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Anaerobic co-digestion of bovine manure and residual sludge from tilapia fish (*Oreochromis niloticus*) breeding ponds

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ABSTRACT

Objetive: Tilapia production was 4.2 million tons in 2016, and almost half of the production came from aquaculture. At the same time, organic waste from breeding increases as the demand for tilapia production. An alternative to using such waste is the production of energy and organic fertilizers. The use of a co-substrate can help to achieve the moisture content necessary to feed the digester. The aim of this study was to determine the effect of the anaerobic co-digestion of bovine manure with residual sludge from tilapia fish breeding ponds in the production of sludge.

Methodology: Methane and CO_2 production, chemical oxygen demand (COD), total solids (TS), total fixed solids (TFS), total volatile solids (TVS), volatile fatty acids (VFA), total nitrogen (TN), total phosphorus (TP) content, and microbiological parameters (fecal coliforms and salmonella) during composting were determined. The organic fertilizer obtained was evaluated by a germination and seedling growth assay.

Results: The results of this study showed that the mixture of bovine manure and residual sludge from tilapia fish breeding ponds (1:1) produced high methane and low CO_2 in the composting process compared to the when these raw materials were composted individually.

Conclusions: Alfalfa germination and seedling growth were significantly boosted by the application of sludge from the mixture of bovine manure with residual sludge from tilapia fish breeding ponds.

Keywords: *Oreochromis niloticus*; composting; germination; sludge; methane.

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INTRODUCTION

The growing demand for energy and food for a growing population has led to the depletion of conventional sources of resources. Alternative technologies have the potential to generate sustainable economies by transforming organic material (livestock manure, agricultural residues, etc.) into biofuels and biofertilizers that help conserve resources and protect the environment[1]. Anaerobic digestion (AD) has become a viable alternative to mitigate the problems caused by organic wastes, such as odor, the large volumes generated,

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the loss of essential nutrients, possibly decreasing pollution[2]. In the anaerobic digestion process, microorganisms consume substrate, either manure or other organic material, resulting in biogas (a mixture of methane and carbon dioxide), and a non-pathogenic fertilizer, which is rich in organic material, humus, nitrogen, phosphorous and potassium, as the end products[3]. Regarding anaerobic codigestion, this is a process that improve the anaerobic digestion performance, optimizing the production of biogas. The world fish production in 2017 was 172.6 million tons. In 2016, the world tilapia production was 4.2 million tons, and almost half of the production came from aquaculture[4]. The increased demand for tilapia is reflected in increased waste that is generated during this process. Economically feasible ways to utilize this waste is to produce biogas and biofertilizers. The wastewater from tilapia breeding ponds after production mainly comprises feces, unconsumed food and bacterial biomass[5]. The conventional production system also produces residual sludge resulting from natural sedimentation. Alternatives have been proposed for the use of the wastewater: to irrigate and fertilize plants, and to be treated for reuse in greenhouses and hydroponic systems [6-11]. The production of fuels such as biogas, using mixtures of livestock manure and aquaculture waste [12-14] could be a clean, renewable, low-cost source of energy, which could replace conventional energy sources. The aim of this study was to determine the effect of the anaerobic co-digestion of manure with residual sludge from tilapia fish (*Oreochromis niloticus*, Linnaeus, 1758) breeding ponds in the production of solid residue. The potential of these solid residue to fertilize was also determined.

MATERIALS AND METHODS

Location of the experiment

The experimental phase of this work was carried out in the Comprehensive Agro-Food Exploitation Laboratory of the Institute of Agricultural Sciences (ICAP, acronym in Spanish) of the Autonomous University of the State of Hidalgo (UAEH, acronym in Spanish).

Obtaining raw material for the experiment

Fresh bovine manure was used, which was provided by the UAEH Ranch, it was kept cold 6 °C until starting the test. The organic solid residue was obtained from an aquaculture farm located in Tezontepec de Aldama, Hidalgo. Fresh residual mud was collected from ponds where tilapia is bred. Before the incubation of the mixtures, wastes in an isolated way were characterized. Proximal composition of residual sludge from Tilapia breeding ponds (RST) was total solids (%) 2.8±0.61, available phosphorus (mg mL^{-1}) 65.04±15.09, calcium (mg mL^{-1}) 595.75±13.7, potassium (mg mL^{-1}) 43.11±6.33, pH 5.61±0.30. Fresh bovine manure (BM) composition was 25.07±5.5 total solids (%), moisture (%) 72.89±12.11, C/N 16.85 pH 6.01±0.45.

Setting up of the experiment and Anaerobic digestion tests

The digestion tests were carried out in 75 mL glass jars at 35±2 °C. In each digester RST and BM were used as inoculum and substrate, both were mixed before being added

to the digesters. To induce an anaerobic environment, each digester was flushed for 5 min (280 mL/min) with inert gas (N_2). Three composting treatments were prepared as followed: a) BM only; b) RST only and c) BMRSM, a 1:1 (V/V) BM and RST mixture. Each treatment had seven replicates, and samples were collected in triplicate on each sampling day (0, 30, 60 and 90 days). The total soluble solids (organic solids) were adjusted to 15%. The composting process was carried out in incubation at 3 5±2 °C for 90 days until solid residue was formed. The initial parameter to determine the samples was the total solids, a balance between the inoculum and the substrate is considered crucial to achieve an optimal production of methane and biogas [15]. The moisture content established was 85%, recommended by [16]. The range of solids used in all tests did not require adding water to reduce the solids loading. Methane and CO_2 production, physicochemical and microbiological parameters during composting were determined. The solid residue obtained was then applied to the biological germination assay, which was subsequently evaluated.

Physicochemical and microbiological parameters during composting

On day 0, 30, 60 and 90 of the incubation, two jars from each treatment were selected at random to determined digestion. The organic samples collected were frozen until characterization. For the characterization and monitoring of the composted organic material samples, the chemical oxygen demand (COD), total solids (TS), total fixed solids (TFS), total volatile solids (TVS), the total organic phosphorus (TP) and nitrogen (TN) content. All the analyses were performed in triplicate.

The total nitrogen content was determined by the Kjendahl method, 0.5 g of sample was weighed on nitrogen-free paper, previously tared. After the sample was placed in the bottom of the Kjendahl flask, 2 g of acceleration mixture and 10 to 15 mL of concentrated sulfuric acid were added later. Then it was placed in the digester flask where it was heated until its complete oxidation points where the mixture turned transparent light green. After the digestion was completed, when the flask was cooled, 200 mL of distilled water was added to dissolve completely. The distillation apparatus was prepared, at the outlet of the refrigerant, a glass tube was adapted which remained immersed in 75 mL of 4% boric acid contained in a 500 mL erlenmeyer flask added with a few drops of wesslow indicator, then 5 mL of 40% NaOH was added to the kjendahl flask, slowly stratifying for each ml of sulfuric acid added during digestion, plus 10 mL of excess due to the possible carbonation of the soda. The distillation was carried out in a kjendahl apparatus, after recovering approximately 250 mL to ensure that all the ammonia has passed. It was titrated with 0.1N HCl solution (turn from green to violet).

For measurement of total calcium and potassium in the samples, 5 g of the samples were digested with 20 mL of concentrated HNO_3 and 10 mL of $HClO_4$, at 170 °C on a hot plate, until the fume changed to white. After cooling, the digested sample was filtered and diluted in the volumetric flask to 100 mL with distilled water. The solution was analyzed for Ca and K by using an atomic absorption spectrometer. For phosphorus determination, the solution was agitated with activated charcoal for 1.0 h and filtered to remove the color. Phosphorus in the solution was analyzed by a molybdate blue colorimetric method. For

extractable Ca, K, Mg and P determination, 5.0 g sample was weighed. A 20 mL mixture of 0.05 mol/L HCl and 0.0075 mol L^{-1} H_2SO_4 was added. After shaking (5 min), the suspension was passed through a filter paper and the filtrate was diluted in the volumetric flask to 100 mL with distilled water. Then, Ca, K, Mg, and P in the solution were analyzed as previously described following the methods described in Standard Methods for the Examination of Water & Wastewater [17]. For the pH measurement, a Thermo Orion pH meter, calibrated with buffer solutions with pH 4 and 7, was used. The sample was shaken for several minutes to obtain a homogeneous mixture, the electrode was introduced, and the reading was recorded once stabilized.

Determination of volatile fatty acids (VFA) by Capillary Electrophoresis

The determination of VFA was carried out in a capillary electrophoresis equipment (Coulter Beckman) with a 47 cm × 75 μm D.I. X 375 μm DE capillary, and an injection length detection of 40 cm. Nitrogen pressure in the capillary was 0.5 psi. The separation was carried out at constant capillary temperature and the UV detector reading was set at 214 nm. The buffer used was a solution of 10mM Benzoic Acid (C_6H_5-COOH), 10 mM Histidine and 1 mM Tetradecyl Trimethyl Ammonium Bromide (TTAB) and adjusting the pH to 6 with 1 M NaOH. A mixture of 50 mg kg^{-1} acetic acid, 50 mg kg^{-1} propionic and 50 mg kg^{-1} butyric acid (1:1:1) was used as a control. The type of VFA was identified based on the migration times of the areas of the chromatograms, and the calculations were performed depending on the concentrations of each of the components of the control [18].

Measuring biogas production

Biogas was collected dairy and measured by water displacement method. Biogas samples were examined by gas chromatography. The gas production was evaluated weekly during incubation. Methane gas was measured using a Perkin Elmer gas chromatograph. A 30 m long Elite Plot-Q capillary column (DVB Plot column) was used. Nitrogen was used as a carrier gas. The injection volume was 0.5 μL. The temperature of the detector was 200 °C, that of the injector 150 °C and that of the column 50 °C [19].

Detection of pathogenic bacteria during the composting process

Multiple tube fermentation tests were used to determine total and fecal coliforms according to the method used by [17]. Salmonella was determined as described by [17] and [20].

Alfalfa (*Medicago sativa* var San Miguel) seed germination assay with solid final residue

The experiment was conducted in green house conditions. Alfalfa seed germination and seedling emergence, sown in biosolids obtained from anaerobic digestion of substrates (BM, RST, BMRSM), were evaluated. Alfalfa seeds were sown in a 100 mm pots containing a sandy soil. The test pots were fertilized with 20 mL of the solid final residue while no solid final residue was added to the control pots. There were three replicates of each treatment.

Seedling emergence rates were recorded. After emergence, only 2 Alfalfa seedlings were kept in each pot. Alfalfa seedlings were watered once weekly. After 20 days, plant height as well as fresh and dry plant weights were measured.

Data analysis

A completely randomized experimental design was used, and a one-way analysis of variance was conducted by performing an analysis in triplicate (n=3). When there were differences of $P<0.05$, the Tukey mean comparison test was used. All data were analyzed using the NCSS 2001, version 5, software (Wireframe Graphics, Kaysville, UT, USA). All experiments were performed in triplicate.

RESULTS AND DISCUSSIONS

Anaerobic co-digestion is an alternative with important results, since it can improve gas production in the anaerobic digestion of solid waste. In this study, this method was used, because the lack of water during fermentation is a limitation, by itself the RST does not have the characteristics of richness of organic material, however it provides the system with high humidity due to the origin of this waste; the mineral richness of RST could activate the enzymatic systems of the microorganisms present in the BM, which functions at the same time as substrate and inoculum for fermentation The two wastes used to carry out the anaerobic co-digestion experiment for the production of biogas and a waste intended to be used as a fertilizer presented relatively high contents of TS and VS, which makes them usable waste in this type of process [21]. Both BM and RSTB residues present different proximal compositions, it is known that the organic fraction of bovine manure is rich in microorganisms [22], on the other hand, the residual sludge from tilapia ponds is rich in micronutrient contents which are found in assimilable forms. 3.1. Chemical Oxygen Demand (COD)

The change in COD in the treatments during the anaerobic digestion is shown in Table 1. This measurement was made as a follow-up to the activity during fermentation. The decrease of COD in the samples at the end of the experiment suggest degradation of organic matter during the composting process. These results coincide with those obtained by [23] in a study on anaerobic digestion; COD reduction was 28.2% after 10 days at temperature of 25 °C in an anaerobic reactor. The variations in COD in the treatments may be related to the chemical characteristics of the organic matter of each sample; degradation of these particles increase the COD [24].

Methane and carbon dioxide production

Figure 1 shows the results regarding the production of methane and CO_2. These results correspond to methanogenesis phase. The highest methane and the lowest CO_2 production were obtained in the BMRSM treatment up to day 60 with about 70% methane produced, but only 43% and 2% methane produced in BM and RST respectively. Methane production depends on the hydrolysis phase: soluble organic matter is produced during hydrolysis, which serves as the substrate in methanogenesis [25]. The formation of soluble organic matter and later methanogenesis were favored in samples from the BMRSM treatment.

Table 1. Chemical oxygen demand (COD) and pH time evolution (90-day trial) during the bio digestion process of bovine manure, residual sludge from tilapia fish aquaculture breeding ponds and 1:1 mixture of both.

Treatment	Time (days)							
	0		30		60		90	
	pH	COD (mg L^{-1})	pH	COD (mg L^{-1})	pH	COD (mg L^{-1})	pH	COD (mg L^{-1})
BM	5.92 ± 0.31^{aA}	90007 ± 310^{dC}	7.30 ± 0.13^{bA}	83795 ± 233^{cC}	7.35 ± 0.12^{bA}	80716 ± 222^{bC}	7.23 ± 0.11^{bA}	76079 ± 173^{aC}
RST	7.26 ± 0.12^{bC}	59193 ± 255^{dB}	7.34 ± 0.18^{bA}	5560 ± 210^{cB}	7.52 ± 0.18^{bA}	48067 ± 197^{bB}	7.75 ± 0.19^{bB}	42606 ± 202^{aB}
BMMR	6.7 ± 0.15^{aB}	51013 ± 184^{dA}	7.49 ± 0.12^{bA}	44853 ± 196^{cA}	7.56 ± 0.11^{bA}	40393 ± 183^{bA}	7.6 ± 0.16^{bB}	28973 ± 192^{aA}

The results are expressed as means standard deviation. Lowercase letters in the same row indicate significant differences ($p<0.05$) among treatments on different analysis day. Different capital letters in the same column indicate significant differences ($p<0.05$) among each treatment on the same analysis days.

The values are like those reported by 1 and 2 that showed results from several experiments, where modifications were made to some factors such as hydraulic retention time, load, type of substrate, pH, among others, obtaining biogas production with methane concentrations between 33-69%. After the bio-oxidative phase, the CO_2 concentration remained stable until the end of the process. This could have occurred because the easily biodegradable compounds were metabolized during the first stage of the process [26] additionally the low content of water content in the reactor results in a rapid accumulation of volatile fatty acids, especially for easily digestible raw materials that hinder the activity of methanogenic bacteria, leading to low biogas production [21].

Volatile Fatty Acids (VFA), Total Solids (TS), Total Volatile Solid (TVS), and Total Fixed Solids (TFS)

Table 2 shows the TS, TVS and TFS content in the different samples. Table 2 shows that of the TS, most were quantified as SV. The initial values of TS and VS of the wastes and the mixtures could be considered as high contents, therefore, its use is attractive for anaerobic

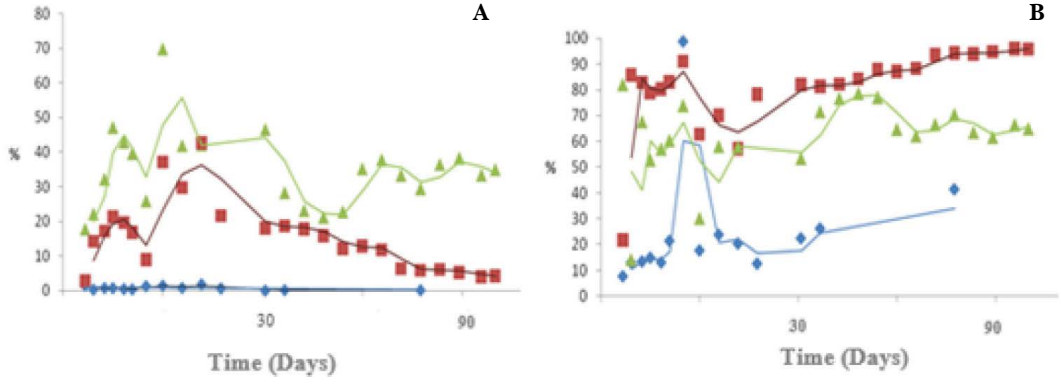

Figure 1. Percentage of (A) methane and B) CO_2 from anaerobic digestion BM, bovine manure (■), RST (residual sludge from tilapia fish Breeding ponds (◆), and BMMR mixture 1:1 BM-RST (▲). Data are the mean of seven jars×three experiments×three measurements by each day of collection. Whole experiment lasted 90 days.

Table 2. Evolution of total solids (TS), total fixed solids (TFS), and total volatile solids (VTS), volatile fatty acids (VFA) and percentages of macronutrients (N, P, C, C/N) during the anaerobic digestion process of substrates.

Time (days)	VFA			Solids			Biosolid composition			
	AA	AP	AB	TS	TFS	VTS	N	P	C	C/N
	$mg{\cdot}L^{-1}$			$g{\cdot}L^{-1}$			%			
BM										
0	2770 ± 320^{b}	960 ± 88^{c}	119.3 ± 21	9.32 ± 0.22^{c}	2.38 ± 0.07^{a}	6.94 ± 0.12^{d}	0.25 ± 0.02^{b}	0.13 ± 0.04^{a}	4.02 ± 0.04^{d}	16.1 ± 0.11^{c}
30	2808 ± 220^{b}	854 ± 71^{c}	ND	8.75 ± 0.13^{b}	3.16 ± 0.09^{b}	5.59 ± 0.20^{c}	0.24 ± 0.03^{ab}	0.12 ± 0.07^{a}	3.24 ± 0.06^{c}	13.5 ± 0.05^{b}
60	2456 ± 174^{ab}	617 ± 53^{b}	ND	8.54 ± 0.25^{ab}	4.13 ± 0.05^{c}	4.41 ± 0.24^{b}	0.22 ± 0.01^{a}	0.12 ± 0.02^{a}	2.56 ± 0.07^{b}	11.6 ± 0.10^{ab}
90	1560 ± 135^{a}	432 ± 31^{a}	ND	8.23 ± 0.23^{a}	4.29 ± 0.06^{c}	3.94 ± 0.15^{a}	0.20 ± 0.03^{a}	0.11 ± 0.09^{a}	2.12 ± 0.10^{a}	10.6 ± 0.09^{a}
RST										
0	420 ± 63^{c}	370 ± 39^{b}	ND	4.43 ± 0.11^{b}	3.78 ± 0.22^{a}	0.65 ± 0.07^{b}	0.06 ± 0.009^{c}	0.04 ± 0.009^{b}	0.38 ± 0.01^{b}	6.3 ± 0.01^{b}
30	$199\pm.35^{b}$	127 ± 38^{a}	ND	4.15 ± 0.12^{a}	3.81 ± 0.15^{a}	0.34 ± 0.11^{a}	0.05 ± 0.003^{bc}	0.03 ± 0.001^{ab}	0.21 ± 0.09^{ab}	4.2 ± 0.08^{a}
60	55 ± 12^{a}	ND	ND	4.10 ± 0.19^{a}	3.85 ± 0.19^{a}	0.25 ± 0.09^{a}	0.04 ± 0.003^{b}	0.03 ± 0.003^{ab}	0.15 ± 0.08^{a}	3.8 ± 0.02^{a}
90	ND	ND	ND	4.05 ± 0.1^{a}	3.84 ± 0.13^{a}	0.21 ± 0.08^{a}	0.03 ± 0.004^{a}	0.02 ± 0.001^{a}	0.13 ± 0.01^{a}	4.3 ± 0.03^{a}
BMMR										
0	11755 ± 21^{d}	434 ± 57^{b}	ND	7.89 ± 0.17^{d}	4.08 ± 0.19^{a}	3.81 ± 0.08^{d}	0.15 ± 0.02^{a}	0.09 ± 0.001^{b}	2.21 ± 0.01^{d}	14.7 ± 0.18^{d}
30	859 ± 96^{c}	165 ± 12^{a}	ND	7.01 ± 0.23^{c}	4.67 ± 0.18^{b}	2.34 ± 0.12^{c}	0.12 ± 0.03^{a}	0.08 ± 0.008^{ab}	1.32 ± 0.03^{c}	11.0 ± 0.13^{c}
60	659 ± 68^{b}	ND	ND	6.35 ± 0.16^{b}	4.77 ± 0.17^{b}	1.58 ± 0.15^{b}	0.11 ± 0.04^{a}	0.07 ± 0.002^{a}	0.91 ± 0.09^{b}	8.3 ± 0.25^{b}
90	318 ± 17^{a}	ND	ND	6.02 ± 0.14^{a}	4.78 ± 0.09^{b}	1.24 ± 0.19^{a}	0.13 ± 0.03^{a}	0.06 ± 0.007^{a}	0.75 ± 0.05^{a}	5.8 ± 0.19^{a}

BM Bovine manure only, RST tilapia breeding ponds residual sludge, MBMR 1:1 mixture of MB and RST, TS-Total solids, TFS-Total fixed solids, VTS-Volatile total solids, VFA-Volatile fatty acids, AA- Acetic acid, AP-Propionic acid, AB-Butyric acid, ND- No detected, N (nitrogen), P (phosphorus), C (carbon), C/N (Carbon/nitrogen relation). Data are the mean of seven jars × three experiments × three measurements by each day of collection. Whole experiment lasted 90 days. [a, b, c] Different letters in columns means statistically different values by Tukey test ($P<0.05$).

co-digestion tests for biogas production [27]. The BM samples had higher concentrations of TS, TVS and TFS compared to other treatments. The high concentration of TS in BM samples was a consequence of TVS and TFS accumulation.

According to [1] TS concentration was 15.6% and 59.5% in bovine and llama manures, respectively, while TVS concentrations were 82.8% and 74.4%. In a previous study, the characteristics of anaerobic digestion of pig manure from different growth stages were investigated. According to growth stage, batch experiments were performed using gestating sow manure (GSM), swine nursery with post-weaned piglet manure (SNM), growing fattening manure (GFM) and mixed manure (MM) as substrates at four substrate concentrations (40, 50, 65 and 80 gVS/L) under mesophilic conditions, the volatile fatty acids/total inorganic carbon (VFA/TIC) ratio increased from 0.10 to 0.89 when loading increased from 40 to 80 gVS/L for GFM [28]. At the beginning of this study, TS was 9.32% in BM, 4.43% in RST and 7.89% in BMRSM. However, the amount TS at the end of the study decreased due to the decomposition of organic matter, which was transformed into methane and CO_2.

According to previous reports, the accumulation of VFA caused a decrease in pH [25]. This contrasts with our results; the pH during digestion remained practically unchanged. An anaerobic digestion process that is carried out efficiently should have a concentration of VFA less than 2000 mg/L [29]. It is normally assumed that part of the hydrolyzed organic matter is converted to VFA and if the concentration of VFA is not high enough to exceed the buffer capacity of the system, the pH remains unchanged, subsequently allowing for methanogenesis[30]. Acetic and propionic acid could be quantified; their concentration increases in the acid-genesis stage then decreases during methanogenesis.

Nitrogen content during digestion

The nitrogen content in BM reported as the minimum necessary for the incorporation into the cell structure of methanogenic bacteria is 0.6% [1]. Conversely, an excess of nitrogen in the substrate leads to the formation of ammonia and lead to decreased methane production since ammonia can alter the pH. The soluble forms of nitrogen are immediately assimilated [1] and [2] the insoluble forms are solubilized before being use by microorganisms during hydrolysis and fermentation [31]. Methanogenic activity decreases with increasing nitrogen concentration[32]. The nitrogen concentration in the BMRSM treatment was highly stable throughout the phases; meanwhile, nitrogen concentration was 20% higher in BM than in BMRSM and RST (Table 2), both having TN content similar to those reported by [1]. In a study carried out by [26] using horse manure mixed with garden waste as substrate, nitrogen content decreased during the first 90 days of the digestion process.

Phosphorus

Phosphorus must be conserved until the end of the co-digestion process since it is an essential nutrient in organic fertilizers. The phosphorus concentration in the BM treatment was 50% higher compared to the BMRSM treatment and around 90% higher

than the RST treatment. The percentage of phosphorus remained practically unchanged throughout the digestion process, being 1.22% in BM, 0.10% in RST and around 0.43% in BMRSM (Table 2).

Carbon content and carbon/nitrogen relation

The carbon content and the relation with nitrogen are important factors in soil since both are crucial elements for the survival of microorganisms. For example, bacteria are known to require 25 to 30 times more carbon than nitrogen. In the assimilation process of this nitrogen compounds part of the C is oxidized to CO_2, so that the concentration of C in the reactor decreases when bacteria recover the nitrogen, then the digestion can continue, but the overall process will be much slower than if the organic material used had a more adequate C/N ratio. If the ratio is low, the C will be depleted before the N, causing the fermentation process to stop and later the material will lose the remaining N [30]. In the case of [26] they found that the C/N ratio showed a slight increase during the first few days of the digestion process, followed by a decrease from 36.7 to 25.8 over a period of 190 days. According to [28] the inhibition of ammonia nitrogen (AN) is a common issue in anaerobic digestion of animal manure and carbon-nitrogen ratio (C/N) is important for the activity of anaerobic microorganisms and performance of anaerobic digestion process, in fact low C/N ratio has the potential risk of leading to ammonia nitrogen inhibition. In this study, the C/N ratio during the co-digestion process showed a significant decrease at the end of the process as reported in another research. Low C/Nm ratio of bovine manure facilitates the balancing of the C/N ratio during fermentation in bioreactors [33].

Microbiological analysis

As can be seen in Table 3, RST had a higher fecal coliform (CF) content compared to BM at the beginning of the composting process. The amount of *Salmonella* detected in all the residues at the beginning was greater than 100 CFU/g. The anaerobic digestion resulted in a 99% elimination of both pathogenic microorganisms. However, composting tilapia residues was very effective in the reduction of fecal coliforms and *Salmonella*. The other two treatments were inefficient in the total inactivation of these bacteria during the co-digestion process. Temperature has been found to be the principal factor that

Table 3. Microbiological profile of the substrates during anaerobic digestion.

Time (days)	BM		RST		BMMR	
	Salmonella	**Faecal coliforms**	***Salmonella***	**Faecal coliforms**	***Salmonella***	**Faecal coliforms**
	CFU/100mL					
0	$11\times10^{3}\pm563^{b}$	$18\times10^{6}\pm773^{d}$	320±245	$21\times10^{4}\pm670^{c}$	$23\times10^{2}\pm321^{b}$	$97\times10^{4}\pm7890^{d}$
30	$40\times10^{2}\pm329^{a}$	$74\times10^{5}\pm512^{c}$	ND	$11\times10^{3}\pm463^{b}$	310 ± 78^{a}	$19\times10^{3}\pm328^{c}$
60	$35\times10^{2}\pm477^{a}$	$30\times10^{4}\pm396^{b}$	ND	$77\times10^{2}\pm378^{a}$	ND	$54\times10^{2}\pm345^{b}$
90	ND	$21\times10^{3}\pm129^{a}$	ND	ND	ND	$11\times10^{2}\pm129^{a}$

BM Bovine manure only, RST tilapia breeding ponds residual sludge, MBMR 1:1 mixture of MB and RST. CFU/mL, colony forming units by milliliter. Data are the mean of seven jars × three experiments × three measurements by each day of collection. Whole experiment lasted 180 days.

determines the inactivation of pathogens during anaerobic digestion [31]. In a study by [32] they determined that an effective reduction of *E. coli* in anaerobic digestion of bovine manure required approximately 60 days at 25 °C and 34 days at 37 °C. Similarly, [34] studied anaerobic co-digestion of swine manure. They observed a significant reduction of pathogens as like *Salmonella enterica* and *Escherichia coli* at 24 °C or 37 °C for 30 days. In contrast, [35] found that after mesophilic anaerobic digestion of dairy manure, pathogens were not completely eliminated and can pose a biosecurity risk. Understanding pathogens behavior in the transformation of manure residues is important to minimize the possible transmission of hazardous microorganisms in crops [36].

Evaluation of the solid residue obtained

The effects of adding sludges from the different treatments to soils on Alfalfa seed germination and growth are presented in Table 4. The samples with sludges showed low seed germination rates, which is probably attributed to the decrease in oxygen tension due to the addition of the solid organic matter. No negative effects were observed regarding the growth parameters of Alfalfa. The height of the plants was significantly higher when BM was applied. It is probable that the microbial community in these samples can promote plant growth [37]. There were no significant differences between the fresh and dry weight of the plants grown in soil to which BM and BMRSM sludges were added. Ash content was higher in plants grown in soil with BMRSM than with RST. The length of the roots was longer when RST was applied compared to the control. Alfalfa germination and seedling growth was significantly improved by the application of sludges [38].

CONCLUSIONS

The results of this study show that using the mixture of bovine manure and residual sludge from tilapia fish breeding ponds (1: 1) yielded a higher production of methane and a lower production of CO_2, which occurred during the first 60 days of the co-digestion process. At the end of the co-digestion process, the stabilization of organic matter was achieved. There was a reduction of more than 99% in the content of pathogenic bacteria in the sludges formed at the end of the co-digestion process.

Table 4. Evaluation of different substrates after anaerobic digestion, on germination of alfalfa (*Medicago sativa* var San Miguel) growth for 20 days.

Treatments	SE	PH (cm)	FW (g)	DW (g)	Ash (mg)	RL (cm
Control	1.04 ± 0.24^{a}	3.16 ± 0.22^{ab}	2.08 ± 0.37^{a}	1.06 ± 0.38^{a}	1.37 ± 0.33^{ab}	1.21 ± 0.16^{b}
BM	0.842 ± 0.15^{a}	2.48 ± 0.19^{a}	3.35 ± 0.25^{b}	2.15 ± 0.36^{b}	1.71 ± 0.24^{b}	0.75 ± 0.26^{a}
RST	0.774 ± 0.19^{a}	3.47 ± 0.17^{b}	2.11 ± 0.56^{a}	1.03 ± 0.23^{a}	1.54 ± 0.18^{b}	1.17 ± 0.30^{b}
BMMR	0.832 ± 0.23^{a}	2.54 ± 0.26^{a}	3.42 ± 0.46^{b}	1.37 ± 0.16^{a}	1.11 ± 0.27^{a}	0.67 ± 0.22^{a}

BM Bovine manure only, RST tilapia breeding ponds residual sludge, MBMR 1:1 mixture of MB and RST. Seedling emergence (plants per day), % Plant height (cm) Fresh weight (g) Dry weight (g), Root length (cm). Data are the mean of 90 plants (three experiments × three measurements) by each treatment. Whole experiment lasted 20 days. $^{a, b, c}$ Different letters mean statistically different values by Tukey test ($P<0.05$).

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