1   # Understanding the fate and impact of capsaicin in anaerobic co-digestion of

2   # food waste and waste activated sludge

3   Mingting Du[a,b], Xuran Liu[a,b], Dongbo Wang[a,b] [*], Qi Yang[a,b], Abing Duan[a,b][*], Hong Chen[c], Yiwen Liu[d], Qilin

4   Wang[d], Bing-Jie Ni[d]

5   [a] College of Environmental Science and Engineering, Hunan University, Changsha 410082, P.R. China

6   [b] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education,

7   Changsha 410082, P.R. China

8   [c] Key Laboratory of Water-Sediment Sciences and Water Disaster Prevention of Hunan Province, School of

9   Hydraulic Engineering, Changsha University of Science & Technology, Changsha 410004, China

10   [d] Centre for Technology in Water and Wastewater, School of Civil and Environmental Engineering, University

11   of Technology Sydney, Sydney, NSW 2007, Australia

12   First author: Mingting Du (dumingting123@163.com)

13   Corresponding author: Dongbo Wang (w.dongbo@yahoo.com) and Abing Duan (duanabing@hnu.edu.cn)

14 ABSTRACT

15   Anaerobic co-digestion is an attractive option to treat food waste and waste activated sludge, which is

16 increasingly applied in real-world situations. As an active component in *Capsicum* species being

17 substantially present in food waste in many areas, capsaicin has been recently demonstrated to inhibit the

18 anaerobic co-digestion. However, the interaction between capsaicin and anaerobic co-digestion are still

19 poorly understood. This work therefore aims to deeply understand the fate and impact of capsaicin in the

20 anaerobic co-digestion. Experiment results showed that capsaicin was completely degraded in anaerobic

21 co-digestion by hydroxylation, O-demethylation, dehydrogenation and doubly oxidization, respectively.

22 Although methane was proven to be produced from capsaicin degradation, the increase in capsaicin

23 concentration resulted in decrease in methane yield from the anaerobic co-digestion. With an increase of

24 capsaicin from 2 ± 0.7 to 68 ± 4 mg/g volatile solids (VS), the maximal methane yield decreased from $274.6 \pm$

25 9.7 to 188.9 ± 8.4 mL/g VS. The mechanic investigations demonstrated that the presence of capsaicin

26 induced apoptosis, probably by either altering key kinases or decreasing the intracellular $NAD^+/NADH$ ratio,

27 which led to significant inhibitions to hydrolysis, acidogenesis, and methanogenesis, especially acetotrophic

28 methanogenesis. Illumina Miseq sequencing analysis exhibited that capsaicin promoted the populations of

29 complex organic degradation microbes such as *Escherichia-Shigella* and *Fonticella* but decreased the

30 numbers of anaerobes relevant to hydrolysis, acidogenesis, and methanogenesis such as *Bacteroide* and

31 *Methanobacterium*.

32 **Keywords:** Capsaicin; Food waste; Waste activate sludge; Anaerobic co-digestion; Methane production

33 1. Introduction

34   Food waste (FW) and waste activated sludge (WAS) are two major bio-solids in the world, which are

35 massively generated. It is documented that more than 1.3 billion tons FW are globally produced each year

36   (Li et al. 2020b, Xu et al. 2018), and the annul amount is estimated to achieve approximately 2.2 billion tons

37   by 2025 (Mehariya et al. 2018). In China, more than 90 million tons of FW is produced each year,

38   accounting for 37-62 % of municipal solid waste (Zhang et al. 2014). As for WAS, its amount in China is

39   over 40 million tons per year, and increases at an annual average rate of 4.75 % (Luo et al. 2020b). The total

40   amount in the world is estimated to increase to 103 million tons by 2025 (Yao et al. 2018).

41   FW and WAS contain substantial degradable substrates (e.g., proteins, carbohydrates, and lipids) and also

42   several recalcitrant or toxic materials such as cellulose, humus antioxidant, antibiotic, and heavy metals (Liu

43   et al. 2020a, Shah et al. 2005, Tao et al. 2020, Wang et al. 2019a). They will pose high risks to the

44   environment if they are not treated appropriately (Li et al. 2020a). As a mature technology to effectively

45   prevent pollution and recover energy concurrently, anaerobic digestion attracts much attention (Liu et al.

46   2020b, Luo et al. 2020a). Compared with the mono-digestion, anaerobic co-digestion of FW and WAS offers

47   complementary benefits such as substrate variability, toxicity dilution, and C/N ratio balance (Sole-Bundo et

48   al. 2017, Zhao et al. 2016). Therefore, it has been recently thought to be a very promising option and

49   growingly executed in field situations (Nghiem et al. 2017).

50   Capsaicin, a major pungent ingredient in varieties of capsicums, is widely used as food additives

51   throughout the world, especially in South East Asian, Latin-American countries and southwest of China

52   (Kurita et al. 2002, Surh and Lee 1995). For instance, the daily consumption of capsicum spices is 5

53   g/person in Thailand (Monsereenusorn 1983) and 20 g/person (one chili pepper) in Mexico (López-Carrillo et

54   al. 1994). The capsaicin content in capsicums usually ranges from 0.1 to 2.5 mg/g (Parrish 1996). This

55   indicates that capsaicin accumulation in FW could be up to 25 g/kg.

56   It is known that capsaicin has a wide variety of antimicrobial effects and can interact at primary sensory

57   neurons exerting the characteristic actions of excitation, desensitization and neurotoxicity (Kurita et al. 2002).

58   Due to this characteristic, several efforts have been carried out to assess the toxicity of capsaicin to

59   microorganisms.   Gu et al. (2019) showed that the minimum inhibitory concentrations of capsaicin on

60   *Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus*, and *Streptococcus sanguis* were 50, 50, 50, 25

61   μg/mL respectively.

62   As anaerobic co-digestion is a biological process with varieties of anaerobes being involved, the presence

63   of capsaicin might also affect these microbes.   Li et al. (2016) demonstrated that when the pungency degree

64   decreased from 1136.1 to 71.5 Scoville Heat Units (i.e., 11.1 to 0.7 g Capsaicin/kg Kitchen Waste), the

65   maximum methane production increased from 9.9 to 13.5 mL/ (g VS h), indicating the detrimental impact of

66   capsaicin on anaerobic digestion.   Yue et al. (2020) recently found that N-Vanillylnonanamide (one type of

67   capsaicin) inhibited anaerobic digestion of glycerol trioleate severely.   As N-Vanillynonanamid addition

68   increased from 0 to 40% wt, the methane yield decreased from 780.21 to 142.1 mL/g Total Volatile Solid.

69   The findings obtained by the previous investigations confirmed the detrimental effect of capsaicin and raised

70   our concerns in terms of the toxicity of capsaicin to anaerobic co-digestion.   Considering the massive

71   quantities of capsaicin-rich wastes treated daily, comprehensive and deep understanding the fate and impact of

72   capsaicin in anaerobic co-digestion is of significance to manipulate the co-digesters in field situations.

73   Despite the excellent work made by Li et al. and Yue et al., details of what happen in capsaicin-rich

74   co-digestion systems remain largely unknown.   For example, capsaicin as an organic, it is unclear about the

75   transformation and metabolism of capsaicin in the process of anaerobic co-digestion and its interaction with

76   anaerobes.   The contribution of capsaicin degradation to methane yield is also not clear.   Moreover, as

77   anaerobic co-digestion contains several processes such as disintegration, hydrolysis, acidogenesis,

78   acetogenesis, and methanogenesis, it is also unknown that the mechanism of capsaicin effects on each process.

79   By clarifying these questions, this work aims to deeply understand the fate and impact of capsaicin in

80   anaerobic co-digestion of FW and WAS. Firstly, the fate of capsaicin in the co-digesters was systematically

81   assessed. Then, the influence of capsaicin at different concentrations on methane production was

82   investigated. Finally, details of how capsaicin affects methane production were explored. To our

83   knowledge, this is the first study revealing the interaction between capsaicin and anaerobic co-digestion.

84   **2. Materials and methods**

85   **2.1 Source and characteristics of FW, WAS, and inocula.**

86   FW used in this work, which was mainly composed of rice, noodles, and vegetables, was withdrawn

87   from a cafeteria in Hunan University (Changsha, China). The indigestible substrates (e.g., inorganic

88   particles, bones, and chopsticks) were first removed before FW was crushed into small particles (1-3 mm) for

89   further use. The main characteristics of FW are as follows: pH 5.8 ± 0.1, total solids (TS) 121.3 ± 5.8 g/L,

90   volatile solids (VS) 116.1 ± 4.3 g/L, total chemical oxygen demand (TCOD) 142.3 ± 7.4 g/L, total proteins

91   31.2 ± 1.4 g COD/L, total carbohydrates 123.5 ± 5.7 g COD/L, and capsaicin content 4 ± 1 mg/g FW. In this

92   study, extra capsaicin, with purity being > 98%, was purchased from Hefei Bomei Biotechnology company.

93   WAS used in this work was taken from the secondary tank of a municipal wastewater treatment plant in

94   Changsha, China. WAS was first filtered with a 2 mm × 2 mm screen and then concentrated in 4 ℃

95   refrigerator for 24 h before use, and the main characteristics of WAS are as follows: pH 6.9 ± 0.1, TS 57.3 ±

96   2.6 g/L, VS 28.3 ± 1.2 g/L, TCOD 36.2 ± 1.6 g/L, total proteins 8.5 ± 0.3 g COD/L, and total carbohydrates

97   1.6 ± 0.1 COD/L.

98   Inocula applied in this work were harvested from a mesophilic anaerobic reactor in our laboratory, which

99   has been operated 130 days with solid retention time of 30 d and WAS as substrate. The main properties of

100   inoculated sludge are as follows: pH 7.2 ± 0.1, TS 46.7 ± 3.5 g/L, and VS 38.6 ± 2.3 g/L.

101   **2.2 Effect of capsaicin on methane production from anaerobic co-digestion.**

102   This batch test was performed in six identical serum bottles, and the working volume of each bottle was

103   500 mL.   Among them, one was set as the blank, and the other five were operated as the experimental

104   reactors.   According to the literature, the mixture ratio of FW to WAS used in this study was set as 1:1 on a

105   VS basis (Mehariya et al. 2018).   Firstly, 1.5 L mixture of FW and WAS was evenly divided into the five

106   experimental reactors, respectively.   Then, different amounts of extra capsaicin were added into the reactors,

107   which led to the initial capsaicin content of 2 ± 0.7, 8 ± 1.2, 20 ± 2.3, 36 ± 2.6, or 68 ± 4.1 mg/g VS.   It

108   should be emphasized that no extra capsaicin was added into the first experimental reactor, and 2 ± 0.7 mg/g

109   VS was the background value in the digestion mixture.   To improve the uniformity of capsaicin, all the

110   mixtures were pretreated in a water-bath shaker (120 rpm, 60 ℃) for 30 min (Huang et al. 2020).   When the

111   mixtures were cooled down to room temperature, each reactor received 50 mL same inocula, as mentioned

112   above.   The blank reactor, which merely contained 50 mL inocula and 300 mL of Milli-Q water, was also

113   conducted to test the methane productivity from the inocula alone.   All the reactors were flushed with high

114   purity nitrogen for 5 min to eliminate oxygen, sealed with rubber stoppers, and placed in an air-bath shaker

115   (120 rpm) at 35 ± 1℃ for 45 d.   pH value in all the reactors was maintained at 7.0 ± 0.1 during the whole

116   digestion period by 4 M hydrochloric acid or 4 M sodium hydroxide with automatic titrators.

117   In this work, all the tests, unless otherwise described, were operated in triplicate.   The data reported

118   below are net values, with the values determined in the blank reactor having been subtracted.   During the

119   entire digestion process, the yield of methane produced was determined periodically by releasing the pressure

120   in the serum bottle using a 300 mL glass syringe to equilibrate with the room pressure according to the

121   method documented in the literature (Liu et al. 2019), and the calculation of the cumulative volume of

122   methane was detailed in our previous publication (Wang et al. 2015).

123   **2.3 Methane production from capsaicin.**

124   Three replicates serum bottles with a working volume of 500 mL each were carried out to assess whether

125   capsaicin can be served as substrates to produce methane in the anaerobic co-digestion process.   Each reactor

126   received 50 mL same inocula.   Among them, one bottle receives 300 mL of Milli-Q water and was set as the

127   control, the other two bottles received 300 mL synthetic medium containing either 36 mg capsaicin/g VS or 68

128   mg capsaicin/g VS as the extra digested substrate.   All the operations were the same as depicted in Section

129   2.2.

130   2.4 Effect of capsaicin on solubilization, hydrolysis, acidogenesis, and methanogenesis.

131   This batch test was performed to assess the effect of capsaicin on digestion steps (i.e., solubilization,

132   hydrolysis, acidogenesis, acetoclastic methanogenesis and hydrogenotrophic methanogenesis) relevant to

133   methane production.   The effect of capsaicin on WAS solubilization was assessed in real WAS by comparing

134   the variations in soluble proteins and soluble polysaccharides in the presence of different capsaicin

135   concentrations, while the impact of capsaicin on other biological processes was evaluated in synthetic media

136   through comparing the specific degradation rates of the model substrates under different concentrations of

137   capsaicin according to the previous study (Wu et al. 2019).   In this test, fifteen replicate serum bottles were

138   performed and divided into five groups (namely Test-I, Test-II, Test-III, Test-IV, and Test-V) with three in

139   each.

140   Test-I: Three reactors with a working volume of 500 mL each were operated.   The three reactors first

141   received 300 mL mixture of FW and WAS, which capsaicin concentrations were 2 ± 0.7, 8 ± 1.2, 36 ± 2.6

142   mg/g VS, respectively.   After being pretreated at 60 ℃ for 30 min, each reactor was fed with 50 mL inocula.

143   All other conditions were the same as those described above.   By measuring the concentrations of soluble

144   proteins and carbohydrates in the initial 2 days, the effect of capsaicin on solubilization could be obtained.

145   Test-II: Three replicate anaerobic digestion reactors were operated.   Each reactor received 50 mL

146 identical inocula and 300 mL same synthetic wastewater containing 5.3 g dextran.   In the anaerobic

147 co-digestion of this study, carbohydrates are the dominate substrate used for hydrolysis, and the carbohydrates

148 in synthetic wastewater were similar to that in the co-digestion substrate.   Afterwards, 0, 11 and 18 mg

149 capsaicin were respectively added into the three reactors, which resulted in the initial capsaicin condition of 0,

150 30 and 50 mg/L, respectively.   All other conditions were the same as those described above.   By measuring

151 the specific degradation rate of dextran, the effect of capsaicin on hydrolysis process could be indicated.

152 　　Test-Ⅲ: This test was operated the same as described in Test-Ⅱ except that the substrate (i.e., dextran) in

153 synthetic wastewater was replaced by 2.7 g glucose, respectively.

154 　　Text-Ⅳ: The operation of this test was performed with the same approach as described in Test-Ⅱ except

155 that 1.25 g sodium acetate was employed to replace dextran in synthetic wastewater.

156 　　Test-V: In this test, three reactors were operated.   Each received 50 mL identical inocula and 300 mL

157 Milli-Q water containing 0, 11 or 18 mg capsaicin.   Afterwards, each reactor was flushed with a mixed gas

158 (40% hydrogen, 10% carbon dioxide and 50% nitrogen) for 5 min to ensure that each was full of synthetic

159 hydrogen-containing gas.   At last, all these reactors were capped with rubber stoppers, sealed, and placed in

160 an incubator (120 rpm) at $35 \pm 1℃$.

161 2.5 Model-based Analysis.

162 　　Methane production was simulated by the modified Gompertz equation (Eq(1)) (Lay et al. 1997), and

163 several kinetic parameters, e.g., Mm (maximum methsane yield potential, mL/g VS or mL/L), Rm (methane

164 production rate, mL/(g VS·d) or mL/d or mL/(L·d)), λ (lag phase time of methane production, d), and t

165 (digestion time, d) were calculated using Origin 7.0 software.

166 　　　　　　　　　　　　　　　　　　　　——　　　　　　　　　　　　(1)

167 　　The effect of capsaicin on each process of anaerobic co-digestion can be assessed by the inhibition

168 constant, which is obtained from Eq (2).

169 (2)

170 Where, X is the reaction rate, subindex "s" is the substrate, subindex "i" is the inhibitor, Ii is the

171 concentration of inhibitor (mg/L), and $K_{s,i}$ is the relevant inhibition constant (mg/L).

172 2.6 Analytical Methods.

173 The analyses of Total Suspended Solid (TSS), Volatile Suspended Solid (VSS), TCOD, and SCOD were

174 conducted in accordance with Standard Methods. Carbohydrate was measured by phenol-sulfuric method

175 with glucose as the standard. Protein was determined by the Lowry-Folin method with BSA as the standard.

176 The composition in biogas was analyzed by gas chromatograph equipped with a thermal conductivity detector

177 according to the method documented in the literature (Wang et al. 2019b).

178 The concentration of capsaicin was determined using HPLC according to the reference (Hwang et al.

179 2017). The samples were first centrifuged at 6500 rpm at 4 ℃ for 10 min and dried at 58 ℃ for 48 h by

180 Vacuum drying oven. Then, 0.2 g dried sample was weighed and dissolved in n-hexane. The dissolved

181 sample was shocked for 5 min before being placed into ultrasonic machine for 10 min. After the sample was

182 re-centrifuged at 2500 rpm for 5min, the normal hexane extract was filtered through 0.22 μm filterable

183 membrane and then injected into HPLC system (Agilent 1200, USA). The mobile phase was methyl alcohol

184 and deionized water (70:30, v/v) with a flow rate 1.0 mL/min. The absorbance was measured at 280 nm.

185 The major metabolic products of capsaicin in the digestion process were measured via liquid

186 chromatography-mass spectrometer/mass spectrometer (Jia et al. 2018). The metabolic products were

187 extracted using solid phase NH_2 Cartridge (6 mL, 500 mg sorbent) and then eluted with methanol. The

188 eluant was filtered through 0.22 μm filterable membrane and injected into LC-MS/MS (LC-MS/MS, Agilent

189 1290 series LC, 6460 Triple Quad LC/MS). The metabolic products were separated using a ZORBAX

190 RRHD Eclipse Plus C18 column (2.1×50mm, 1.8 μm) and the mass spectrometers were performed in

191 positive electron-spray ionization (ESI+) mode.   The samples preparation and determination method of

192 LC-MS/MS were detailed in Supplementary Information (SI).

193   For determining the activity of key enzymes, 25 mL sample was taken out from the reactors, cleaned

194 using 100 mM sodium phosphate buffer (pH 7.4), sonicated at 20 kHz at 4 ℃ for 10 min, and finally

195 centrifuged at 12000 rpm at 4 ℃ for 15 min to remove the debris.   The extracts were kept on the ice before

196 analyzing.   The relative activities of function enzymes (mch, F420, AK, PTA, Coenzyme A, acetyl-CoA

197 decarbonylase/synthase complex (ACDS) and Coenzyme M) relevant to methane production were analyzed

198 using previously reported methods (Grahame and DeMoll 1996, Li et al. 2015, Liu et al. 2015, Wang et al.

199 2018a).

200   The membrane fluidity of microbial cells was determined by flow cytometry using Annexin V-FITC

201 fluorescence dye according to the reference (Luo et al. 2016).   Briefly, the samples were centrifuged in a 50

202 mL tube at 6000 rpm for 5 min, with the pellet being suspended by pre-cooled phosphate buffer saline (PBS).

203 Then the suspension was heated at 60 ℃ in a water bath for 30 min and re-suspended by pre-cooled PBS.

204 After filtering the suspension using 500 Nylon mesh and stained, the samples were determined using BD

205 Biosciences AccuriC6 flow cytometer (Bacton Dickinson Immunocytometry Systems, San Jose, CA, USA),

206 with the staining process being available in SI.

207   For microbial community analysis, the collected samples were first centrifuged for 5 min at 10000 rpm.

208 Then, the total DNA was extracted from the samples using the Fast DNA kit (MoBio Laboratories) according

209 to the instruction from manufacture. The quantity and purity of DNA were analyzed with a Nanodrop

210 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).   The high-throughput sequencing (Illumina

211 Miseq) was conducted by Majorbio co., Ltd. (Shanghai, China).   The primers 515F

212   (5′-GTGCCAGCMGCCGCGG-3′) and 806R (5'-GGACTACHVGGGTWTCTAAT-3') targeting the V4

213   regions of bacterial 16S DNA genes were used.

214   **2.7 Statistical Analysis.**

215   All the batch experiments were performed in triplicate. An analysis of variance (ANOVA) with least

216   significant difference test was used to assess the significance of results, and $p < 0.05$ was considered

217   statistically significant.

218   **3. Results and discussion**

219   **3.1 Capsaicin variation in anaerobic co-digestion.**

220   Fig. 1A shows the variations in HPLC chromatogram of extracted mixtures taken from the reactor fed

221   with 68 ± 4.1 mg/g VS capsaicin at different digestion times. According to HPLC chromatogram tested with

222   the standard capsaicin, the absorption peak of capsaicin appears at near 4.5 min (Fig. S1). It can be seen that

223   the absorption peak gradually declined with the digestion time, which indicates that capsaicin was degraded in

224   the anaerobic co-digestion process. Quantitative calculation exhibits that the concentration of capsaicin in

225   digestion mixture also decreased with the digestion. With the digestion time increase from 0 to 9 d,

226   capsaicin concentration decreased from 68 ± 4.1 to 14.2 ± 2.4 mg/g VS. On 12 d digestion, less than 1 mg/g

227   VS capsaicin was measured in the system, suggesting that capsaicin was almost degraded in the co-digestion

228   completely. The results further suggests that the specific degradation rate of capsaicin in this co-digestion

229   system is 4.1 mg/(g VS)·d. Similar observations were also made on other reactors fed with other capsaicin

230   levels.

231   It is reported that capsaicin could be metabolized by crude enzyme preparations from body, midgut and

232   Malpighian tubule of *Helicoverpa armigera* and *H. assulta* (Zhu et al. 2020). Through a CO_2 Evolution Test,

233   Wang et al. (2014) demonstrated that capsaicin could be biodegraded by microbes and bio-converted to CO_2

234 and H_2O under aerobic conditions.　When a filamentous fungus i.e., *Aspergillus oryzae*, was incubated with

235 red pepper powder in potato dextrose broth, Lee et al. (2015a) found that capsaicin was degraded with five

236 metabolites being obtained.　Despite these significant researches, this is the first study proving the

237 degradation of capsaicin in anaerobic co-digestion of FW and WAS.

238　3.2 Identification of the Main Metabolites of Capsaicin Degradation.

239　　LC-MS/MS was used to identify the metabolites of capsaicin in anaerobic co-digestion process.　Since

240 the fragment ions of MS/MS spectrometry occurred at 10.496 min are the same to those produced from

241 standard capsaicin (Fig. S2), the peak occurred at 10.496 min is considered to be capsaicin.　On 0 d, one peak

242 was detected at 10.496 min (M) in LC/MS chromatogram extracted the sample withdrawn from the digester

243 fed with 68 ± 4.1 mg/g VS.　On 9 d, this peak decreased largely, and meanwhile three new peaks occurred,

244 with their retention time being 6.708 min (M1), 8.362 min (M2), and 12.331 min (M3), respectively (Fig. 2).

245 This confirms again that capsaicin was degraded in the anaerobic co-digestion and suggests that three major

246 metabolic products were generated from capsaicin degradation.　The proposed structure and details of

247 metabolites are shown in Table S1.

248　　To understand capsaicin biodegradation and identify these three metabolites, the precursor ions $[M + H]^+$,

249 $[M1 + H]^+$, $[M2 + H]^+$, $[M3 + H]^+$, and their fragmentation patterns were further determined.　The

250 precursor ion peak of $[M + H]^+$ at *m/z* 306 indicates that the molecular weight of capsaicin was 305 (Fig. 3A).

251 Besides, three fragment ions at *m/z* 137, 170, and 182 were also found in Fig. 3A.　The fragment ion at *m/z*

252 137 was reported to be vanillyl portion of capsaicin resulting from cleavage of C7-N8, while the product ions

253 at *m/z* 170 and 182 were respectively derived from fragmentation of alkyl chain at C7-N8 and C1-C7 (Reilly

254 et al. 2003).

255   Analogous to capsaicin (M), the molecular weight of M1 was 321 due to the precursor ion peak of

256   [M1+H]$^+$ at m/z 322 (Fig. 3B).   Apart from the precursor ion peak, there are other four fragment ions being

257   observed in Fig. 3B.   The presence of fragment ion at m/z 137 indicated that vanilloid ring of capsaicin was

258   not modified.   Shifts in the product ions at m/z from 182 and 170 to 198 and 186 may be owing to addition of

259   oxygen atom (16 amu) to the alkyl chain.   The product ion at m/z 181 was likely due to loss of hydroxyl

260   group from fragment ion at m/z 198 while the appearance of fragment ion at m/z 304 was probably due to loss

261   of terminal CH_2O from m/z 322.   Based on these analyses, M1 (m/z 322) was inferred to alkyl hydroxylated

262   metabolite of capsaicin, with its molecular structure being detailed in Fig. 3B.   It was reported that the alkyl

263   side chain of capsaicin was susceptible to enzymatic oxidation (Reilly and Yost 2006), and M1 was also

264   detected as a capsaicin metabolite by *Aspergillus oryzae* (Lee et al. 2015a).

265   Four fragment ions were observed in the MS/MS spectrum of M2 (Fig. 3C).   The precursor ion peak at

266   m/z 292 [M2+H]$^+$ indicated that the molecular weight of M2 was 291.   The appearance of fragment ion at

267   m/z 123 was probably owing to a net loss of 14 amu from the vanilloid ring (m/z 137) through demethylation

268   of methoxy group.   Gonzalez-Gil et al. (2019) demonstrated that tramadol and trimethoprim can transformed

269   to O-desmethyltramadol and 4-desmethyltrimethoprim by demethylation of methoxy groups in anaerobic

270   digestion.   The existences of fragment ions at m/z 170 and 182, however, suggested that the alkyl chain was

271   not modified.   Therefore, M2 was inferred to be an O-demethylation metabolite of capsaicin, which was

272   previously detected in capsaicin degradation catalyzed by P450 enzyme as well (Reilly et al. 2003).

273   The precursor ion peak at m/z 336 [M3+H]$^+$ indicated the molecular weight of M3 being 335 (Fig. 3D),

274   which was 30 amu larger than that of capsaicin.   It can be seen that the fragment ion at m/z 170 still existed,

275   suggesting that the alkyl chain at C7-N8 was unchanged.   However, the fragment ion at m/z 137 disappeared,

276   which suggested that the vanilloid portion of capsaicin was modified.   Two new fragment ions at m/z 184

277 and 140 were detected. The fragment ion at m/z 184 was likely produced by cleavage of alkyl chain at

278 N8-C9 of capsaicin, while the fragment ion at m/z 140 may be due to loss of -CH_2NO from the fragment ion

279 m/z 184. Thus, M3 was probably a doubly oxidized metabolite of dehydrogenated capsaicin, and this was

280 also determined in capsaicin bioconversion by *H. armigera* (Tian et al. 2019).

281 3.3 Could methane be produced from capsaicin degradation?

282 The analyses above showed that in anaerobic co-digestion, capsaicin was first degraded into M1, M2,

283 and M3 by hydroxylation, O-demethylation, dehydrogenation and doubly oxidization, respectively.

284 According to the literature, these three metabolites would be further hydrolyzed into amino and carboxyl

285 compounds by amidases, with the possible degradation pathway of capsaicin being proposed in Fig. 4 (Cho et

286 al. 2014, Wang et al. 2018, Yue et al. 2020). However, it remains unknown whether these metabolic

287 hydrolysates could be used for methane production in anaerobic co-digestion, requiring to be further clarified.

288 To figure out this possibility, one batch test was operated using either inocula or inocula + capsaicin as

289 the digestion substrate, with the experimental details being shown in Section 2.3. It was found that about

290 31.0 ± 0.9 mL methane was produced from the sole inocula whereas the corresponding value was 44.3 ± 1.3

291 mL from inocula + 36 mg capsaicin/g VS and 59.9 ± 1.8 mL from inocula + 68 mg capsaicin/g VS (Fig. S3).

292 This indicates that capsaicin could be served as substrate for methane production in anaerobic digestion.

293 According to Eq (3), it can be calculated that 0.59 g (or 826 mL) of methane will be produced from 1 g

294 capsaicin if it is completely digested, suggesting that 60 and 87 mL methane should be generated from inocula

295 + 36 mg capsaicin/g VS and inocula + 68 mg capsaicin/g VS, respectively. However, the measured data

296 from the two inocula + capsaicin digesters were much lower than the theoretical values. The possible

297 reasons might be due to 1) incomplete utilization of capsaicin degradation intermediates and 2) inhibition of

298 capsaicin to the activity of inocula.

299    $C_{18}H_{27}NO_3 + 10.5\ H_2O \rightarrow 11.25\ CH_4 + 6.75\ CO_2 + NH_3$ (3)

300   3.4 Effect of capsaicin on methane production.

301   The cumulative methane yield during anaerobic co-digestion in the presence of capsaicin at different

302   concentrations is shown in Fig. 5.   It can be observed that methane yield in the reactor without extra

303   capsaicin addition (i.e., 2 ± 0.7 mg/g VS capsaicin reactor) increased with the digestion time from 1 to 34 d,

304   and no signification increase was observed after that day 34 (p > 0.05).   The optimum digestion time for this

305   reactor was therefore 34 d, with the maximum methane yield of 274.6 ± 9.7 mL/g of VS being measured.

306   From Fig. 5, it was also found the increase of capsaicin affected methane production obviously.   With an

307   increase of capsaicin concentration from 2 ± 0.7 to 68 ± 4.1 mg/g VS, methane yield decreased from $274.6 \pm$

308   9.7 to 188.9 ± 8.4 mL/g VS.   Apart from methane yield, the optimum digestion time was also affected by

309   capsaicin.   For example, 68 ± 4.1 mg/g VS of capsaicin increased the optimum digestion time to 58 d.

310   To further understand the effect of capsaicin on anaerobic digestion from the aspect of model, the

311   modified Gompertz equation was employed to estimate three kinetic parameters, i.e., maximum methane yield

312   potential (*Mm*), methane production rate (*Rm*), and lag phase time (λ).   It can be found that the Gompertz

313   equation described the experimental data well, with $R^2 > 0.99$ in all the scenarios (Fig. 5).   When capsaicin

314   concentration increased from 2 ± 0.7 to 68 ± 4.1 mg/g VS, the determined *Mm* and *Rm* decreased respectively

315   from 257.4 mL/g VS and 17.8 mL/(g VS·d) to 189.9 mL/g VS and 6.6 mL/(g VS·d), whereas the calculated λ

316   increased from 6.7 to 14.7 d.   This indicates that the increase of capsaicin not only decreased biochemical

317   methane potential but also inhibited the rate of methane production.

318   According to the analyses above, it was demonstrated that the addition of capsaicin reduced rather than

319   improved methane yield, though the addition of capsaicin provided extra substrate for anaerobes to produce

320   methane.   This suggests that anaerobic co-digestion from original substrates was largely inhibited by

321 capsaicin, which was in accordance with the results reported previously (Yue et al. 2020). Based on the

322 experimental data obtained in the current work, it was estimated that 50% inhibitory concentration of

323 capsaicin to anaerobic co-digestion was around 36.26 mg capsaicin/g VS (Fig. S4). Since anaerobic

324 co-digestion includes several processes such as solubilization, hydrolysis, acidogenesis, and methanogenesis,

325 the mechanism of how capsaicin affects methane production were explored in the following text.

326 3.5 Mechanism of How Capsaicin Inhibits Anaerobic Co-digestion.

327 It was found that the concentrations of soluble proteins and carbohydrates increased with capsaicin

328 concentration (Fig. 6A and Fig. 6B). For example, the concentrations of soluble proteins and carbohydrates

329 in the reactor fed with 2 ± 0.7 mg/g VS capsaicin were respectively 882.7 ± 23.2 and 2695.4 ± 25.5 mg

330 COD/L, while the corresponding concentrations in the reactor fed with 68 ± 4.1 mg/g VS capsaicin were

331 1505.8 ± 29.1 and 5530.2 ± 53.2 mg COD/L, respectively. According to the literature, an increase in the

332 fluorescence intensity indicates an increase in soluble organics in digestion liquid (Fig. 6C) (Xu et al. 2017).

333 There are two peaks, which are respectively located at Ex/Em of 200-250/290-320 and 250-280/<380 nm,

334 being found in all the samples (Fig. 6C). The fluorescence intensity of both the peaks increased with the

335 addition of capsaicin, suggesting that an increase of capsaicin enhanced increases in soluble organics. All

336 these facts confirmed that the presence of capsaicin improved rather than reduced solubilization, indicating

337 that capsaicin may inhibit the bio-processes involved in anaerobic co-digestion.

338 Table 1 summarizes the experiment value and model-simulation value of inhibition of capsaicin on

339 dextran, glucose, acetate and H_2. It can be seen that the specific degradation rates of dextran, glucose, acetate,

340 and hydrogen in the blank (i.e., 0 mg/L capsaicin) were respectively 8.07 ± 0.26, 7.00 ± 0.30, 2.85 ± 0.14, and

341 0.24 ± 0.01 mg/g VS·h, and these values were considered the original activities of microbes relevant to

342 hydrolysis, acidogenesis, acetotrophic methanogenesis, and hydrogenotrophic methanogenesis, respectively.

343 When 50 mg/L capsaicin was added, these values decreased respectively to 6.19 ± 0.62, 3.30 ± 0.66, $1.12 \pm$

344 0.03, and 0.17 ± 0.02 mg/g VS·h.   This suggests 50 mg/L capsaicin reduced the relative activities of

345 microbes (expressed as % of the original) relevant to hydrolysis, acidogenesis, acetotrophic methanogenesis,

346 and hydrogenotrophic methanogenesis by 23%, 27%, 61%, and 29%, respectively.   Similar observations

347 were also made at 30 mg/L capsaicin.

348 From Table 1, it was also found that inhibition constant ($K_{s,i}$) of capsaicin to the degradation of these

349 substrates was in the order of acetate > H_2 > glucose > dextran, suggesting that the inhibition of capsaicin to

350 these bioprocesses was in the sequence of acetotrophic methanogenesis > hydrogenotrophic methanogenesis >

351 acidogenesis > hydrolysis.   All the results showed that although capsaicin enhanced solubilization, it

352 significantly inhibited the bioprocesses of hydrolysis, acidogenesis, and methanogenesis, especially

353 acetotrophic methanogenesis.   It can be understood why capsaicin inhibited methane production from

354 anaerobic co-digestion.

355 It was reported that capsaicin could induce apoptosis by either altering key kinases (Pramanik and

356 Srivastava 2012), or decreasing the intracellular NAD^+/NADH ratio by binding to quinone binding site of

357 NADH dehydrogenase 1 (NDH-1) (Lee et al. 2015b, Yagi 1990).   Furthermore, Torrecillas et al. (2015)

358 found capsaicin molecule could establish a molecular interaction with cell membrane, where the nine-carbon

359 alkyl chain of capsaicin was aligned with the phospholipid acyl chains, perturbing the cooperative behavior of

360 the phospholipid and inducing apoptosis.   When capsaicin entered into the anaerobic co-digestion systems, it

361 could contact with the membrane and key enzymes of anaerobes, or even enter into the intracellular cells.

362 These behaviors may result in inactivation of functional enzymes, reduction in conversion between NAD^+ and

363 NADH, or even cell lysis (Fig. 7A).

364   This deduction can be supported by flow cytometry, which is usually used to reflect the cell functional

365   state.   The physiological status of microorganism cells can be divided into four regions.   Among them,

366   viable cells are shown in AV^-/PI^- (Q_4), early apoptotic cells are shown in AV^+/PI^- (Q_3), necrotic (or late

367   apoptotic) cells are shown in AV^+/PI^+ (Q_2), and debris and damaged cells are shown in AV^-/PI^+ (Q_1).   It can be

368   seen that with an increase of capsaicin from 0 to 50 mg/L the fluorescence percentage of viable cells

369   decreased from 96.7% to 78.7%, while the fluorescence percentage of early apoptotic cells increased from 0.9%

370   to 16.0% (Fig. 7B). This is the major reason for capsaicin enhancing solubilization but reducing hydrolysis,

371   acidogenesis and methanogenesis.

372   According to the data shown in Table 1, it can be found that the inhibition of capsaicin to acetotrophic

373   methanogenesis, one major pathway responsible for methane production, was much severer than that to

374   hydrogenotrophic methanogenesis, the other methane production pathway.   Thus, one might want to know

375   why capsaicin caused different inhibitions to these two methane production pathways.   In acetotrophic

376   methanogenesis, acetate can be either converted into acetyl-phosphate and acetyl-CoA catalyzed by AK and

377   PTA in turn or directly degraded to acetyl-CoA catalyzed by CoA.   The generated acetyl-CoA is then

378   converted into 5-methyl-THMPT under the catalysis of ACDS.   In hydrogenotrophic methanogenesis, H_2 and

379   CO_2 can be converted into 5-methyl-THMPT, with mch and F_{420} being as the key enzymes (Fig. 8A).   As the

380   same intermediate in the two pathways, 5-methyl-THMPT then undergoes the succession steps of

381   5-methyl-THMPT $\rightarrow$ methyl-CoM $\rightarrow$ CH_4.   It was found that although the presence of capsaicin inhibited

382   the activity of all these enzymes in acetotrophic and hydrogenotrophic methanogenesis, its inhibition to CoA

383   and ACDS was much severer than that to others (Fig. 8B).   This may explain why capsaicin caused severer

384   inhibition to acetotrophic methanogenesis than hydrogenotrophic methanogenesis.

385   When ACDS catalyzes the step of acetyl-CoA to 5-Methyl-THMPT, two electrons would be generated

386   (Grahame and DeMoll 1996).   In anaerobic digestion, the generated electrons are generally transferred to

387   ferredoxin by 4Fe-4S clusters ligands on ACDS (Ferry 2011).   Previous publication demonstrated that

388   capsaicin could be activated to an electrophilic intermediate, and this intermediate could compete for the

389   electrons available for ferredoxin utilization (Fig. 8C) (Surh and Lee 1995).   This causes reductions in the

390   electrons transferred by 4Fe-4S clusters ligands on ACDS, which might be the reason for capsaicin severely

391   inhibiting the activity of ACDS.   Moreover, it is reported that the amino group of capsaicin can be subjected

392   to dehydration condensation reaction with carboxyl group of CoA, inhibiting the metabolic activity of CoA

393   (Yue et al. 2020).

394   **3.6 Effect of capsaicin on microbial community.**

395   Illumina Miseq 16S-rRNA genes sequencing was performed to investigate the effect of capsaicin on

396   microbial community by comparing the structure and abundance of microbial community between the control

397   and experimental reactors.   The control reactor was fed with 2 mg/g VS capsaicin co-substrates, while the

398   experimental reactor was fed with 68 mg/g VS capsaicin co-substrates.   The number of operational

399   taxonomic units was 1074 in the control reactor and 1050 in the experimental digester, with 886 being shared

400   (Fig. S5).   The Alpha diversity results (Table S2) showed that PD index was similar in the two reactors,

401   while Chao index in the control was greater than that in the experiment reactor, suggesting that the increase of

402   capsaicin reduced the microbial diversity but did not change largely the microbial structure.

403   At the phylum level, the most predominant bacteria in the two reactors were *Firmicutes*, *Bacteroidetes*,

404   *Proteobacteria*, and *Actinobacteria* (Fig. S6).   It is reported that several anaerobes in these phyla can degrade

405   organic compounds (e.g., proteins and carbohydrates) (Wei et al. 2019), and many bacteria affiliated to

406   *Firmicutes* and *Proteobacteria* were volatile fatty acid producers (Wang et al. 2017).   *Euryarchaeota* was the

407 only archaeal phylum in the two reactors (Fig. S6), which was known as methanogens and detected in several

408 anaerobic digesters (Wei et al. 2019).

409   Fig. 9 displays the genus-level distributions of bacteria and archaea abundances in the two reactors.

410 Seven genera of bacteria, which were responsible for hydrolysis and acidogenesis, were detected in the two

411 reactors.   For example, the abundance of *Bacteroide*, which was reported to degrade carbohydrates and

412 organic acids (Pang et al. 2020), was measured to be 17.6% in the control, but it was completely washed out

413 in the experiment reactor.   The abundances of *Dysgonomonadacea*, *Petrimonas*, and *Macellibacteroides*,

414 which were able to degrade various polysaccharides and proteins (Maspolim et al. 2015, Murakami et al. 2018,

415 Xu et al. 2019), reduced from 6.5%, 5.3%, and 7.9% in the control to 3.2%, 2.4%, and 1.2% in the experiment

416 reactor, respectively (Fig. 9A).   Further calculation showed that the total abundance of these genera was 42.6%

417 in the control and 14.8% in the experiment reactor, indicating that capsaicin reduced largely the number of

418 anaerobes relevant to hydrolysis and acidogenesis.

419   It can be also seen from Fig. 9A that the abundance of *Escherichia-Shigella*, a resistant genus having

420 ability to degrade aromatic organic pollutants (Cui et al. 2017, Vasiliadou et al. 2018), increased from 1.0% in

421 the control to 10.7% in the experiment reactor.   Moreover, the abundance of several other contaminant

422 degradation microbes such as *Fonticella*, *Pirellulaceae*, *Enterococcus* and *Herbinix* (Cao et al. 2018, Fraj et al.

423 2013, Koeck et al. 2016, Tong et al. 2017), increased with capsaicin addition as well.   The total abundance of

424 potential complex organic degradation microbes increased from 20.7% in the control to 42.4% in the

425 experiment reactor, suggesting that these microbes might be capsaicin decomposers in such anaerobic

426 co-digestion systems.

427   Fig. 9B shows the distribution of archaea community in the two reactors.   The total sequences of

428 methanogens decreased from 16313 in the control to 7592 in the experiment reactor, suggested that the

429 addition of capsaicin reduced the total archaea populations. Among them, the abundance of

430 *Methanobacterium*, decreased from 73.4% in the control to 64.7% in the experiment reactor. However, the

431 abundances of *Methanobrevibacter* and *Methanosphaera* increased from 2.0% and 0.2% in the control to 9.1%

432 and 2.2%, respectively. This suggest that the effect of capsaicin on different types of archaea is different,

433 and manipulation of co-digesters to enrich *Methanobrevibacter* and *Methanosphaera* could effectively

434 mitigate the inhibition of capsaicin to methane production. The accurate reason for this different impact is

435 unclear at the current stage, more efforts are required in the future.

436 **4. Conclusions**

437   This study evaluated the degradation of capsaicin in anaerobic co-digestion of FW and WAS and

438 explored the effect of capsaicin on methane production as well as the underlying mechanisms of capsaicin

439 affecting methane yield. The findings obtained not only advance the understanding of capsaicin-involved

440 digestion but also guide engineers to develop effective strategies in the future to manipulate anaerobic

441 co-digestion of FW and WAS. The main conclusions are:

442   (1) Capsaicin can be degraded by some microbe and used as substrate to produce methane.

443   HPLC-MS/MS analysis that hydroxylation, O-demethylation, dehydrogenation and doubly

444   oxidization are involved in capsaicin degradation.

445   (2) The presence of capsaicin not only slowed the process of anaerobic co-digestion but also decreased

446   methane yield. With the increase of capsaicin from $2 \pm 0.7.$ to 68 ± 4 mg/g VS, the maximal methane

447   yield decreased from 274.6 ± 9.7 to 188.9 ± 8.4 mL/g VS, while the methane production rate

448   decreased from 17.76 to 6.63 mL/(g VS·d).

449   (3) Mechanism investigations revealed that the presence of capsaicin induced apoptosis, which led to

450   significant inhibitions to hydrolysis, acidogenesis, and methanogenesis, especially acetotrophic

451   methanogenesis.

452   (4) The presence of capsaicin enhanced the populations of complex organic degradation microbes such

453   as *Escherichia-Shigella* and *Fonticella* but decreased the numbers of anaerobes relevant to hydrolysis,

454   acidogenesis, and methanogenesis such as *Bacteroide* and *Methanobacterium*.

458   Appendix A. Supplementary data

459   This file contains Text S1-S3, Table S1-S2 and Fig. S1-S6.

460

References

461 **References**

462 Cao, J., Wang, C., Dou, Z., Liu, M. and Ji, D. (2018) Hyphospheric impacts of earthworms and arbuscular mycorrhizal fungus on soil

463   bacterial community to promote oxytetracycline degradation. J. Hazard. Mater. 341, 346-354.

464   Cho, S., Moon, H.-I., Hong, G.-E., Lee, C.-H., Kim, J.-M. and Kim, S.-K. (2014) Biodegradation of capsaicin by Bacillus

465   licheniformis SK1230. J. Korean. Soc. Appl. Bi. 57(3), 335-339.

466 Cui, D., Shen, D., Wu, C., Li, C., Leng, D. and Zhao, M. (2017) Biodegradation of aniline by a novel bacterial mixed culture AC. Int.

467   Biodeter. Biodegr. 125, 86-96.

468   Ferry, J.G. (2011) Fundamentals of methanogenic pathways that are key to the biomethanation of complex biomass. Curr. Opin.

469   Biotechnol. 22(3), 351-357.

470   Fraj, B., Ben Hania, W., Postec, A., Hamdi, M., Ollivier, B. and Fardeau, M.-L. (2013) Fonticella tunisiensis gen. nov., sp. nov.,

471   isolated from a hot spring. Int. J. Evol. Microbiol. 63(Pt_6), 1947-1950.

472   Gonzalez-Gil, L., Krah, D., Ghattas, A.K., Carballa, M., Wick, A., Helmholz, L., Lema, J.M. and Ternes, T.A. (2019)

473   Biotransformation of organic micropollutants by anaerobic sludge enzymes. Water Res. 152, 202-214.

474   Grahame, D.A. and DeMoll, E. (1996) Partial reactions catalyzed by protein components of the acetyl-CoA decarbonylase synthase

475   enzyme complex from Methanosarcina barkeri. J. Biol. Chem. 271(14), 8352-8358.

476 Gu, H., Yang, Z., Yu, W., Xu, K. and Fu, Y.-f. (2019) Antibacterial Activity of Capsaicin against Sectional Cariogenic Bacteria. Pak. J.

477   Zool. 51(2).

478   Huang, X., Liu, X., Chen, F., Wang, Y., Li, X., Wang, D., Tao, Z., Xu, D., Xue, W., Geng, M. and Yang, Q. (2020) Clarithromycin

479   affect methane production from anaerobic digestion of waste activated sludge. J. Clean. Prod. 255, 120321.

480   Hwang, I.M., Choi, J.Y., Nho, E.Y., Lee, G.H., Jamila, N., Khan, N., Jo, C.H. and Kim, K.S. (2017) Characterization of Red Peppers

481   (Capsicum annuum) by High-performance Liquid Chromatography and Near-infrared Spectroscopy. Anal. Lett. 50(13),

482   2090-2104.

483   Jia, Y., Khanal, S.K., Shu, H., Zhang, H., Chen, G.H. and Lu, H. (2018) Ciprofloxacin degradation in anaerobic sulfate-reducing

484   bacteria (SRB) sludge system: Mechanism and pathways. Water Res. 136, 64-74.

485   Koeck, D.E., Hahnke, S., Zverlov, V.V. and Zverlov, V.V. (2016) Herbinix luporum sp. nov., a thermophilic cellulose-degrading

486   bacterium isolated from a thermophilic biogas reactor. Int. J. Syst. Evol. Microbiol. 66(10), 4132-4137.

487 Kurita, S., Kitagawa, E., Kim, C.H., Momose, Y. and Iwahashi, H. (2002) Studies on the antimicrobial mechanisms of capsaicin using

488   yeast DNA microarray. Biosci. Biotechnol. Biochem. 66(3), 532-536.

489   López-Carrillo, L.; Hernández Avila, M. and Dubrow, R. (1994) Chili pepper consumption and gastric cancer in Mexico: a

490   case-control study. Am. Epidemiol. 139(3), 263-271.

491   Lay, J.-J., Li, Y.-Y. and Noike, T. (1997) Influences of pH and moisture content on the methane production in high-solids sludge

492   digestion. Water Res. 31(6), 1518-1524.

493   Lee, M., Cho, J.-Y., Lee, Y.G., Lee, H.J., Lim, S.-I., Park, S.-L. and Moon, J.-H. (2015a) Bioconversion of Capsaicin by Aspergillus

494   oryzae. J. Agr. Food. Chem. 63(26), 6102-6108.

495   Lee, Y.-H., Chen, H.-Y., Su, L.J. and Chueh, P.J. (2015b) Sirtuin 1 (SIRT1) Deacetylase Activity and NAD+/NADH Ratio Are

496   Imperative for Capsaicin-Mediated Programmed Cell Death. J. Agr. Food. Chem. 63(33), 7361-7370.

497   Li, Y., Xu, Q., Liu, X., Wang, Y., Wang, D., Yang, G., Yuan, X., Yang, F., Huang, J. and Wu, Z. (2020a) Peroxide/Zero-valent iron (Fe0)

498   pretreatment for promoting dewaterability of anaerobically digested sludge: A mechanistic study. Journal of Hazardous Materials

499   400, 123112.

500   Li, Y., Zhu, Y., Wang, D., Yang, G., Pan, L., Wang, Q., Ni, B.-J., Li, H., Yuan, X., Jiang, L. and Tang, W. (2020b) Fe(II) catalyzing

501   sodium percarbonate facilitates the dewaterability of waste activated sludge: Performance, mechanism, and implication. Water

502   Research 174, 115626.

503   Li, X., Chen, Y., Zhao, S., Chen, H., Zheng, X., Luo, J. and Liu, Y. (2015) Efficient production of optically pure L-lactic acid from

504   food waste at ambient temperature by regulating key enzyme activity. Water Res. 70, 148-157.

505   Li, Y., Jin, Y., Li, J., Li, H. and Yu, Z. (2016) Effects of pungency degree on mesophilic anaerobic digestion of kitchen waste. Appl.

506   Energ. 181, 171-178.

507   Liu, K., Chen, Y., Xiao, N., Zheng, X. and Li, M. (2015) Effect of humic acids with different characteristics on fermentative

508   short-chain fatty acids production from waste activated sludge. Environ. Sci. Technol. 49(8), 4929-4936.

509   Liu, X., Du, M., Yang, J., Wu, Y., Xu, Q., Wang, D., Yang, Q., Yang, G. and Li, X. (2020a) Sulfite serving as a pretreatment method for

510   alkaline fermentation to enhance short-chain fatty acid production from waste activated sludge. Chem. Eng. J. 385.

511   Liu, X., Huang, X., Wu, Y., Xu, Q., Du, M., Wang, D., Yang, Q., Liu, Y., Ni, B.-J., Yang, G., Yang, F. and Wang, Q. (2020b) Activation

512   of nitrite by freezing process for anaerobic digestion enhancement of waste activated sludge: Performance and mechanisms.

513   Chemical Engineering Journal 387, 124147.

514   Liu, X., Xu, Q., Wang, D., Wu, Y., Yang, Q., Liu, Y., Wang, Q., Li, X., Li, H., Zeng, G. and Yang, G. (2019) Unveiling the mechanisms

515   of how cationic polyacrylamide affects short-chain fatty acids accumulation during long-term anaerobic fermentation of waste

516   activated sludge. Water Res. 155, 142-151.

517   Luo, J., Chen, Y. and Feng, L. (2016) Polycyclic Aromatic Hydrocarbon Affects Acetic Acid Production during Anaerobic

518   Fermentation of Waste Activated Sludge by Altering Activity and Viability of Acetogen. Environ. Sci. Technol. 50(13),

519   6921-6929.

520 Luo, J., Huang, W., Guo, W., Ge, R., Zhang, Q., Fang, F., Feng, Q., Cao, J. and Wu, Y. (2020a) Novel strategy to stimulate the food

521   wastes anaerobic fermentation performance by eggshell wastes conditioning and the underlying mechanisms. Chemical

522   Engineering Journal 398, 125560.

523 Luo, J., Zhang, Q., Zhao, J., Wu, Y., Wu, L., Li, H., Tang, M., Sun, Y., Guo, W., Feng, Q., Cao, J. and Wang, D. (2020b) Potential

524   influences of exogenous pollutants occurred in waste activated sludge on anaerobic digestion: A review. J. Hazard. Mater. 383,

525   121176.

526 Maspolim, Y., Zhou, Y., Guo, C., Xiao, K. and Ng, W.J. (2015) The effect of pH on solubilization of organic matter and microbial

527   community structures in sludge fermentation. Bioresour. Technol. 190, 289-298.

528 Mehariya, S., Patel, A.K., Obulisamy, P.K., Punniyakotti, E. and Wong, J.W.C. (2018) Co-digestion of food waste and sewage sludge

529   for methane production: Current status and perspective. Bioresour. Technol. 265, 519-531.

530 Monsereenusorn, Y. (1983) Subchronic toxicity studies of capsaicin and capsicum in rats. Res. Commun. Chem. Pathol. Pharmacol.

531   41(1), 95-110.

532 Murakami, T., Segawa, T., Takeuchi, N., Barcaza Sepulveda, G., Labarca, P., Kohshima, S. and Hongoh, Y. (2018) Metagenomic

533   analyses highlight the symbiotic association between the glacier stonefly Andiperla willinki and its bacterial gut community.

534   Environ. Microbiol. 20(11), 4170-4183.

535 Nghiem, L.D., Koch, K., Bolzonella, D. and Drewes, J.E. (2017) Full scale co-digestion of wastewater sludge and food waste:

536   Bottlenecks and possibilities. Renew. Sust. Energ. Rev. 72, 354-362.

537 Pang, H., Ma, W., He, J., Pan, X., Ma, Y., Guo, D., Yan, Z. and Nan, J. (2020) Hydrolase activity and microbial community dynamic

538   shift related to the lack in multivalent cations during cation exchange resin-enhanced anaerobic fermentation of waste activated

539   sludge. J. Hazard. Mater. 398, 122930.

540 Parrish, M.J.J.o.A.I. (1996) Liquid Chromatographic Method for Determining Capsaicinoids in Capsicums and Their Extractives:

541   Collaborative Study. J. Aoac. Int. 79(3), 738-745.

542   Pramanik, K.C. and Srivastava, S.K. (2012) Apoptosis Signal-Regulating Kinase 1ΓÇôThioredoxin Complex Dissociation by

543   Capsaicin Causes Pancreatic Tumor Growth Suppression by Inducing Apoptosis. Antioxid. Redox. Sign. 17(10), 1417-1432.

544   Reilly, C.A., Ehlhardt, W.J., Jackson, D.A., Kulanthaivel, P., Mutlib, A.E., Espina, R.J., Moody, D.E., Crouch, D.J. and Yost, G.S.

545   (2003) Metabolism of capsaicin by cytochrome P450 produces novel dehydrogenated metabolites and decreases cytotoxicity to

546   lung and liver cells. Chem. Res. Toxicol. 16(3), 336-349.

547   Reilly, C.A. and Yost, G.S. (2006) Metabolism of Capsaicinoids by P450 Enzymes: A Review of Recent Findings on Reaction

548   Mechanisms, Bio-Activation, and Detoxification Processes. Drug Metab. Rev. 38(4), 685-706.

549 Shah, A.G., Pierson, J.A. and Pavlostathis, S.G. (2005) Fate and effect of the antioxidant ethoxyquin on a mixed methanogenic culture.

550   Water Res. 39(17), 4251-4263.

551 Sole-Bundo, M., Eskicioglu, C., Garfi, M., Carrere, H. and Ferrer, I. (2017) Anaerobic co-digestion of microalgal biomass and wheat

552   straw with and without thermo-alkaline pretreatment. Bioresour. Technol. 237, 89-98.

553 Surh, Y.J. and Lee, S.S. (1995) Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential. Life Sci.

554   56(22), 1845-1855.

555 Tao, Z., Wang, D., Yao, F., Huang, X., Wu, Y., Du, M., Chen, Z., An, H., Li, X. and Yang, Q. (2020) The effects of thiosulfinates on

556   methane production from anaerobic co-digestion of waste activated sludge and food waste and mitigate method. J Hazard Mater

557   384, 121363.

558 Tian, K., Zhu, J., Li, M. and Qiu, X. (2019) Capsaicin is efficiently transformed by multiple cytochrome P450s from Capsicum

559   fruit-feeding Helicoverpa armigera. Pestic. Biochem. Physiol. 156, 145-151.

560 Tong, J., Lu, X., Zhang, J., Sui, Q., Wang, R., Chen, M. and Wei, Y. (2017) Occurrence of antibiotic resistance genes and mobile

561   genetic elements in enterococci and genomic DNA during anaerobic digestion of pharmaceutical waste sludge with different

562   pretreatments. Bioresour. Technol. 235, 316-324.

563 Torrecillas, A., Schneider, M., Fernandez-Martinez, A.M., Ausili, A., de Godos, A.M., Corbalan-Garcia, S. and Gomez-Fernandez, J.C.

564   (2015) Capsaicin Fluidifies the Membrane and Localizes Itself near the Lipid-Water Interface. ACS. Chem. Neurosci. 6(10),

565   1741-1750.

566 Vasiliadou, I.A., Molina, R., Martinez, F., Melero, J.A., Stathopoulou, P.M. and Tsiamis, G. (2018) Toxicity assessment of

567   pharmaceutical compounds on mixed culture from activated sludge using respirometric technique: The role of microbial

568   community structure. Sci. Total. Environ. 630, 809-819.

569 Wang, D., Duan, Y., Yang, Q., Liu, Y., Ni, B.J., Wang, Q., Zeng, G., Li, X. and Yuan, Z. (2018a) Free ammonia enhances dark

570   fermentative hydrogen production from waste activated sludge. Water Res. 133, 272-281.

571 Wang, D., He, D., Liu, X., Xu, Q., Yang, Q., Li, X., Liu, Y., Wang, Q., Ni, B.J. and Li, H. (2019a) The underlying mechanism of

572   calcium peroxide pretreatment enhancing methane production from anaerobic digestion of waste activated sludge. Water Res. 164,

573   114934.

574 Wang, D., Liu, X., Zeng, G., Zhao, J., Liu, Y., Wang, Q., Chen, F., Li, X. and Yang, Q. (2018b) Understanding the impact of cationic

575   polyacrylamide on anaerobic digestion of waste activated sludge. Water Res. 130, 281-290.

576 Wang, D., Zhao, J., Zeng, G., Chen, Y., Bond, P.L. and Li, X. (2015) How Does Poly(hydroxyalkanoate) Affect Methane Production

577   from the Anaerobic Digestion of Waste-Activated Sludge? Environ. Sci. Technol. 49(20), 12253-12262.

578 Wang, J., Shi, T., Yang, X., Han, W. and Zhou, Y. (2014) Environmental risk assessment on capsaicin used as active substance for

579   antifouling system on ships. Chemosphere. 104, 85-90.

580   Wang, Y., Wang, D., Chen, F., Yang, Q., Li, Y., Li, X. and Zeng, G. (2019b) Effect of triclocarban on hydrogen production from dark

581   fermentation of waste activated sludge. Bioresour. Technol. 279, 307-316.

582   Wang, Y., Wang, D., Liu, Y., Wang, Q., Chen, F., Yang, Q., Li, X., Zeng, G. and Li, H. (2017) Triclocarban enhances short-chain fatty

583   acids production from anaerobic fermentation of waste activated sludge. Water Res. 127, 150-161.

584   Wei, W., Huang, Q.S., Sun, J., Dai, X. and Ni, B.J. (2019) Revealing the Mechanisms of Polyethylene Microplastics Affecting

585   Anaerobic Digestion of Waste Activated Sludge. Environ. Sci. Technol. 53(16), 9604-9613.

586   Wu, Y., Wang, D., Liu, X., Xu, Q., Chen, Y., Yang, Q., Li, H. and Ni, B. (2019) Effect of poly aluminum chloride on dark fermentative

587   hydrogen accumulation from waste activated sludge. Water Res. 153, 217-228.

588   Xu, F., Li, Y., Ge, X., Yang, L. and Li, Y. (2018) Anaerobic digestion of food waste - Challenges and opportunities. Bioresour Technol

589   247, 1047-1058.

590   Xu, Q., Li, X., Ding, R., Wang, D., Liu, Y., Wang, Q., Zhao, J., Chen, F., Zeng, G., Yang, Q. and Li, H. (2017) Understanding and

591   mitigating the toxicity of cadmium to the anaerobic fermentation of waste activated sludge. Water Res. 124, 269-279.

592   Xu, Q., Liu, X., Wang, D., Liu, Y., Wang, Q., Ni, B.J., Li, X., Yang, Q. and Li, H. (2019) Enhanced short-chain fatty acids production

593   from waste activated sludge by sophorolipid: Performance, mechanism, and implication. Bioresour. Technol. 284, 456-465.

594   Yagi, T. (1990) Inhibition by capsaicin of NADH-quinone oxidoreductases is correlated with the presence of energy-coupling site 1 in

595   various organisms. Arch. Biochem. Biophys. 281(2), 305-311.

596   Yao, Z., Su, W., Wu, D., Tang, J., Wu, W., Liu, J. and Han, W. (2018) A state-of-the-art review of biohydrogen producing from sewage

597   sludge. Int. J. Energ. Res. 42(14), 4301-4312.

598   Yue, L., Cheng, J., Zhang, H., Yuan, L., Hua, J., Dong, H., Li, Y.-y. and Zhou, J. (2020) Inhibition of N-Vanillylnonanamide in

599   anaerobic digestion of lipids in food waste: Microorganisms damage and blocked electron transfer. J. Hazard. Mater. 399.

600   Zhang, C., Su, H., Baeyens, J. and Tan, T. (2014) Reviewing the anaerobic digestion of food waste for biogas production. Renew. Sust.

601   Energ. Rev. 38, 383-392.

602   Zhang, J., Li, W., Lee, J., Loh, K.-C., Dai, Y. and Tong, Y.W. (2017) Enhancement of biogas production in anaerobic co-digestion of

603   food waste and waste activated sludge by biological co-pretreatment. Energy. 137, 479-486.

604   Zhao, J., Zhang, C., Wang, D., Li, X., An, H., Xie, T., Chen, F., Xu, Q., Sun, Y., Zeng, G. and Yang, Q. (2016) Revealing the

605   Underlying Mechanisms of How Sodium Chloride Affects Short-Chain Fatty Acid Production from the Cofermentation of Waste

606   Activated Sludge and Food Waste. ACS Sustain. Chem. Eng. 4(9), 4675-4684.

607   Zhu, J., Tian, K., Reilly, C.A. and Qiu, X. (2020) Capsaicinoid metabolism by the generalist Helicoverpa armigera and specialist H.

608   assulta: Species and tissue differences. Pestic. Biochem. Physiol. 163, 164-174.

609

632   PTA: phosphotransacetylase; ACDS: acetyl-CoA decarbonylase/synthase; mch: methenyl-THMPT

633   cyclohydrolase; CoM: coenzyme-M.   Error bars represent standard deviations of triplicate tests.

634   **Fig. 9.** The distribution of bacteria (A) and archaea (B) abundances at genus level in the two reactors.

1 **Table 1**

2 Experiment and model values of capsaicin inhibition to dextran, glucose, acetate and H_2 conversions.

Substrate	Experiment values[a]			$X_{s,o}$[b]	Model values		$K_{s,i}$[d]
	0 mg/L	30mg/L	50 mg/L		$X_{s,i}$[c]		
					30 mg/L	50 mg/L	
Dextran	8.07±0.26	5.99±0.19	6.19±0.62	3.01	1.98	1.39	48.5
Glucose	7.00±0.30	5.56±0.35	5.12±0.66	2.00	1.41	1.05	35.9
Acetate	2.85±0.14	1.44±0.13	1.12±0.03	0.79	0.35	0.33	13.7
H_2	0.24±0.01	0.19±0.02	0.17±0.02	1.90	1.45	1.34	33.4

3 [a] Results are the average and their standard deviations of triplicate tests, and the unit is mg/g VS·h

4 [b] $X_{s,0}$ is the degradation of the substrate without capsaicin addition, and the unit is g/(L·d) or g/L.

5 [c] $X_{s,i}$ is the degradation of the substrate when different capsaicin are added, and the unit is g/(L·d) or g/L.

6 [d] $K_{s,i}$ is the related inhibition constant of capsaicin calculated by Eq (2), and the unit is mg/L.
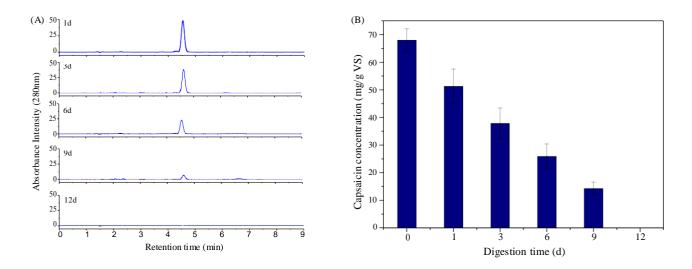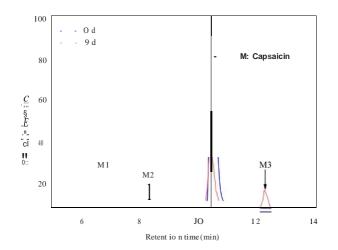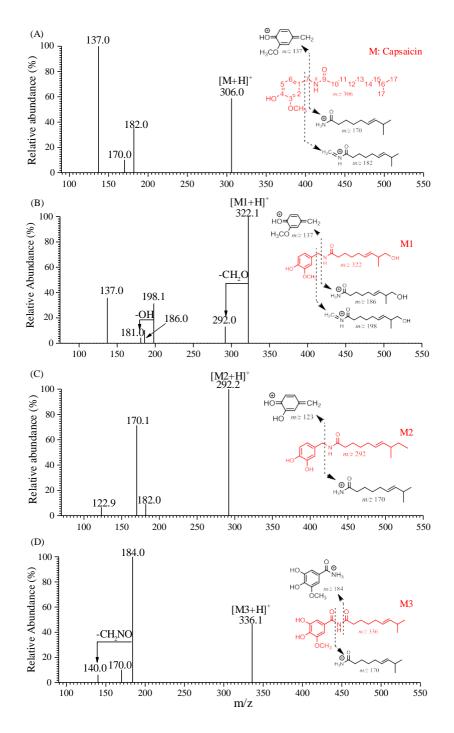
1

2   **Fig. 1.** Variations in HPLC chromatogram (A) and capsaicin concentration (B) at different digestion times.

3

Fig. 2. Full scan mode LC/MS chromatogram of capsaicin and metabolites produced in digestion process

extracted samples withdrawn from the digester fed with 68 ± 4.1 mg/g VS.

8

Fig. 3. Representative MS/MS spectra of M (A), M1 (B), M2 (C), and M3 (D). The structures and

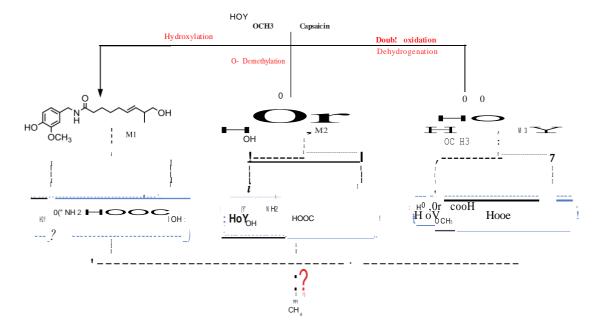fragmentation patterns of key diagnostic ions are presented in the figure.

Fig. 4. The possible pathways of capsaicin biodegradation during anaerobic co-digestion. The pathways shown in dash line were not demonstrated in this work and proposed according to the literature (Cho et al. 2014, Yue et al. 2020).
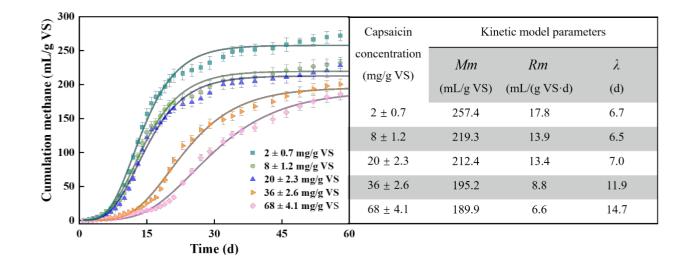
17

Capsaicin concentration (mg/g VS)	Kinetic model parameters		
	Mm (mL/g VS)	*Rm* (mL/(g VS·d)	*λ* (d)
2 ± 0.7	257.4	17.8	6.7
8 ± 1.2	219.3	13.9	6.5
20 ± 2.3	212.4	13.4	7.0
36 ± 2.6	195.2	8.8	11.9
68 ± 4.1	189.9	6.6	14.7

18 **Fig. 5.** Measured and simulated (using modified Gompertz equation) methane production from anaerobic

19 co-digestion in the presence of capsaicin at different concentrations.   Symbols represent experimental data

20 and lines represent model fit.   Error bars represent standard deviations of triplicate tests.
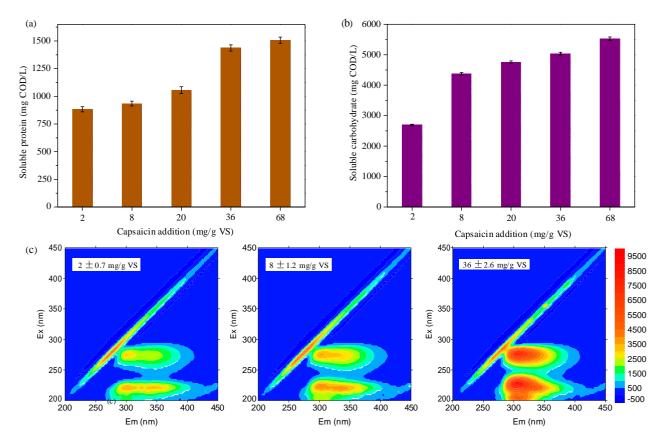
21

Fig. 6. The soluble proteins (a), soluble carbohydrates (b), and EEM spectra (c) of digestion liquid. Samples were collected on 2 d digestion. Error bars represent standard deviations of triplicate tests. The samples used to determine fluorescence EEM were diluted by 50 times.
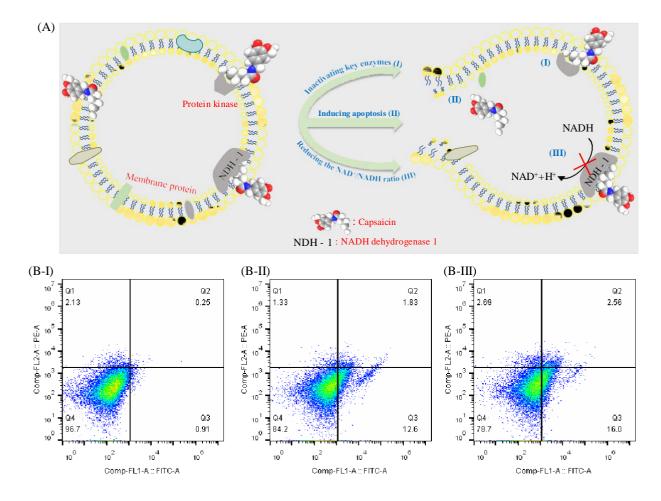
27

Fig. 7. The schematic diagram of interaction between capsaicin molecule and cell membrane (A), and flow

cytometric analysis of physiological status of microorganism cell (B). B-I: 0 mg/L capsaicin (The control)

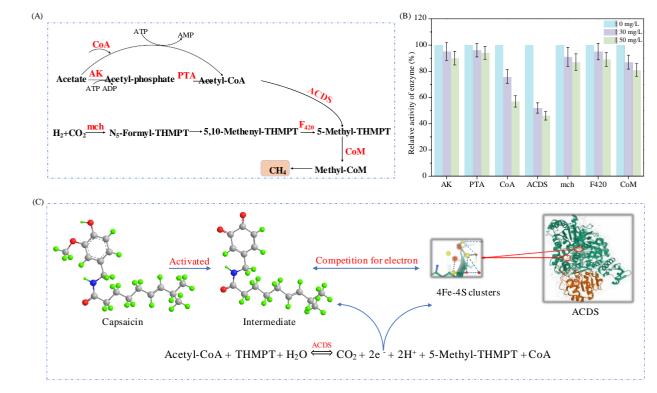(A), B-II: 30 mg/L capsaicin (B), and B-III: 50 mg/L capsaicin.

31

Fig. 8. Metabolic pathways of acetotrophic and hydrogenotrophic methanogenesis (A), the impact of capsaicin

on the relative activities of key enzymes involved in the two pathways (B), and schematic diagram of

capsaicin inhibiting the activity of ACDS (C).   THMPT: tetrahydromethanopterin; AK: acetate kinase; PTA:

phosphotransacetylase; ACDS: acetyl-CoA decarbonylase/synthase; mch: methenyl-THMPT cyclohydrolase;

CoM: coenzyme-M.   Error bars represent standard deviations of triplicate tests.

40 **Fig. 9.** The distribution of bacteria (A) and archaea (B) abundances at genus level in the two reactors.