain et al. (2003) discuss that temperatures differences can hinder horizontal gene transfer, which would account for differences in gene concentration when treated by thermophilic digestion. They report that when genes are transferred from a mesophilic to a thermophilic environment, the mesophilic proteins may become inactive, while proteins from thermophiles may not have enough energy for catalysis when in mesophilic environments (Jain et al., 2003). The higher temperatures at thermophilic environments may also make naked DNA more labile, thereby “hampering the circulation of mesophilic DNA lacking thermal protective mechanisms within high temperature environments.” (Jain et al., 2003). Genetic engineering of thermophiles has reported difficulty in modification of bacteria. Thermophiles do not contain necessary DNA uptake systems to allow for foreign DNA transformation (Taylor et al., 2011). Because of this, ARG transfer to thermophilic bacteria is likely less than ARG transfer to mesophilic bacteria. This may account for the commonly reported reduction in ARG concentrations in thermophilic digester as compared to mesophilic digestion. Given the variable responses of ARGs to AD presented in this study, further work should investigate the effect of AD on ARG concentrations across multiple types of resistance and ARGs.
Figure 10: ARG concentration in feed and effluents of Control and Ceftiofur treatments over 50 day experiment period
Figure 11: Ratio of ARG concentration to 16S concentration in feed and effluent of Control and Ceftiofur treatments over 50 day experiment period.
3.4.2 Effect of Ceftiofur on ARG Concentrations

The concentration of each ARG in the effluent over time can be seen in Figures 12, 13, 14, and 15 for 16S, cfx(A), mef(A), and tet(Q), respectively. As seen in Figure 13, the concentration of cfx(A) genes increased on day 15 and 18 for the Ceftiofur treatment. Afterwards, both treatments experienced a 2 log reduction in cfx(A) concentration. This drop may be caused by the death of the ARG harboring bacteria after 2 HRTs of digestion, or due to the storage of the manure at 4°C before use in the digesters. The cold storage was used to minimize organics degradation but the effect of cold temperatures on ARGs has not been studied (Miller et al., 2016). Since the concentration from both treatments dropped at that time, it appears for the drop to not be related to the treatment of cows with Ceftiofur. Figure 14 shows the change in concentration of mef(A) over time. In contrast to cfx(A), mef(A) concentrations increase steadily for both treatments until day 36, where they decrease again until the end of the experiment. The concentration of mef(A) increased by 1 log for the Ceftiofur treatment between day 27 and day 30. This may be due to the transfer of the gene among the microbes within the digester (Miller et al., 2016; Sun et al., 2016). When looking at Figure 16 with the concentration of mef(A) normalized to the concentration of 16S genes, the ratio increases throughout the experiment for the Ceftiofur treatment. This demonstrates that the number of mef(A) genes within the reactor is increasing relative to the number of bacterial species. Therefore, resistance to mef(A) likely increases in response to anaerobic digestion when in the presence of Ceftiofur, which has been shown to affect the microbial community of the cow fecal metagenome and likely expands to affect the community of the digester (Chambers, et al., 2015). Tet(Q) gene concentration (Figure 15) peaked within the 3rd HRT for both treatments but immediately decreased afterwards. This may be due to the change in microbial community during this time (Lu et al., 2014). The two treatments in this study show similar trends in ARG concentration change over time, but with mef(A) displaying significantly higher concentrations in the presence of Ceftiofur. No previous studies have investigated the impact of Ceftiofur on ARG concentration during anaerobic digestion, but a previous study which anaerobically digested sulfamethoxazole and silver nanoparticles also report gene specific differences. Miller et al. (2013) reports similar ratios of ARGs for intI1, tet(W) and tet(O) between test digesters and the thermophilic control digester, and ratios of sulI, sulII, which were lower in test digesters compared to the control. These results and previous
literature studies show that anaerobic digestion may help in the reduction of ARGs, but are gene specific and the mechanism of ARG development must be investigated to further understand the cause of ARG change. Further research on the effect of Ceftiofur on the microbial community and the community transfer of ARGs in thermophilic conditions is necessary to fully understand the effect of antibiotics and ARGs in anaerobic digestion.

Overall, we conclude that anaerobic digestion is a successful technology in reducing two genes of interest, cfx(A), which encodes beta-lactamase resistance (Chambers et al., 2015), and tet(Q), which encodes for tetracycline resistance (Whittle, et al., 2003). The increase in concentration of the mef(A) gene, which encodes for macrolide resistance, needs to be analyzed further in order to fully understand the potential impact (Grosso et al., 2002). The efflux pump created by expression of the mef(A) gene provides low and moderate resistance to macrolides which are 14 and 15 membered (Grosso et al., 2002). There are varying reports of the frequency of mef(A) in macrolide resistant strains of bacteria. Grosso et al. (2002) states that within S. pnemoniae, mef(A) is more common than the ermB gene for conferring macrolide resistance. Ojo et al. (2004) reports that erm(B) is more common than mef(A) in gram-positive bacteria (60% and 9% respectively), but mef(A) is more common in gram-negative bacteria (19% for erm(B), 54% for mef(A)). Soge et al. (2008) explain that the mef(A) is carried less in Clostridium perfringens than tet genes and erm(B), but mef(A) has been identified in Bacteroides and Fusobacterium, which are anaerobic bacteria. These groups are commonly reported in metagenomics studies for presence in thermophilic anaerobic digesters, and thereby may carry the mef(A) gene. Future research into the transfer of mef(A) through the environment will help determine the appropriate end use for digestate to minimize the effect of this macrolide resistant gene.
Figure 12: Concentration of 16S genes in Control and Ceftiofur reactors over time

Figure 13: Concentration of cfx(A) genes in Control and Ceftiofur reactors over time
Figure 14: Concentration of mef(A) genes in Control and Ceftiofur reactors over time

Figure 15: Concentration of tet(Q) genes in Control and Ceftiofur reactors over time
Figure 16: Ratio of *mef(A)* concentration and 16S concentration in Control and Ceftiofur reactors over time.
4 CONCLUSIONS

Based on the results of this study, it can be concluded that Ceftiofur has an effect on the gas production of anaerobic digesters. Ceftiofur is found as the parent molecule in the feed manure but is significantly reduced during anaerobic digestion. The amount of Ceftiofur in the reactors also decreases as time continues. The change in ARGs is gene dependent, with concentrations of $cfx(A)$ and $tet(Q)$ reducing significantly in the effluent as compared to the feed, in both treatments, but the concentration of $mef(A)$ increased. The concentration of ARGs in the presence of Ceftiofur treatment is also gene specific, as only $mef(A)$ was reported higher in Ceftiofur treatment. Further research needs to be performed to understand the mechanism of gene proliferation in digesters.

Antimicrobial resistance is a worldwide problem which poses threats to food, water, the environment, and health structures for both animals and people. The FAO Action Plan on Antimicrobial Resistance (AMR) (FAO, 2016) uses five objectives to minimize the impact of AMR globally. While these five objectives including awareness training and surveillance, monitoring and record keeping are best used to minimize the generation of resistance, it is good practice to use engineering controls to mitigate the spread of resistance when eliminating it is impossible. AD has been shown to be effective against $tet$ ARGs but further research on a variety of ARGs is needed in order to truly investigate the mechanism of transfer and expression of AMR through ARGs. This research provides additional information to the varied results in literature about the effect of antibiotics on gas production during anaerobic digestion. It can also be concluded that anaerobic digestion is an economical treatment for the reduction of antibiotics in manure, to prevent environmental damage and the spread of antibiotic resistance.
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