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# Assessment of pretreatment and digestion temperature on anaerobic digestion of whiskey byproducts and microbial taxonomy

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ABSTRACT

The effects of steam and sulfuric acid pretreatment on anaerobic digestion (AD) of whiskey byproducts (including draff, thin and thick stillage) were investigated in order to improve the digestion performance under both mesophilic and thermophilic temperatures. The results of biomethane potential assays suggested that thermophilic AD facilitated the release of free ammonia (ca. 1200 mg/L) from byproducts, resulting in strong ammonia inhibition and volatile fatty acid accumulation. In contrast, no free ammonia inhibition (ca. 700 mg/L) was observed under mesophilic AD; the methane yield from mesophilic AD was between 375.3 ± 13.6 mL/g volatile solid (VS; acid-treated sample) and 389.1 ± 8.5 mL/g VS (untreated sample). Although acid pretreatment (2% acid under 135 °C for 15 min) did not improve the methane yield from mesophilic AD, it reduced the digestion time by 14.3% compared to that of the untreated sample. Microbial community analysis showed that irrelevant of pretreatment, hydrogenotrophic methanogens of *Methanobrevibacter* (28.9%–49.8% in abundance) and *Methanoculleus* (26.0%–55.9% in abundance) were the dominant archaeal genus under mesophilic AD. In comparison, hydrogenotrophic *Methanothermobacter* (over 97% in abundance) were dominant in thermophilic AD. This study could be exploited to aid in decarbonizing the whiskey industry by optimizing the biogas process in a circular economy system.

## 1. Introduction

The agri-food and beverage industry including whiskey and beer production is an important economic sector and a significant asset for countries such as Ireland [1] and Scotland (where it is termed whisky) [2]. The industry's heavy reliance on fossil fuels such as fuel oil and natural gas adds to its carbon footprint [3]. The pursuit of a sustainable, low-carbon-emission and circular economy should drive the transition from a fossil fuel-based industrial system to a renewable energy-based one [4]. Indeed, renewable energy produced from agri-food and beverage byproducts can provide a promising opportunity to substitute the use of fossil fuels and subsequently diminish carbon emissions from this industry [5].

Along with the production of whiskey, a significant quantity of solid and liquid byproducts, respectively termed as draff (or spent grain) and pot ale (or stillage), are also produced [6]; the characteristics of these byproducts (high carbohydrates and chemical oxygen demand (COD) concentration) make them suitable for anaerobic digestion (AD) [7]. The resultant biogas after upgrading, predominately methane, can substitute the consumption of natural gas in distilleries, reducing its energy-related greenhouse gas (GHG) emissions.

The digestion of thin stillage could meet 60% of the daily energy demand of a bioethanol plant [8]. If used in AD, distillery liquid byproduct was reported to reduce annual $CO_2$ emissions by 1000 tonnes (equivalent to 25% of the $CO_2$ emissions from the distillery) [9]. Recent studies showed that 42%–54% of GHG emissions from distilleries could be reduced by replacing natural gas with renewable biogas [4,10]. Additionally, the digestate, a byproduct from AD, can be used as a biofertilizer to support the growth of grain crops used for whiskey production, reducing fossil fertilizer use and again reducing GHG emissions in a circular bioeconomy system [11]. It is reported that with the addition of trace elements and other element-containing materials to anaerobic digestate, the digestate fertility can be increased by 5–8% [12,13,14].

As shown in Table 1, most research has focused on the mesophilic

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**Nomenclature**

| | |
|---|---|
| AD | Anaerobic Digestion |
| AM | Acetoclastic Methanogenesis |
| BMP | Biomethane Potential |
| BI | Biodegradability Index |
| COD | Chemical Oxygen Demand |
| sCOD | Soluble Chemical Oxygen Demand |
| C/N ratio | Carbon to Nitrogen Ratio |
| DDGS | Distillery Dried Grain with Solubles |
| FAN | Free Ammonia Nitrogen |
| GHG | Greenhouse Gas |
| GC | Gas Chromatography |
| HRT | Hydraulic Retention Time |
| HM | Hydrogenotrophic Methanogenesis |
| SAO | Syntrophic Acetate Oxidation |
| TS | Total Solids |
| VS | Volatile Solids |
| VFAs | Volatile Fatty Acids |
| WBM | Whiskey Byproducts Mixture |

digestion of distillery liquid byproducts (such as pot ale, thin stillage, and whole stillage). These liquid byproducts are easily degradable, the digestion of which requires a short retention time with a high COD removal efficiency and high methane production [15]. However, the digestion efficiency of the solid byproducts is relatively lower due to its inherent complex lignocellulosic structure, which would typically need a longer hydraulic retention time of 30 days [16].

Various pretreatment technologies have been developed to break down the rigid structure of lignocellulosic biomass with an ambition to improve biogas/biomethane yield and speed up the digestion process [17]. Steam pretreatment is a commonly used physical pretreatment technique for biomass deconstruction. Steam pretreatment can remove a significant part of hemicellulose from solid lignocellulosic feedstock and makes cellulose more accessible to microorganisms during AD. Chemical and thermochemical pretreatments with dilute chemical solutions (such as alkaline and acid solutions) have received wide research interest due to their high efficiency in altering biomass structure and improvement in biomethane yield [18,19,20]. The methane yield from brewery spent grains (solid byproducts from the brewery industry) increased by 27.8% after microwave-assisted hydrothermal pretreatment at 140 °C [5]. The methane yield from alkaline and mechanically pretreated whiskey byproducts increased from 130 mL/g volatile solid (VS) to 250 mL/g VS, equivalent to an increase of 92.3% [21].

Acid pretreatment using $H_2SO_4$, $HNO_3$, and HCl at mild temperatures can effectively dissolve hemicellulose and cellulose, increasing the concentration of resulting monomers [22]. When pretreated with 4% $H_2SO_4$ at room temperature for 2 days, the grass *Salvinia molesta* exhibited an increased methane production by 81.8% with a shortened digestion time [23]. Pretreatment of wheat plant (1% $H_2SO_4$ at 121 °C for 120 min) led to 91.5% solubilization of xylan, 15.2% lignin removal, and an increase in methane yield of 15.5% (as compared to untreated wheat plant) [24]. When estimating a practical application, the authors' previous work assumed that the 20% increase in methane production from draff would reduce the economic payback time from 5 years to 4 years, with an energy-related GHG emission reduction by 62% [25]. To conclude, opportunities for pretreatment of distillery spent grains for a higher digestion efficiency are promising [7] to achieve greater sustainability.

**Table 1**
Prior work assessing the AD of distillery byproducts with/without pretreatment and its related microbial community analysis.

| Substrate for AD | Pretreatment | Digestion mode and temperature | HRT (days) | Methane yield (L/kg $VS_{added}$) | Microbial community | Reference |
|---|---|---|---|---|---|---|
| Whole stillage | No | Batch and continuous, mesophilic 35 °C | 22 (batch); 30–60 (continuous) | 401–458 (batch); 213–508 (continuous) | N/A | [26] |
| | | Batch and continuous, thermophilic 55 °C | 22 (batch); 60 (continuous) | 429-693[a] (batch); 433 (continuous) | | |
| Pot ale | No<br>Deproteination | Batch, Mesophilic 37 °C | 28–42 | 520<br>551 (+6%) | N/A | [27] |
| Whole stillage<br>Thin stillage<br>Syrup<br>Wet cake | No | Batch, mesophilic 35 °C | N/A | 469<br>500<br>470<br>425 | N/A | [28] |
| Pot ale | No<br>Combined alkali (1 M NaOH) and beating for 15 min | Batch, mesophilic 35 °C | 21 | 400 mL biogas/g VS<br>510 mL biogas/g VS (+27.5%) | N/A | [21] |
| Pot ale and spent grain (5:1 by weight) | No<br>Combined alkali (1 M NaOH) and beating for 15 min | Batch, mesophilic 35 °C | 21 | 207 mL biogas/g VS<br>279 mL biogas/g VS (+34.8%) | N/A | [21] |
| Spent grain | No<br>Microwave assisted hydrothermal | Continuous, mesophilic 37 °C | 67 | 386.5<br>493.7 (+27.7%) | N/A | [5] |
| Spent grain | Enzymatic | Continuous, mesophilic 35 °C | 4.3–2.5 | 245 L/kg $COD_{added}$ | Bacterial: *Cytophaga*, *Clostridium*, *Bacillus* Archea: Methanosaeta harundinacea | [29] |
| DDGS | Biological with effluent | Continuous, mesophilic 38 °C | Ca. 30 | 288.3 L/kg TS (+7.7%) | Bacterial: Bacteroidetes, Archea: *Methanosarcina* | [30] |
| Distillery byproducts mixture | No | Continuous, mesophilic 37 °C | 117 | 330 | Bacterial: *Firmicutes*, Archea: *Methanomicrobiales* and *Methanosarcinales* | [31] |
| | | Continuous, thermophilic 55 °C | 124 | 365 (+10.6%) | Bacterial: *Firmicutes*, Archea: Methanomassiliicoccus | |

HRT: hydraulic retention time; DDGS: distillery dried grain with solubles. N/A: Not analyzed.
[a] The high methane yield is due to the presence of a high COD content, which was not accounted for in the VS measurement.

To date, studies on the effects of pretreatment with/without the addition of acids on the methane yield and AD performance of whiskey byproducts are scarce. Organic acids including acetic acid are reported to enhance the hydrolysis efficiency of lignocellulosic biomass in pretreatment and the subsequent methane production in AD [32]. Therefore, the hypothesis of this study is that whiskey liquid byproducts with an acidic nature (low pH value around 3.5) may provide a potential opportunity to serve as an acid catalyst for the pretreatment of draff. In addition, as shown in Table 1, data are scarce in terms of comparing mesophilic and thermophilic digestion of pretreated distillery byproducts mixture, and its related microbial community analysis. Such a comprehensive investigation could contribute to making decisions on the process control for a better AD performance.

The objectives of this study are to (1) evaluate the feasibility of anaerobic co-digestion of whiskey solid and liquid byproducts, namely draff, thin and thick stillage, (2) investigate and compare the effects of steam and sulfuric acid pretreatment on the AD performance under mesophilic and thermophilic conditions, and (3) illustrate the effects of different pretreatments and digestion temperatures on microbial taxonomy. This study should add to the limited existing information in relation to the application of pretreatment and anaerobic co-digestion technologies to whiskey byproducts, with a goal to achieve green alcohol production and build a sustainable alcohol industry within a circular bioeconomy.

## 2. Material and methods

### 2.1. Material and inoculum

The whiskey byproducts (draff, thin and thick stillage) were sourced from a large distillery in Ireland. The contents of total solids (TS) and VS, and other characteristics of the whiskey byproducts and the mesophilic and thermophilic inocula are shown in Table 2. Prior to the experimental process (including pretreatment and AD), a mixture consisting of draff, thin stillage, and thick stillage in the ratio of 1:7:6 based on wet weight (with data provided by the distillery) was generated and thoroughly mixed. This mixture is named as whiskey byproducts mixture (WBM). The content of C, N, and the C/N ratio of the mixture were 47.12 ± 0.10%, 4.69 ± 0.17%, and 10.06, respectively. The C/N ratio was lower than the recommended optimal ratio (20–30) for AD [33]. This suboptimal C/N ratio might lead to ammonia inhibition, resulting in a low biomethane production from the substrates. It should be pointed out that the mixture was separately prepared for mesophilic and thermophilic batch AD assays as they were conducted at different times; this does not affect the physicochemical properties of the samples; refer to supplementary data.

The inoculum used in mesophilic and thermophilic AD was sourced from an existing biogas plant in Ireland, which operates under mesophilic condition and is fed with food waste with high protein content. This has led to elevated levels of free ammonia nitrogen (FAN) in the inocula (see Table 2). The inoculum was directly used for mesophilic AD while the thermophilic inoculum was adapted from the mesophilic inoculum for one month. To maintain high microbial activity, both inocula were fed with cellulose and protein. The inocula were degassed to remove the residual gas before the biomethane potential (BMP) assays.

### 2.2. Pretreatment of whiskey byproducts

Depending on the pretreatment conditions, the byproducts mixture was grouped into three: (1) untreated group, (2) steam-treated group, and (3) dilute $H_2SO_4$ treated group (the final concentration of $H_2SO_4$ was 2% v/v). The pretreatment group without the addition of $H_2SO_4$ (steam pretreatment) was set to investigate the effect of low pH of thin and thick stillage during the steam heating process. Each treatment was prepared in quadruplicate. The pretreatment was conducted in an autoclave at 135 °C for 15 min [22]. After pretreatment, all the samples were cooled to room temperature, and then 1 M NaOH solution was used to adjust the pH to 6.5–7.0 for methanogenesis. One of the quadruplicates was stored at 4 °C for further analysis.

### 2.3. Biomethane potential assays

The Bioprocess Control™ automatic methane potential test system (AMPTS II) was employed to conduct BMP assays in triplicate using the pretreated and untreated samples. Cellulose powder (Sigma Aldrich) was used as a standard substrate to assess the inoculum activity. Both mesophilic and thermophilic AD systems employed 15 bottles, each of 650 mL total volume (400 mL working volume). The temperature for mesophilic and thermophilic digestion was set and maintained at 37 ± 0.5 °C and 55 ± 0.5 °C respectively, using a water bath. In mesophilic BMP assays, a calculated quantity of substrate (5.76 g VS) and inoculum (11.52 g VS) was initially added to each bottle; in thermophilic BMP assays, a calculated quantity of substrate (5.60 g VS) and inoculum (11.20 g VS) was initially added to each bottle; see supplementary data. This calculation was based on a 2:1 inoculum-to-substrate ratio of VS [34]. All data are recorded automatically on a bespoke software package. The BMP assays ran for 30 days.

**Table 2**
Chemical compositions of whiskey byproducts and inocula for mesophilic and thermophilic anaerobic digestion.

| Parameter (Unit)* | Draff | Thin stillage | Thick stillage | Byprodcuts mixture [C] | Inoculum (Mesophilic) | Inoculum (Thermophilic) |
|---|---|---|---|---|---|---|
| pH | / | 4.04 | 3.87 | 3.89 | 8.13 | 7.55 |
| Total solids (%) | 29.66 ± 0.11 | 3.19 ± 0.00 | 8.87 ± 0.03 | 7.54 | 5.17 ± 0.07 | 5.74 ± 0.12 |
| Volatile solids (%) | 97.71 ± 0.17 | 93.30 ± 0.21 | 95.62 ± 0.11 | 95.72 | 69.56 ± 0.31 | 59.64 ± 0.40 |
| C (%) | 48.87 ± 0.17 | 44.65 ± 0.15 | 49.39 ± 0.16 | 48.24 | / | / |
| N (%) | 4.74 ± 0.10 | 5.14 ± 0.08 | 4.74 ± 0.05 | 4.82 | / | / |
| O (%) | 40.04 ± 0.17 | 44.48 ± 0.26 | 39.27 ± 0.24 | 40.59 | / | / |
| H (%) | 6.36 ± 0.10 | 5.73 ± 0.06 | 6.60 ± 0.04 | 6.35 | / | / |
| C:N ratio | 10.32 ± 0.23 | 8.70 ± 0.12 | 10.42 ± 0.07 | 10.0 | / | / |
| tCOD (g/L) | / | 52.0 ± 0.7 | 90.8 ± 3.6 | / | / | / |
| sCOD (g/L) | / | 36.3 ± 0.4 | 39.7 ± 0.0 | / | 9.3 ± 0.1 | 13.3 ± 0.1 |
| FAN (mg/L)[A] | / | / | / | / | 628.1 ± 4.2 | 420.3 ± 0.0 |
| $NH_3$-N (mg/L) | / | / | / | / | 4260.0 ± 28.3 | 3460.0 ± 0.0 |
| Crude protein (%)[B] | 29.63 | 32.06 | 29.63 | 30.15 | / | / |
| Glucan (%) | 21.82 ± 0.57 | / | / | / | / | / |
| Xylan (%) | 18.71 ± 0.20 | / | / | / | / | / |
| Araban (%) | 8.31 ± 0.00 | / | / | / | / | / |
| Lignin (%) | 17.61 ± 0.07 | / | / | / | / | / |

*Note*: * Total solids are based on the wet matter (% w/w), all other parameters are based on dry matter (% w/w); [A] FAN represents free ammonia; [B] Protein content was calculated based on the elemental N content: Crude protein = Nitrogen content × 6.25; [C] for whiskey byproducts mixture, the pH value is measured, while other parameters are calculated based on a separate sample added.

### 2.4. Analytical methods

The elemental composition of draff, thin and thick stillage was analyzed using an elemental analyzer with the method described in a previous study [22]. The concentration of reducing sugars in the hydrolysate before and after pretreatment was analyzed using the 3,5-dinitrosalicylic acid method [35]. Samples were taken from all the digesters at the beginning and the end of the experiments for analysis of pH value, ammonia nitrogen ($NH_3$-N), and soluble COD (sCOD). The samples were filtered (0.45 mm pore size) to analyze sCOD and volatile fatty acids (VFAs). The COD, TS, and VS analyses were carried out following standard methods [11]. The pH value of thin and thick stillage, and the digestate before and after digestion was analyzed using an F20 pH meter (METTLER TOLEDO, Switzerland). Samples for VFAs analysis were stored at −20 °C. Before VFAs analysis, the samples were thawed, and the pH of the samples was adjusted to <2.5. After centrifugation at 10,000 rpm for 10 min, the samples were filtered using 0.45-mm syringe filter. VFAs (acetate, propionate, butyrate and valerate) were analyzed and quantified by a gas chromatography (GC).

The sCOD removal efficiency is calculated based on the following equation (Eq. (1)):

$$\text{sCOD removal efficiency} = \left(\text{sCOD}_{\text{input}} - \text{sCOD}_{\text{output}}\right)/\text{sCOD}_{\text{input}} \times 100\% \quad (1)$$

where $\text{sCOD}_{\text{input}}$, $\text{sCOD}_{\text{output}}$ is the amount of sCOD of untreated and pretreated samples before and after AD, respectively. All the COD mentioned in this study refers to sCOD.

The concentration of FAN, dependent on pH and temperature, was calculated as per Eq. (2) according to Hansen et al. [36]:

$$NH_3(\text{Free}) = \text{TAN}/\left(1 + \left(10^{-\text{pH}}\right)/\left(10^{-(0.09018+2729.92/T)}\right)\right) \quad (2)$$

where TAN is the concentration of ammonia nitrogen ($NH_3$-N, mg/L) in this study; $T$ is the temperature in Kelvin for mesophilic and thermophilic digestion.

Statistic significant differences of data were analyzed by using the SPSS 22.0 software with the ANOVA method at the significance level of 0.05.

### 2.5. Microbial analysis

After the BMP assays, liquid samples were taken from all the digesters for microbial analysis to investigate the effect of pretreatment on the bacteria and archaea community. These samples were stored at −20 °C prior to further analysis. The detailed and standard procedure for microbial community structure analysis can be found in the literature [37]. Significant differences in microbial community compositions between two samples based on the Fisher's exact test at 0.05 level and principal component analysis were analyzed using the Majorbio Cloud Platform (www.majorbio.com). The raw sequence data were submitted into NCBI Sequence Read Archive under accession code PRJNA658255.

## 3. Results and discussion

### 3.1. Effects of pretreatment and digestion temperature on biomethane production

Acid pretreatment can break down the recalcitrant lignocellulosic structure to release the water-soluble sugars and boost the hydrolysis process. The concentration of reducing sugars and sCOD in the hydrolysate (the liquid fraction) before and after pretreatment is shown in Table 3. The reducing sugar concentration of the raw substrate was 1.26 ± 0.06 g/L (1.23 ± 0.07 g/L from samples used for mesophilic AD and 1.29 ± 0.03 g/L from samples used for thermophilic AD). Both steam pretreatment and sulfuric acid pretreatment had significant effects on the production of reducing sugars ($p < 0.01$). Steam pretreatment increased reducing sugars yield to 6.47 ± 0.83 g/L (5.71 ± 0.12 g/L from samples used for mesophilic AD and 7.22 ± 0.05 g/L from samples used for thermophilic AD), while acid pretreatment significantly increased sugars yield to 32.80 ± 0.31 g/L (32.54 ± 0.07 g/L from samples used for mesophilic AD and 33.05 ± 0.19 g/L from samples used for thermophilic AD). Correspondingly, the sCOD also increased after steam (37.0 g/L) and acid (59.9 g/L) pretreatment, compared with that of raw samples (32.7 g/L).

The increased concentration of reducing sugars after pretreatment can be attributed to the hydrolysis of hemicellulose from draff as it is the most vulnerable component during the steam and sulfuric acid pretreatment [24,38]. Deng et al. [22] found that the concentration of reducing sugars in the hydrolysate from 2% sulfuric acid pretreated

**Table 3**
Characteristics of methane production from mesophilic and thermophilic anaerobic digestion of whiskey byproducts.

| Parameters | Mesophilic digesters | | | Thermophilic digesters | | |
|---|---|---|---|---|---|---|
| | Raw material | Steam-treated samples | Acid-treated samples | Raw material | Steam-treated samples | Acid-treated samples |
| Pretreatment hydrolysate | | | | | | |
| Reducing sugars (g/L)* | 1.23 ± 0.07[a] | 5.71 ± 0.12[b] | 32.54 ± 0.07[c] | 1.29 ± 0.03[a] | 7.22 ± 0.05[b] | 33.05 ± 0.19[c] |
| sCOD (g/L) | 32.5 ± 0.1[a] | 36.9 ± 0.1[b] | 61.8 ± 1.1[d] | 32.8 ± 0.0[a] | 37.2 ± 0.3[b] | 58.0 ± 0.3[c] |
| Anaerobic digestion | | | | | | |
| BMP results (mL/g VS) | 389.1 ± 8.5 | 379.7 ± 8.1 | 375.3 ± 13.6 | 110.9 ± 22.1 | 215.2 ± 9.5 | 78.1 ± 4.6 |
| BI (%)[A] | 75.3 | 73.5 | 72.6 | 21.5 | 41.6 | 15.1 |
| $Y_m$ (mL/g VS)[B] | 394.9 | 384.7 | 381.7 | N. A | N. A | N. A |
| $R_m$ (mL/d/g VS)[C] | 30.4 | 29.5 | 31.8 | N. A | N. A | N. A |
| λ (d)[D] | 1.49 | 1.30 | 1.57 | N. A | N. A | N. A |
| $R^2$ | 0.997 | 0.997 | 0.984 | N. A | N. A | N. A |
| $T_{80}$ (days)[E] | 14 | 14 | 12 | 28 | 22 | 25 |

*Note*: * Reducing sugars and sCOD concentration are analyzed from the hydrolysate before and after pretreatment; [abc] means significant difference with $p < 0.05$.

[A] Biodegradability index (BI) is the ratio of specific methane yield to theoretical methane yield.

[B] $Y_m$ is the simulated maximum specific methane yield.

[C] $R_m$ is the simulated maximum daily methane yield.

[D] λ is the lag phase time of digestion.

[E] $T_{80}$ means the time necessary to obtain the 80% of the total methane yield.

grass silage (pretreatment condition: 2% $H_2SO_4$, 135 °C for 15 min) was the highest among other pretreatments using sulfuric acid concentrations in the range of 1%-4%. The authors also found that the main components in reducing sugars were xylose and arabinose hydrolyzed from hemicellulose, which accounted for 87% of the total reducing sugars [22]. It has been reported that hydrolysis of complex substrates is the bottleneck for efficient digestion, thus the increased concentration of reducing sugars and COD can facilitate substrate digestion.

*3.1.1. Biomethane production from mesophilic and thermophilic digestion*

The cumulative and daily methane yield from untreated and pretreated WBM under mesophilic and thermophilic digestion are presented in Fig. 1. The respective methane yield from cellulose under mesophilic and thermophilic AD was 310.9 ± 8.0 mL/g VS and 326.2 ± 2.1 mL/g VS, suggesting the healthy status of the inocula.

As shown in Fig. 1 (A) and (C) for mesophilic AD, pretreatment had a significant effect on daily methane yield but did not significantly affect the cumulative methane yield compared to the untreated samples. The theoretical methane production from WBM is 516.8 ± 0.7 mL/g VS based on the calculation of elemental analysis from Table 2 using the Buswell equation [39].

For untreated samples, the daily methane yield peaked at day 1 and day 9, at 30.7 ± 1.3 mL/g VS/d and 32.1 ± 1.5 mL/g VS/d, respectively; the cumulative methane yield was 389.1 ± 8.5 mL/g VS, corresponding to a biodegradability index (BI) of 75.3%. The peak of daily methane yield at day 1 could be explained by the conversion of easily digestible fractions such as monosaccharides, organic acid, and proteins, while the subsequent peak of daily methane yield might be attributed to the relatively recalcitrant fraction of structure-carbohydrates [40–42]. The high BI suggested that the substrate was easily degraded during AD [43].

For steam-treated samples, the daily methane yield also peaked at day 1 and day 9, at 34.7 ± 1.1 mL/g VS/d and 32.5 ± 1.3 mL/g VS/d respectively, a respective increase of 13.0% and 1.2% compared to the untreated samples; the cumulative methane yield was 379.7 ± 8.1 mL/g VS, corresponding to a BI of 73.5%, which showed no significant difference in comparison with that of untreated samples ($p > 0.05$). This suggests that steam pretreatment applied in this study had no significant effect on the methane yield from whiskey byproducts.

For sulfuric acid-treated samples, the daily methane yield peaked at day 1 and day 10, at 43.3 ± 0.9 mL/g VS/d and 35.9 ± 1.8 mL/g VS/d, respectively, a respective increase of 41.0% and 11.8% compared to the untreated samples. The improved maximum daily methane yield from the digestion of sulfuric acid-treated samples might be attributed to the high concentration of reducing sugars as it could be effortlessly converted into methane during the digestion process [22,41]. The cumulative methane yield from sulfuric acid-treated samples was 375.3 ± 13.6 mL/g VS, with a BI of 72.6%, not significantly different from that of untreated samples ($p > 0.05$). This suggests that sulfuric acid pretreatment had no positive effect on the cumulative methane yield from whiskey byproducts.

Table 3 presents the kinetic study results of methane production from the untreated and pretreated WBM fitted by the modified Gompertz equation [13]. The model showed good fitting results as the $R^2$ was >0.98 in all cases under mesophilic AD. The lag phase for all samples was around 1.5 days and the time needed to achieve 80% of the cumulative methane yield ($T_{80}$) from untreated, steam-treated, and sulfuric acid-treated samples was 14, 14, and 12 days, respectively; this suggested that although sulfuric acid pretreatment did not significantly improve the methane yield of whiskey byproducts, it reduced digestion time by 14.3%. These results are in good agreement with previous studies; Deng et al. [22] and Us et al. [44] both reported that the methane production rate significantly increased from sulfuric acid pretreated grass silage and greenhouse residues, respectively, although the final methane yield was not significantly enhanced.

Under thermophilic digestion, the daily methane yield and cumulative methane yield from untreated and pretreated samples are shown in Fig. 1 (B) and (D). For untreated samples, the daily methane yield peaked at day 1 and day 27, at 19.7 ± 0.5 mL/g VS/d and 10.4 ± 0.9 mL/

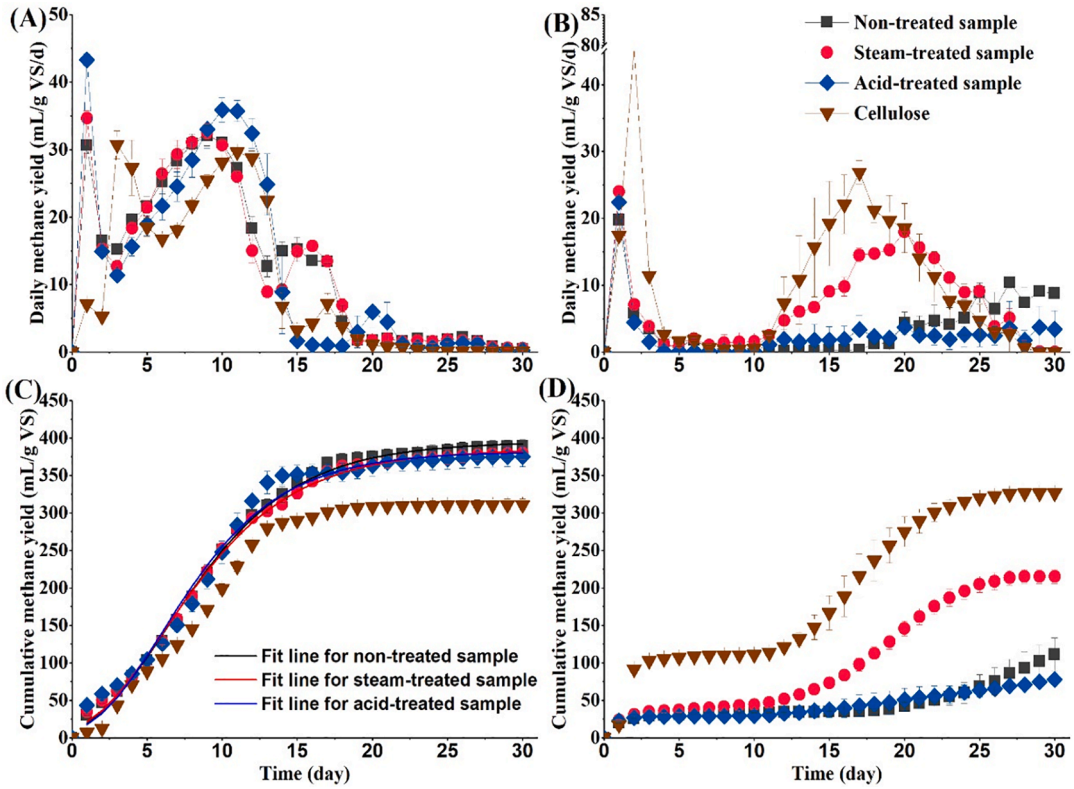


**Fig. 1.** Daily and cumulative methane yield from untreated and pretreated whiskey byproducts under mesophilic (A, C) and thermophilic (B, D) anaerobic digestion.

g VS/d, respectively; the cumulative methane yield was only 110.9 ± 22.1 mL/g VS, corresponding to a low BI of 21.5%, which indicated that methanogenesis was inhibited.

For steam-treated samples, the daily methane yield peaked at day 1 and day 20, at 24.0 ± 0.6 mL/g VS/d and 18.0 ± 0.1 mL/g VS/d, respectively, a respective increase of 21.8% and 73.1% compared to the untreated samples. The cumulative methane yield was 215.2 ± 9.5 mL/g VS, corresponding to a BI of 41.6%, an increase in methane yield of 94.0% compared to that from untreated samples; however, this figure was only 56.7% of that under mesophilic digestion.

For sulfuric acid-treated samples, the daily methane yield also peaked at day 1, at 22.4 ± 0.2 mL/g VS/d, an increase of 13.7% compared to the untreated samples. The cumulative methane yield from sulfuric acid-treated samples was only 78.1 ± 4.6 mL/g VS, with a BI of only 15.1%, suggesting that digestion of sulfuric acid-treated samples was strongly inhibited.

#### 3.1.2. Discussion on biomethane production

The modified Gompertz model failed to fit the BMP results from thermophilic digestion presumably due to the inhibition effect during digestion. The time necessary to obtain 80% of the total methane yield ($T_{80}$) for all cases was twice that of mesophilic digestion, demonstrating that the WBM was not suitable for thermophilic digestion. Eskicioglu et al. [26] investigated the effects of digestion temperature and inoculum-to-substrate ratio on the biodegradability, and biomethane production from whole stillage (obtained from an ethanol plant); the BMP results showed that there was no significant lag phase for both mesophilic and thermophilic AD of whole stillage, and the VS removal efficiency under all cases was between 82% and 97%, with a methane yield from 401 to 693 mL/g VS. The result of the work by Eskicioglu et al. [26] suggested that whole stillage is a suitable substrate for digestion (within the confines of a BMP) with an inoculum-to-substrate ratio in the range 1:1 to 4:1. Therefore, given the methane yield and digestion performance from the WBM assessed here under different digestion temperatures, it may be stated that the WBM is not suitable for thermophilic digestion due to its low C/N ratio, and the likely ammonia inhibition and subsequent accumulation of VFAs as described in work by Masse et al. [45].

### 3.2. Effects of pretreatment and digestion temperature on process parameters

Process parameters, such as VFAs profile and ammonia concentration (including free ammonia and ammonia nitrogen), were evaluated to further understand the effects of pretreatment and digestion temperature on the digestion of the WBM. The VFAs profile over time for mesophilic and thermophilic AD is shown in Fig. 2.

#### 3.2.1. VFA profiles of mesophilic and thermophilic digestion

From Fig. 2 (A), (B), and (C), it was obvious that acetic acid was the main organic acid of total VFAs under mesophilic digestion. For untreated samples, the maximum total VFAs concentration was 6254.1 ± 1354.1 mg/L obtained at day 4, of which the concentration of acetic acid was 4858.4 ± 1569.5 mg/L, accounting for 80% of the total VFAs; there was no accumulation of VFAs as it was gradually converted to methane and carbon dioxide over time (Fig. 2(A)). For steam-treated samples, the maximum total VFAs concentration was 5013.6 ± 174.6 mg/L obtained at day 5, of which the concentration of acetic acid was 3672.4 ± 157.2 mg/L, accounting for 73% of the total VFAs (Fig. 2(B)). For sulfuric acid-treated samples, the maximum total VFAs concentration was 6771.5 ± 542.7 mg/L obtained at day 4, of which the concentration of acetic acid was 5214.2 ± 422.4 mg/L, accounting for 77% of the total VFAs (Fig. 2 (C)). There was no VFAs accumulation for both steam-treated and acid-treated samples, which was almost completely consumed by day 14, in agreement with the aforementioned $T_{80}$. It was noted that propionic acid was completely degraded at the end of the digestion process.

As mentioned before, it was observed that the thermophilic digestion of the WBM was strongly inhibited, which might be attributed to (1) build-up of VFAs, which decreased the pH value of the system, resulting in reduced activity of the methane-producing consortium [46]; (2) the

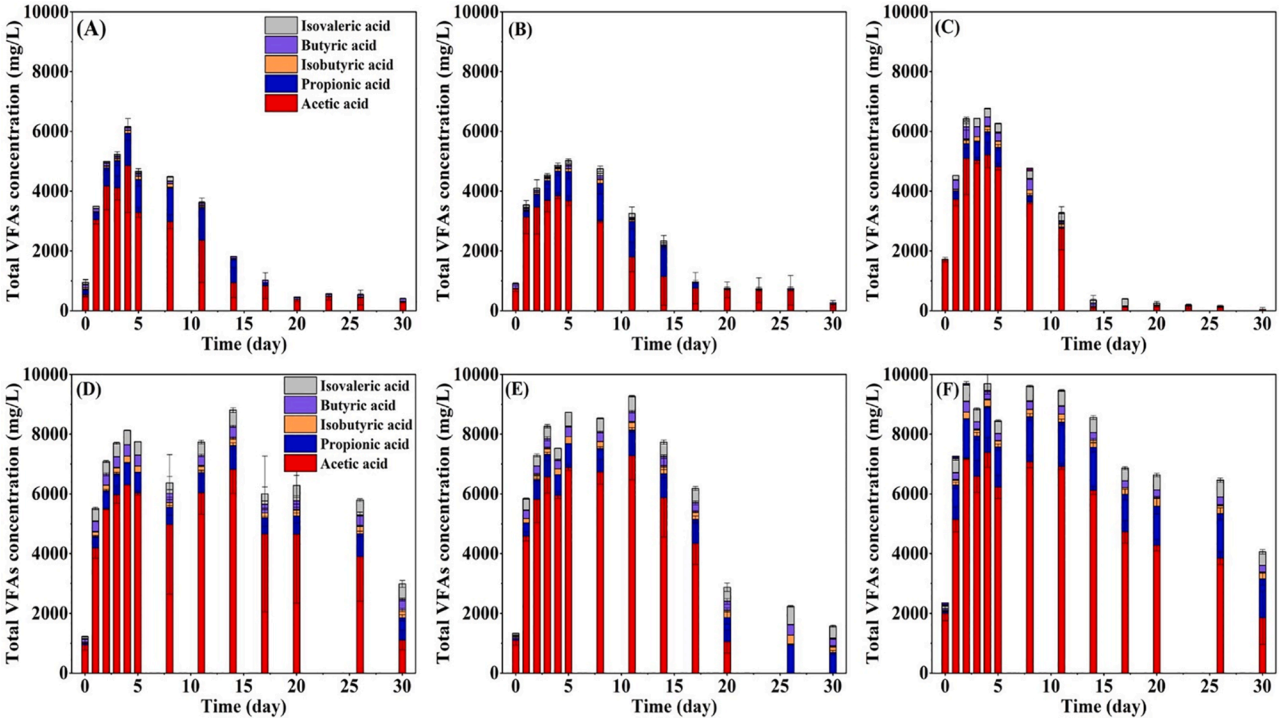


**Fig. 2.** Variations of volatile fatty acids concentration of untreated and pretreated whiskey byproducts under mesophilic and thermophilic anaerobic digestion (AD). (A), (B) and (C) represent no pretreatment, steam pretreatment, and sulfuric acid pretreatment of whiskey byproducts under mesophilic AD; (D), (E), and (F) represent no pretreatment, steam pretreatment, and sulfuric acid pretreatment of whiskey byproducts under thermophilic AD.

increased concentration of free ammonia and ammonia nitrogen, which resulted from rapid hydrolysis of proteins, leading to inactivity of methane-producing consortium. As shown in Fig. 2 (D), (E), and (F), acetic acid was also the main organic acid of the total VFAs. For untreated samples, the concentration of total VFAs ranged from 5514.2 ± 460.9 mg/L to 8125.2 ± 23.8 mg/L before day 11; this increased to 8802.6 ± 1064.8 mg/L at day 14, of which the concentration of acetic acid was 6819.3 ± 802.0 mg/L, corresponding to 77.5% of the total VFAs (Fig. 2 (D)). At the end of the digestion trial of the untreated samples, the concentration of total VFAs was 2985.7 ± 1.6 mg/L, including 743.7 ± 103.8 mg/L of propionic acid and 348.6 ± 50.6 mg/L of butyric acid, indicating the build-up of VFAs during the AD process. For steam-treated samples, the concentration of total VFAs reached 9268.5 ± 963.9 mg/L on day 11, of which the concentration of acetic acid was 7279.1 ± 804.5 mg/L, corresponding to 78.5% of the total VFAs (Fig. 2 (E)). At the end of the digestion trial of the steam-treated samples, the concentration of total VFAs was 1572.4 ± 161.4 mg/L, including 688.9 ± 70.1 mg/L of propionic acid and 257.7 ± 27.4 mg/L of butyric acid, indicating the slight build-up of VFAs during the AD process.

For acid-treated samples, the maximum concentration of total VFAs was 9697.8 ± 40.7 mg/L at day 2, of which the concentration of acetic acid was 7184.3 ± 31.5 mg/L, corresponding to 74.1% of the total VFAs (Fig. 2 (F)). The total VFAs concentration remained over 6462.3 ± 497.0 mg/L, and ended at a level of 4061.0 ± 1204.0 mg/L, including 1855.7 ± 891.1 of acetic acid, 1301.3 ± 210.2 mg/L of propionic acid, and 235.1 ± 1.1 mg/L of butyric acid.

Wang et al. [47] reported that the activity of the methane-producing consortium was strongly inhibited when the concentration of propionic acid was over 900 mg/L in the AD system. Indeed Siegert and Banks[46] suggested that under mesophilic conditions, the activity of the methane-producing consortium could be slightly inhibited with a total VFAs concentration above 4000 mg/L, which would impact all these trials [48]. It may be plausible to infer from this work that in thermophilic digestion of nitrogenous feedstocks, the build-up of total VFAs concentration to over 8,000 mg/L along with high proportions of propionic acid reduced the activity of the methane-producing consortium; this led to a significantly lower methane yield as compared to that achieved in mesophilic digestion.

### 3.2.2. *Variations of pH value, COD and ammonia concentration*

Fig. 3 displays the variations in pH value, sCOD concentration, $NH_3$-N, and FAN concentration during digestion of untreated and pretreated samples. Irrelevant of the pretreatment method, the pH value in both mesophilic and thermophilic AD was around 8 before and after digestion, indicating an overall stable digestion process [49,50].

As shown in Fig. 3 (A), under mesophilic digestion, the sCOD concentration for the digester with untreated samples (the effluent of mixed inoculum and samples) at the very beginning (day 0) was 14.2 ± 1.0 g/L; this was 14.5 ± 0.5 g/L for the digester with steam-treated samples, which showed no significant difference compared to that of untreated samples ($p > 0.05$). The sCOD concentration for the digester with acid-treated samples increased to 21.4 ± 1.2 g/L, which was consistent with the results of reducing sugar and sCOD concentration after acid pretreatment (Table 3); this confirmed the promotional effect of acid pretreatment on the hydrolysis of carbohydrates. This is in agreement with Deng et al. [22] who reported that the sCOD concentration of grass silage digestate before digestion trials was significantly increased by sulfuric acid pretreatment (2% $H_2SO_4$ at 135 °C for 15 min). At the end of the digestion period in this work, the sCOD concentration of

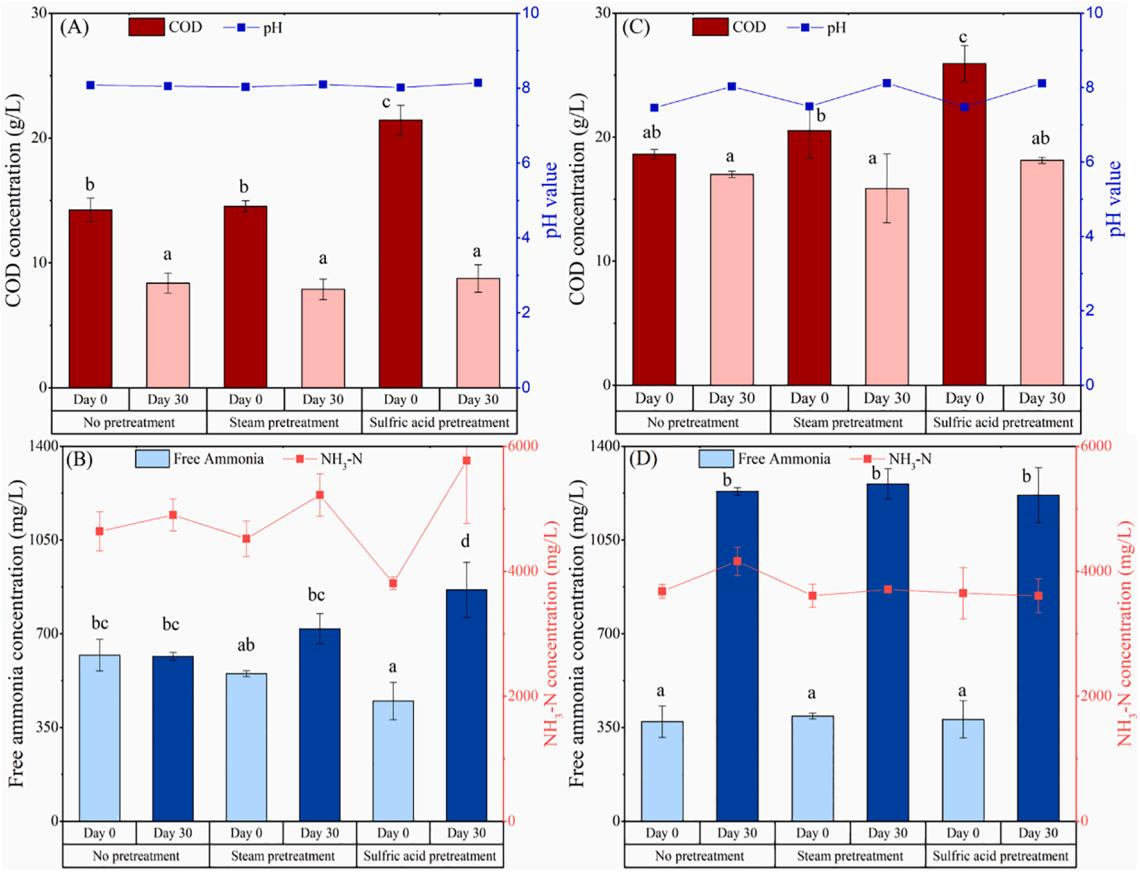


**Fig. 3.** COD, free ammonia and $NH_3$-N concentrations of the effluent from anaerobic digestion of untreated and pretreated whiskey byproducts under mesophilic (A, B) and thermophilic (C, D) conditions. abcd is the statistical significance with a level of 0.05.

untreated, steam-treated, and acid-treated samples was 8.4 ± 0.8, 7.9 ± 0.8, and 8.8 ± 1.1 g/L, respectively, with a respective sCOD removal efficiency of 57.0%, 62.9%, and 72.6%.

Before AD (day 0), the initial FAN and $NH_3$-N concentration in the digester with untreated samples was 620.3 ± 59.0 mg/L and 4640.0 ± 311.1 mg/L, respectively. These levels changed very slightly to 615.8 ± 14.5 mg/L and 4900.0 ± 254.6 mg/L by day 30 (shown in Fig. 3 (B)), exhibiting no significant difference ($p > 0.05$) compared to the initial concentration.

For the digester with steam-treated samples, the FAN concentration significantly increased from 550.9 ± 10.9 mg/L (day 0) to 718.6 ± 56.8 mg/L after 30 days of digestion while the $NH_3$-N concentration increased from 4520.0 ± 282.8 mg/L to 5220.0 ± 339.4 mg/L (shown in Fig. 3 (B)). For the digester with acid-treated samples, the FAN concentration significantly increased from 448.7 ± 69.4 mg/L (day 0) to 863.9 ± 103.1 mg/L after 30 days of digestion, while the $NH_3$-N concentration significantly increased from 3810.0 ± 99.0 mg/L to 5770.0 ± 1004.1 mg/L (shown in Fig. 3 (B)).

Compared to that of untreated samples, the FAN concentration in the digestion system of steam-treated and acid-treated samples increased by 16.7% and 40.3%, respectively; this demonstrated that pretreatment improved the hydrolysis efficiency of proteins. The mechanism of ammonia inhibition is that FAN may diffuse passively into cells resulting in a proton imbalance and/or potassium deficiency [51,52]. The FAN concentration for all samples under mesophilic AD was above the reported inhibition threshold of thin stillage (460 mg/L) [53,54]. However, it did not inhibit the methanogenesis in the present study; this could be attributed to the high tolerance of the inocula to a high FAN concentration after a long-term incubation as it contains 628.1 mg/L of FAN and 4260 mg/L of $NH_3$-N (Table 2), respectively. A previous study concluded that by slowly increasing the organic loading rate in an AD system, inocula with high tolerance to a high FAN concentration could be accumulated [51], thereby leading to a more stable methanogenesis process. It was noticed that although the FAN concentration in the digester of steam-treated and acid-treated samples was higher than that of untreated samples, the biomethane production from mesophilic AD of untreated, steam-treated and acid-treated samples was not significantly different.

As shown in Fig. 3 (C), under thermophilic digestion, the sCOD concentration in the digester with untreated samples before AD (day 0) was 18.6 ± 0.4 g/L; this was 20.5 ± 2.2 g/L in the digester with steam-treated samples, which also showed no significant difference compared to that of untreated samples ($p > 0.05$). The sCOD concentration in the digester with acid-treated samples increased to 25.9 ± 1.5 g/L. It should be noted that the sCOD concentration at day 0 was different for mesophilic and thermophilic AD; this is because of the different sCOD concentrations in mesophilic and thermophilic inoculum as shown in Table 2. With a substrate to inoculum ratio of 1:2, the sCOD of the sample and the inoculum (in a VS ratio of 1:2) are measured not just the sample. The sCOD concentration of the untreated and pretreated samples is assessed and presented in Table 3. At the end of the digestion period, the sCOD concentration of untreated, steam-treated, and acid-treated samples was 17.0 ± 0.3, 15.9 ± 2.8, and 18.1 ± 0.3 g/L, respectively, with a respective sCOD removal efficiency of 17.6%, 28.1%, and 47.1%, which again confirmed the inhibition of thermophilic AD.

Before AD (day 0), the initial FAN and $NH_3$-N concentration in the digester with untreated samples were 371.7 ± 0.5 mg/L and 3680.0 ± 113.1 mg/L, respectively; these were 1231.5 ± 207.4 mg/L and 4160.0 ± 226.3 mg/L after 30 days of digestion (shown in Fig. 3 (D)), a respective increase of 231.3% and 13.0%. The FAN concentration in the digester with steam-treated samples significantly increased from 392.6 ± 37.0 mg/L (day 0) to 1259.6 ± 68.6 mg/L after 30 days of digestion, corresponding to an increase of 220.8%. The FAN concentration in the digester with acid-treated samples significantly increased from 380.1 ± 37.2 mg/L (day 0) to 1217.4 ± 129.9 mg/L after digestion, corresponding to an increase of 220.3%; however, there was no difference for the $NH_3$-N concentration before and after digestion of steam-treated and acid-treated samples (Fig. 3 (D)).

These results illustrated that under thermophilic AD, temperature significantly affected the hydrolysis of proteins more so than the pretreatment method; this resulted in the increase in FAN concentration and subsequent inhibition of methanogenesis. This is in agreement with previous studies when using nitrogen-rich materials as substrates to produce methane by AD technology [55–57]. Lauterbock et al. [58] reported ammonia inhibition of the mesophilic AD of slaughterhouse wastewater, where the FAN concentration was 1000–1200 mg/L. Nakakubo et al. [59] reported that the thermophilic digestion of pig manure could be inhibited even with acclimatized inocula.

It should be pointed out that the thermophilic AD of untreated and pretreated WBM was not completely inhibited; this could be explained by the "inhibitory steady state" of AD, resulting from the interaction between FAN, VFAs, and pH, in which the AD process was running steadily but with a low methane production [60]. Li et al. [61] also reported an "inhibitory steady state" when using feedstocks rich in reducing sugars and proteins for anaerobic co-digestion, and the authors elucidated the inhibitory conditions for anaerobic methane production under different scenarios. Therefore, it may be concluded that ammonia inhibition, especially FAN inhibition, led to the low methane production in an inhibitory steady state from the WBM under thermophilic digestion.

### 3.3. Microbial community analysis

Free ammonia can affect the methanogenic pathways under mesophilic and thermophilic AD of nitrogen-rich feedstock such as chicken manure [62]. Therefore, microbial structure analysis by high-throughput sequencing was conducted to provide insights into the effect of pretreatment and digestion temperature on the digestion performance of the WBM.

#### 3.3.1. Bacterial structure in mesophilic and thermophilic digestion

Irrelevant of pretreatment and digestion temperature, the bacterial community was largely composed of the phyla *Firmicute* (65.3%-82.1%), *Cloacimonetes* (2.6%-10.3%), *Bacteroidetes* (0.9%-11.0%), and *Proteobacteria* (1.5%-8.4%); as can be seen in supplementary data. It is reported that *Firmicute* are mainly responsible for the degradation of organic matter, while *Proteobacteria* play an important role in the consumption of long-chain fatty acids and glucose [50,63].

As shown in Fig. 4, on genus level, the dominant bacteria (with relative abundance over 0.5%) were *Cloacamonas* (10.3%), *Ercella* (5.9%), *Syntrophaceticus* (2.7%), *Gallicola* (1.3%), and *Anaerobaculum* (1.2%) in the mesophilic digester of the untreated samples; this was similar to the bacterial community of the inocula. The relative abundance of genera *Syntrophaceticus*, *Gallicola,* and *Anaerobaculum* increased to 11.3%, 1.6%, and 4.1% in the steam-treated mesophilic digester, and 3.6%, 2.3%, and 4.5% in the acid-treated mesophilic digester, respectively; however, the relative abundance of genus *Cloacamonas* decreased to 2.4% and 4.2%, respectively.

*Ercella* (family *Ruminococcaceae*) function in converting carbohydrates to acetic acid, hydrogen, and succinic acid [25]. *Syntrophaceticus* (family *Thermoanaerobacteraceae*) are reported to play a crucial role in syntrophic acetate oxidation (SAO). Coupled with hydrogenotrophic methanogenesis (HM), the resulting $H_2$ and $CO_2$ from SAO can be converted into methane under a high acetate concentration [64,65]. The increased relative abundance of these genera may contribute to the increased methane production rate from AD of steam-treated and acid-treated samples in this study. *Gallicola* and *Anaerobaculum* are mentioned as playing a part in transforming sugars and amino acids to acetic acid, $H_2$, and $CO_2$ [66,67]. *Cloacamonas* (phyla *Cloacimonetes*) are believed to ferment amino acids and produce $H_2$ [68]. The varied relative abundance of these genera illustrated that the hydrolytic and

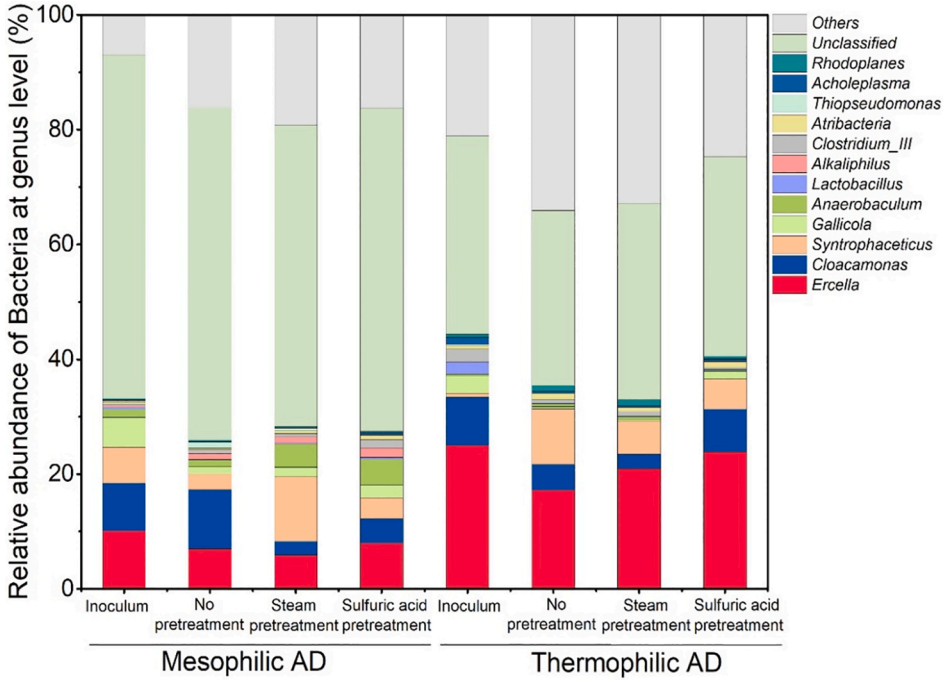


**Fig. 4.** Taxonomic classification of bacterial communities at the genus level under mesophilic and thermophilic anaerobic digestion (AD).

acidogenic pathways of carbohydrates and proteins may have shifted after steam/acid pretreatment.

The dominant bacteria in thermophilic digesters were different from those in mesophilic ones on the genus level. The bacteria mainly consisted of *Ercella* (17.2%), *Syntrophaceticus* (9.7%), and *Cloacamonas* (4.5%) in thermophilic digestion of untreated samples. The relative abundance of these bacteria was 20.9%, 5.7%, and 2.6% in the thermophilic steam-treated digester, and 23.9%, 5.3%, and 7.4% in thermophilic acid-treated digestion, respectively, indicating that increased digestion temperature favored the growth of *Ercella* and pretreatment negatively affected the SAO as the relative abundance of *Sytrophaceticus* decreased compared to that in mesophilic digesters. This may harm the efficiency of the SAO-HM pathway under standard conditions (Eqs. (3) to (5)), resulting in low biomethane production under thermophilic conditions in comparison to that under mesophilic conditions.

SAO-HM pathway:

$$\boldsymbol{SAO}: CH_3COO^- + H^+ + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad \Delta G = +94.78 kJ/mol \tag{3}$$

$$\boldsymbol{HM}: CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \Delta G = -130.69 kJ/mol \tag{4}$$

$$\boldsymbol{SAO-HM}: CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad \Delta G = -35.91 kJ/mol \tag{5}$$

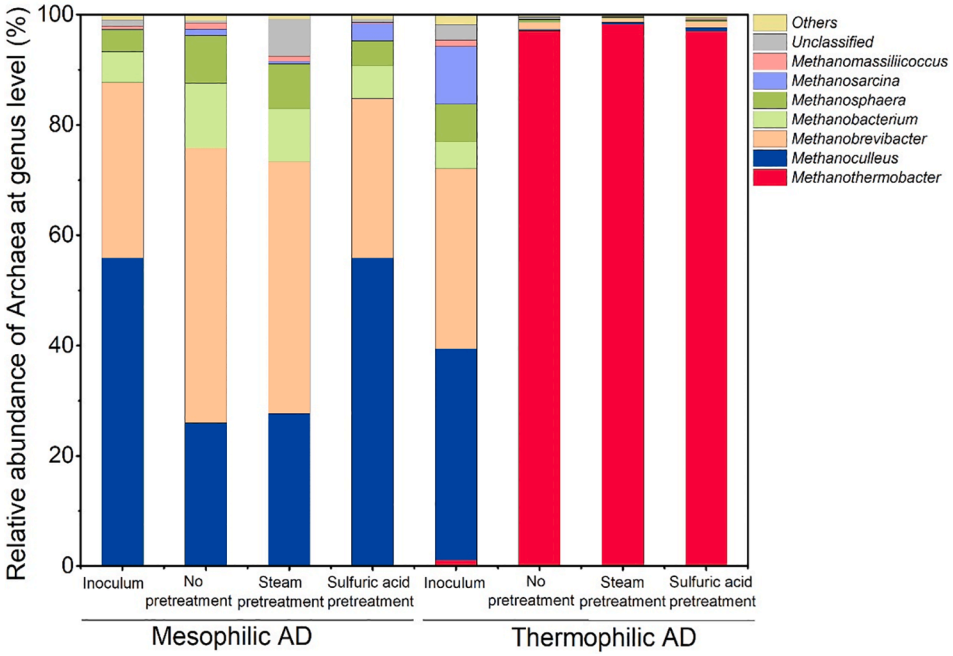


**Fig. 5.** Taxonomic classification of archaeal communities at the genus level under mesophilic and thermophilic anaerobic digestion (AD).

*3.3.2. Archaeal structure in mesophilic and thermophilic digestion*

The archaea composition in mesophilic digesters was similar to that in the initial inocula. The mesophilic archaeal community was composed of the genus *Methanoculleus*, *Methanobrevibacter*, *Methanobacterium,* and *Methanosphaera* (shown in Fig. 5). The relative abundance of these genera was 26.0%, 49.8%, 11.7%, and 8.7% in the mesophilic digester of untreated samples; and 27.6%, 45.7%, 9.6%, and 8.1% in the mesophilic digester of steam-treated samples. This indicates that the effect of steam pretreatment on the archaea community was relatively insignificant.

However, in the digester of acid-treated samples, the respective relative abundance of genera *Methanoculleus* and *Methanosarcina* increased to 55.9% and 3.1%, while the respective relative abundance of genera *Methanobrevibacter*, *Methanobacterium,* and *Methanosphaera* decreased to 28.9%, 5.9%, and 4.5%. This suggests that the dominant genus in methanogenesis of the WBM changed after acid pretreatment.

AM pathway:

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad \Delta G = -35.91 kJ/mol \tag{6}$$

*Methanosarcina* serve to convert acetate to methane via the aceto-clastic methanogenesis (AM) pathway (Eq. (6)) [69]. *Methanoculleus* are tolerant to high ammonia concentrations [70] and are an important and common hydrogenotrophic methanogenic species, typically accompanied by SAO bacteria under mesophilic conditions [53,71]. *Methanoculleus* can grow well at low hydrogen partial pressures [62,72]. *Methanobrevibacter*, *Methanobacterium,* and *Methanosphaera* (family *Mathanobacteriaceae*) were reported to produce methane through the reduction of $CO_2$ via $H_2$ [71–74]. The foregoing demonstrates that methanogenesis of untreated and steam-treated samples was dominated by hydrogenotrophic methanogens. The methanogenesis step for acid-treated digestate samples was also dominated by hydrogenotrophic methanogens but with the assistance of acetotrophic methanogens via the AM pathway. Ziganshin et al. [75] investigated the bacteria and archaea composition in mesophilic digestion of DDGS, a by-product from ethanol production; the results showed that *Methanoculles* were the dominant genus, indicating that hydrogenotrophic methanogenesis was the dominant pathway [75].

In thermophilic digesters, irrelevant of pretreatment methods, the genus *Methanothermobacter* (abundance 97.0% to 98.3% of the family *Mathanobacteriaceae*) predominated the archaea community while *Methanobrevibacter* (0.6%–1.2%) presented the minor genus (Fig. 5). *Methanothermobacter* are thermophilic methanogen that grow best at temperatures in the range of 55–65 °C; they use $CO_2$ and $H_2$ as substrates to produce methane [65] following the SAO as per Eqs. (3) to (5).

Similar findings were observed by other researchers when digesting high nitrogen content substrate [59]. In comparing the methanogenic pathway (using stable carbon isotope analysis) for methane production from mesophilic and thermophilic digestion of chicken manure, Yin et al. [62] found that under high FAN and VFAs concentrations, the hydrogenotrophic partner shifted in SAO under mesophilic and thermophilic conditions; the authors reported that *Methanoculles* (94%) were the important hydrogenotrophic partner in SAO in mesophilic AD, while *Methanothermobacter* (96%) were the important hydrogenotrophic partner in thermophilic AD [62]. The high level of genus *Methanothermobacter* in thermophilic digesters suggested this methanogen is of great importance in the SAO-HM pathway of the thermophilic AD process. This agreed with the present work that *Methanoculles* (26.0–55.9%) and *Methanobrevibacter* (28.9–49.8%) were important hydrogenotrophic partners in SAO in mesophilic AD, while *Methanothermobacter* (96%) were the important hydrogenotrophic partner in thermophilic AD. Based on the aforementioned discussion, it is plausible to propose the metabolic pathway of the digestion of WBM in this study, which is shown in Fig. 6.

## 4. Conclusions

Mesophilic and thermophilic AD of untreated and steam/sulfuric acid treated whiskey byproducts were compared. Both steam and acid pretreatment showed no promotional effect on biomethane production, but acid pretreatment speeded up the mesophilic AD process by 14.3% due to the release of more easily digestible sugars. The highest methane yield (389.1 ± 8.5 mL/g VS) was from the digestion of untreated samples, suggesting a high biological degradability of the raw material.

Thermophilic AD of whiskey byproducts was strongly inhibited by high free ammonia concentrations (over 1000 mg/L) and VFAs accumulation (over 4000 mg/L) due to the high protein content and low C/N ratio, resulting in a low methane yield and low sCOD removal efficiency.

Microbial community analysis revealed that the dominant methanogens in mesophilic AD were *Methanoculleus* (26.0–55.9% in abundance) and *Methanobrevibacter* (28.9–49.8% in abundance), while thermophilic AD was dominated by *Methanothermobacter* (97.0–98.3% in abundance); this indicated that methane produced from both mesophilic and thermophilic AD of the whiskey byproducts mixture was primarily through the hydrogenotrophic methanogenesis pathway.

## CRediT authorship contribution statement

**Xihui Kang:** Writing - original draft, Data curation, Formal analysis. **Richen Lin:** Conceptualization, Validation, Supervision, Writing - review & editing. **Lianhua Li:** Supervision, Writing - review & editing. **Benteng Wu:** Validation, Writing - review & editing. **Chen Deng:** Investigation, Writing - review & editing. **Richard O'Shea:** Validation, Writing - review & editing. **Yongming Sun:** Supervision, Funding acquisition, Writing - review & editing. **Jerry D. Murphy:** Validation,

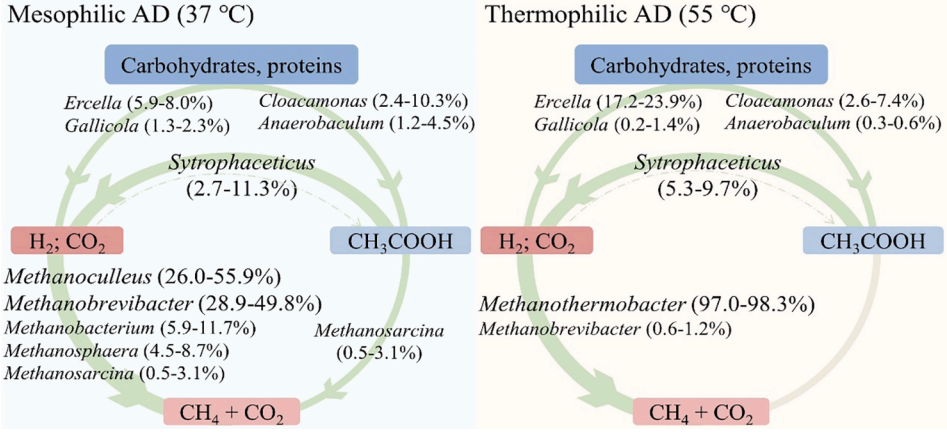


**Fig. 6.** Proposed metabolic pathways of mesophilic and thermophilic anaerobic digestion (AD) of whiskey byproducts.

Supervision, Funding acquisition, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.enconman.2021.114331.

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