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14	Optimization of orange peel waste ensiling for sustainable anaerobic digestion
15	
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17	
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22	
23	Abstract
24	
25	Today, orange peel waste (OPW) is mainly used as cattle feed, often after ensiling. This storage
26	phase can increase the efficiency of anaerobic digestion, since it allows both a better management
27	of possible co-digestion and a reduction in the high content of essential oils (mainly composed of d-
28	Limonene a well-known inhibitor of anaerobic digestion). The effects of ensiling on the methane
29	potential of OPW have been little studied, particularly its microbiological profile. This study has
30	simulated, at laboratory scale, OPW ensiling under three different conditions. Ensiled OPW
31	samples were then either directly anaeobically digested or subjected to simple pretreatments aiming
32	at the further removal of d-Limonene. The microbiota evolution during ensiling and the species of
33	microorganisms present during the aforementioned process were also identified. After ensiling, up
34	to over 70% of the initial d-Limonene content of OPW was removed and biomethane yield was
35	preserved up to about 90%.

Keywords: anaerobic digestion; d-Limonene; ensiling; microbiota; molecular identification;
orange peel waste.

40 *Highlights*:

41

42	•	OPW ensiling under dry or uncontrolled conditions are the most suitable techniques
43	•	During ensiling up to 63% of volatile solids in OPW are lost
44	•	Ensiling allows d-Limonene removal up to 75%
45	•	Up to about 90% of the methane potential of fresh OPW is preserved by ensiling
46	•	The microbiological population shows a high biodiversity.

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48 **1. Introduction**

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Orange peel waste (OPW), the residue of orange juice production, is about 50-60% of the processed 50 fruit (in weight). The specific physical and chemical properties of OPW (the high amount and 51 seasonal nature of the production, the low pH, the high water content, the presence of essential oils -52 53 mainly d-Limonene, 80-95% of the total) make its management difficult [1,2]. OPW has a high biorefining potential [1] both for the extraction of added-value products (e.g., pectin and bioactive 54 compounds) and biofuels (e.g. biogas from anaerobic digestion - AD) [3-9]. However, the 55 biomethane yield of OPW is curbed due to its high content of d-Limonene [6], which is highly toxic 56 to microorganisms [2,10]. Three alternatives are available to overcome this limitation: (i) the 57 preliminary removal of d-Limonene [5,7,11–13]; (ii) the co-digestion with other substrates [14–18]; 58 (iii) the digestion of OPW alone adopting moderate organic loading rates (OLRs) and/or using 59 additives [6,8,9]. 60

Since the advanced removal of d-Limonene from OPW is expensive and the digestion of OPW alone due the aforementioned problems reduces the overall economic convenience of the process, co-digestion is a more promising management option. However, its present application for energy

conversion of OPW is limited, since AD plants located in citrus production areas are not able to treat the high amounts of residues produced during the limited time of the harvesting season (from November to April in the Mediterranean climate) and long distance transportation is economically unsustainable. Therefore, OPW is traditionally used as animal feed [19–22] and ensiling [17,23] is commonly used, as for forages, for conservation throughout the year.

The ensiling process is commonly divided into subsequent four steps [24–26]: (1) an aerobic phase, beginning immediately after process start, when aerobic bacteria and yeasts predominate, thanks to the air entrapped in the biomass; (2) a fermentation phase, when anaerobic and facultative microorganisms use the available substrates for their metabolism, producing mainly organic acids; (3) a steady storage phase in the silage silo, when the reduced pH after the previous phase allows the substrate preservation; and (4) the feed-out phase, when the material is exposed to air for the subsequent use (the latter stage is not considered in this paper.

During ensiling, the properties of raw OPW (e.g. pH and volatile solids) are quickly made stable due to a spontaneous lactic fermentation. Stabilisation is normally completed in about two weeks [27,28], when pH becomes slightly higher than three. The changes are also macroscopically evident: in 10-20 days OPW can not be visually recognizable, since the original substrate becomes a dense homogeneous slurry and only the seeds remain intact (Figure 1 - SI).

According to the literature, the main product of fermentation is lactic acid and, secondarily, ethanol and acetic acid.

However, until now the effects of ensiling on the methane potential of AD of OPW have been little studied, despite the potential increases in methane yields that can be expected. Previous results of experimental tests of AD of ensiled OPW [7,28,29] have shown, beside the viability of the process, that the methane production per unit of digested biomass weight is similar to the energy yield of the raw substrate. The d-Limonene is partially removed during the process, but a noticeable loss of volatile solids (VS) is observed. Overall, ensiling provides preliminary homogenization, hydrolysis and acidification of OPW. However, until now OPW ensiling has not been optimized in view of using the ensiled material as a substrate for AD, whose objective is the maximization of the methane yield. Therefore, more research is needed in order to identify the most sustainable ensiling technique to be used as OPW pre-treatment in AD plants. Moreover, little has been reported in the literature about microbiota of OPW fermentation during ensilage [27].

To fill these gaps, this study explores a set of possible conditions and treatments for OPW ensiling, 95 targeted to maximise d-Limonene removal and, at the same time, limiting the biomass loss. In more 96 detail, the ensiling process is simulated at the laboratory scale under (i) natural, (ii) wet (adding 97 20% water to raw OPW), and (iii) dry (in a drainage system purposely prepared) conditions. In 98 order to remove as much d-Limonene possible, all samples of ensiled OPW are then subjected to (i) 99 simple centrifugation and (ii) ethanol extraction and centrifugation. Moreover, the microbiota 100 evolution of OPW and the species of microorganisms involved in the ensiling process are evaluated. 101 102 Finally, the overall loss of VS and the bio-methane potential (BMP) of the samples have been evaluated. 103

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105 **2. Materials and methods**

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107 2.1. OPW sampling
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OPW was sampled from an orange processing factory in Reggio Calabria (Southern Italy) and immediately frozen (-20°C). According to [30], freezing is not expected to affect the biological activity of the biomass. Before starting ensiling, the samples of OPW were thawed at room temperature

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114 2.2. OPW ensiling

OPW was ensiled in hermetically sealed batches. Each batch (made of glass, with a volume of 1.1 116 L) is provided with a central neck, closed with a stopper, and two side openings closed with rubber 117 septa that allow the biogas withdrawal. Three ensiling conditions were tested: (i) natural conditions 118 (hereinafter indicated as "ENS"), as usually carried out by agro-farms of the Mediterranean Basin 119 (ii) "wet" conditions, where water (20% w/w) was added to OPW ("WET") to try to improve d-120 Limonene leaching; (iii) "dry" conditions ("DRY"), placing OPW over a drainage system (quartz 121 gravel), in order to remove by gravity the liquid released by the biomass in order to reduce the 122 moisture and speed the stabilization process. 123

For each ensiling condition, six batches were prepared: three batches were opened after 7, 14 and 21 days respectively, in order to evaluate the changes (weight loss, TS, VS, COD, microbiota) in OPW throughout the process. The remaining three batches were opened after 28 days, when ensiling was stopped. In fact, in previous studies a substantial stability of the ensiled biomass was observed after 2-4 weeks [25–27]. In these three batches, the volume of the biogas produced during ensiling was measured three times per week using a graduated 100-mL syringe.

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131 2.3. Treatments on ensiled OPW

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After ensiling, the OPW was extracted from the batches and subjected to two treatments₇ in order to further remove d-Limonene: (i) a chemical treatment followed by centrifugation and (ii) a simple centrifugation. As regards the first treatment, each sample of ensiled OPW (under ENS, WET and DRY conditions) was chemically treated (hereinafter the treated samples were referred as "CHEM") using ethanol as solvent for d-Limonene extraction and then centrifuged at 9000 rpm for three minutes. Solvent was dosed at 10% w/w with a contact time of one hour under continuous mixing in a rotary shaker, Stuart Scientific Rotator Drive STR/4). Moreover, OPW after ensiling (also in this case under ENS, WET and DRY conditions) was also subjected to simple centrifugation (CEN) only (that is, without a previous treatment with ethanol), in order to evaluate the efficiency of d-Limonene removal by the chemical treatment.

In both treatments, the liquid from the centrifuge was disposed of, while the solid biomass was usedas substrate for BMP tests.

The OPW samples were weighed before and after ensiling, and before and after each treatment, in order to estimate the various mass flows. Table 1 reports a scheme of the experimental tests carried out on the nine samples.

148

Table 1. Acronyms of the OPW samples subjected to the experimental tests (ENS - naturally
ensiled OPW; WET - OPW ensiled in wet conditions; DRY - OPW ensiled in dry conditions).

151

			Treatments	
		Natural ensiling	Centrifugation	Chemical (ethanol addition)
	Natural	ENS	ENS+CEN	ENS+CHEM
Condition	Wet	WET	WET+CEN	WET+CHEM
	Dry	DRY	DRY+CEN	DRY+CHEM

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153

154 2.4. Physico-chemical measurements

155

Before and after ensiling and treatments, pH, contents of total (TS) and volatile (VS) solids, and chemical oxygen demand (COD) of OPW were measured following standard methods [31]. As suggested in [32,33], we cared to prevent the loss of as much of volatile compounds as possible, such as some components of the essential oils (EO), acetic acid and, if present, ethanol and other alcohols. To this aim, during TS measurement we usually limit oven temperature at 60 °C. Under
 this temperature, water evaporation can be considered complete when stable weight is reached.

For COD measurement first each OPW sample was dried. Subsequently, it was milled and the powder was then mixed to distilled water. Finally, COD was measured by the potassium dichromate method using pre-dosed cell tests (WTW 114555) the method complies with the DIN ISO 15705 and is similar to APHA 5220 D method.

As regards the determination of the concentration of d-Limonene before and after the experimental 166 tests, the analysis is difficult for OPW, since the concentration is strongly influenced by the 167 extraction conditions and the degradation level of the substrate. Moreover, the complexity increases 168 169 if the possible inhibition of AD process must also be measured. Since during ensiling the biomass was homogenised, presumably most of the EO was released throughout the process due to the 170 breaking of the small flavedo sacs which contain it. Following previous tests [9], which used a 171 172 "mild" EO extraction only the d-Limonene that was available immediately after substrate feeding was determined . 173

174 d-Limonene was extracted from the biomass by mixing 1.5 g of sample with 3 mL of a solution of toluene (Sigma-Aldrich, St. Louis, MO, USA) and cyclohexane (0.1M, Sigma-Aldrich, St. Louis, 175 MO, USA), which was used as internal standard, for two hours. This blend was then injected into a 176 gas chromatograph (Agilent 6890) equipped with a wide-bore capillary column and a flame 177 ionization detector (FID), the latter set at 250 °C. The capillary column (J&W DB-WAXetr 50 m x 178 320 mm x 1 mm) used nitrogen as gas carrier with a flow rate of 10 mL/min. The temperature, 179 initially kept 50 °C for 8 min, was then raised to 230 °C (at 5 °C/min) for 2 min and finally set at 180 240 °C for 4 min during the post run. 181

The liquid recovered after centrifugation of ENS and WET samples and that collected at the bottom of the DRY ensiling reactor were analysed for propionic, butyric and lactic acids. The liquid samples were filtered (1.2 μ m) twice and then 2.5 mL of filtrate were mixed with 2.5 mL of ethyl acetate (Sigma-Aldrich) and shacked to allow organic acids extraction.

186	The amount of organic acids extracted was determined with a gas chromatograph (Agilent 6890)
187	equipped with a wide-bore capillary column (CPWAX52CB, 50 m, i.d. 1/4 0.53 mm) and a flame
188	ionization detector (FID). The injector was settled at 250°C. The temperature program started at
189	50°C, held for 5 min, the temperature was raised to 230°C at 5°C/min, held for 8 min, raised to
190	240°C and held for 2 min during the post run.

192 2.5. Biochemical methane potential (BMP) tests

193

Three series of BMP tests were carried out in triplicate for each sample under mesophilic conditions (35 ± 0.5 °C) as follows (Figure 1):

196

197 1. BMP1: ENS; WET; DRY.

- 198 2. BMP2: ENS+CEN; WET+CEN; DRY+CEN.
- 199 3. BMP3: ENS+CHEM; WET+CHEM; DRY+CHEM.
- 200

In each series the blank (that is a batch to assess the biogas production of the inoculum) and, as additional internal controls, two other batches were added; the first fed with cellulose and the other with raw OPW. The cellulose-fed reactor is suggested by UNI/TS 11703:2018 (Italian standard procedure for BMP tests) in order to verify inoculum activity. The second was designed as an internal control to verify the response of the different inocula to the substrate.

As inoculum of the AD process, a liquid digestate was used collected in three separate sampling operations from a full-scale anaerobic digester fed with cattle manure and agro-industry residues. After collection, the inoculum was sieved and stored for less than a week at 35 °C to reduce nonspecific biogas production (i.e. the production of the inoculum itself). The TS of the inoculum of the three BMP tests was $5.5\pm0.2\%$, the VS $69.5\pm2.4\%$ and the pH was 7.5 ± 0.05 . For each BMP test 1.1-L bottles with a central neck and two other lateral necks equipped with perforable septa (WTW-Germany) were used. Each bottle was placed on a magnetic stirrer, and the digestion blend was continuously mixed in a thermostatic cabinet kept at a preset temperature $(35\pm0.5 \text{ }^{\circ}\text{C}).$

In each batch the substrate was mixed with 200 mL of inoculum at a ratio (on a VS basis) equal to 215 0.3, this value being in the range suggested by UNI/TS 11703:2018. According to the same 216 regulation three nutrient solutions were also added, to supply nutrients and micronutrients for the 217 bacterial metabolism. The three solutions (indicated as A, B and C) contained KH2PO4, 218 Na2HPO4·12H2O, NH4Cl (A, 5% final volume), CaCl2·2H2O, MgCl2·6H2O, FeCl2·4H2O (B, 219 5% of final volume) and MnCl2·4H2O, H3BO3, ZnCl2, CuCl2, Na2MoO4·2H2O, CoCl2·6H2O, 220 NiCl2·6H2O, Na2SeO3 (C, 1% of final volume). Finally, water was added to the batch, in order to 221 reach final volume (600 mL) and to keep the TS content at about 35 gTS/L, which is consistent with 222 223 the limits (10-50 gTS/L) required by the aforementioned UNI/TS regulation. In accordance with this regulation, the BMP tests were stopped when the daily methane production of a batch was 224 225 lower by 1% than the cumulated volume from the process start.

226 About three times per week, the biogas produced in each batch was withdrawn using a 100 mL syringe and transferred with care into an alkaline trap through a tube. After the injection, the carbon 227 dioxide in the biogas was absorbed by an alkali solution (NaOH 3M), while the methane bubbles, 228 229 increasing the pressure in the trap, displaced the same volume of the alkali solution, measured in a graduated cylinder. The test was stopped when daily production was lower that 1% of the 230 cumulated value since test start. The net specific methane production (that is, the methane volume, 231 normalised to standard conditions, per unit of VS depurated by the blank production) was calculated 232 as follows: 233

234
$$BMP = \frac{(v_{CH_{4,S}} - v_{CH_{4,blank}})}{v_{S_n} \cdot v_n}$$
 [1]

- where:
- 237
- $-V_{CH4,s} =$ final cumulated methane production (NmL_{CH4})
- $-V_{CH4,blank} = final cumulated methane production of the blank (NmL_{CH4})$
- 240 VS_s = initial VS concentration of the substrate (g_{VS} ·L⁻¹)
- 241 V_s =total volume of the batch (L)
- 242
- 243 According to the aforementioned Italian standard procedure, the test was accepted, if the batch fed
- with cellulose in the same BMP series produced 335 $\text{NmL} \cdot \text{g}_{\text{VS}}^{-1} \pm 25\%$.
- 245



Figure 1. Experimental scheme of the BMP (I, II and III) tests carried out after OPW (orange peel
waste) ensiling subjected to different ensiling conditions and treatments (ENS - naturally ensiled
OPW; WET - OPW ensiled in wet conditions; DRY - OPW ensiled in dry conditions).

The net specific cumulative methane production of each BMP test was modelled using the modified Gompertz equation [34], in order to verify its prediction capacity under the experimental conditions:

255
$$B = P \cdot exp\left\{-exp\left[\frac{R_m \cdot e}{p} \cdot (\lambda - t) + 1\right]\right\}$$
[2]

256

where:

258 - $B (\text{NmL} \cdot \text{g}_{\text{VS}}^{-1}) =$ specific methane production at time t (d)

259 -
$$P(\text{NmL}\cdot g_{\text{VS}}^{-1}) = \text{specific methane production at } t = \infty$$

260 -
$$R_m$$
 (NmL·(g_{VS}·d)⁻¹) = maximum methane production rate

261 -
$$\lambda$$
 (d) = lag phase duration

262

263 *P*, R_m and λ were calculated using iteratively the least square method of the routine "Solver" of 264 Microsoft Excel until to the highest r^2 between the modelled and experimental data.

265

267

First, the statistical significance of the final values of the weight loss as well as TS and VS contents after the ensiling tests was investigated using the t-test (at p < 0.05).

Then a two-way Analysis Of Variance (ANOVA) along with Tukey's test (designed for the pairwise comparisons) was used to evaluate the statistical significance of the net cumulated specific methane yields of the batches, assuming as variability factors: (i) the ensiling conditions (ENS, WET and DRY); (ii) the treatment (raw OPW, natural ensiling, chemical treatment and centrifugation); (iii) reciprocal interaction of ensiling condition and treatment. At p < 0.05 level of significance was adopted. It was not necessary to perform data transformations for the analysis.
ANOVA assumes normality and this assumption was checked using QQ-plots.

All the statistical analyses on the samples were carried out using the XLSTAT (release 2017) software.

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280 2.8. Microbiological analyses and strains isolation

281

The microbiota associated with raw and ensiled OPW (ENS, WET and DRY modes) was analysed 282 at day 0 (raw OPW), 7, 14 and 28 of the ensiling period, the leachate collected during DRY ensiling 283 was also considered. To this end, each type of solid sample was firstly homogenized to allow the 284 microorganisms release from the solid matrix; more specifically, 10 g of each solid OPW sample 285 was homogenized in a solution of 0.9% NaCl. Then, the obtained homogenates and the leachate of 286 287 DRY OPW were diluted ten-fold and inoculated by spread-plate method in triplicate onto Petri plates, containing: (i) Plate Count Agar (PCA) (Sigma-Aldrich), for total microbial count (TMC); 288 (ii) de Man-Rogosa-Sharpe (MRS) agar (VWR International srl, Italy), supplemented with 15 289 mg/L cycloheximide (Oxoid), to count lactic acid bacteria (LAB); and (iii) Yeast Peptone Dextrose 290 (YPD) agar (VWR, International srl, Italy), supplemented with 100 mg/L chloramphenicol 291 (Liofilchem Diagnostici, Italy), to count yeasts. All the plates were incubated at 30 °C for two days 292 under aerobic conditions for yeasts and TMC, and under anaerobic conditions for LAB. At day 0, 7, 293 14, 21, and 28 during ensilage, the colonies grown on YPD and MRS agar were randomly picked 294 from the highest dilution sample [35]; then, the isolates were purified by streaking on the 295 corresponding isolation medium and stored as glycerol stock at - 80 °C until use. The isolated 296 bacteria were tested for catalase and for Gram by KOH method [36]. 297

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299 2.9. Restriction analyses and sequencing

DNA from overnight grown yeasts (101 isolates) and bacteria (97 isolates), isolated throughout the 301 ensilage and from the different treatments, was extracted by InstaGene Matrix (Bio-Rad 302 Laboratories, USA), according to the manufacturer's instructions. Then, yeasts were analyzed by 303 PCR of the 5.8S-ITS regions using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and 304 ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and amplification conditions, according to [37], and 305 bacteria were analysed by PCR of the 16S rRNA gene, using the Y1 (5'-306 TGGCTCAGAACGAACGCTGGCGGC-3') and Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-307 3') primers, according to [38]. Firstly, yeasts and bacteria were grouped by Restriction Fragment 308 Length Polymorphism (RFLP) of the 5.8S ITS rRNA region (HaeIII and HinfI restriction enzymes) 309 310 and Amplified Ribosomal DNA Restriction Analysis (ARDRA) of the 16S rRNA gene (HaeIII and AluI restriction enzymes), respectively. Then, three samples for each PCR-RFLP and PCR-ARDRA 311 profile were chosen to sequence the 26S D1/D2 rRNA region (NL1 and NL4 primers) and 16S 312 313 rRNA regions (fD1 and rD1 primers) for yeasts and bacteria, respectively [39]. The obtained amplicons were purified and sequenced by Sanger method (Eurofins Genomics, Germany). The 314 sequences were analyzed and compared with the sequences of the National Center for 315 Biotechnology Information (NCBI) using BLASTN [40]. To differentiate the genotypically closely 316 related Lactobacillus plantarum, Lactobacillus pentosus, and Lactobacillus paraplantarum, the 317 318 multiplex PCR of *recA* gene was carried out, according to [41].

319

320 3. Results

321

322 *3.1. Ensiling and subsequent treatments*

323

During ensiling the OPW lost weight (minimum $2.72\% \pm 0.81\%$ in DRY mode, maximum $3.15\% \pm 0.27\%$ for ENS mode) (Table 2). This weight loss was fast-until the 10^{th} day and subsequently slower (Figure 2).

328 **Table 2.** Main physico-chemical properties of the OPW (orange peel waste) subjected to different ensiling conditions and treatments.

-	
- 2	20
3	27

	Loss	r	ΓS		VS	
OPW	(% on fresh	(% on fro	esh weight)	initial		final
	weight)	initial	final	(% c	n TS)	(% on raw biomass)
RAW		17.8 ± 0.53		96.9 ± 0.01		
(non-ensiled)	-	17.8 ± 0.55	-	90.9 ± 0.01		
ENS	3.15 ± 0.27 a	17.8 ± 0.53	11.1 ± 0.28 ab	96.9 ± 0.01	94.4 ± 0.06 a	10.5 ± 0.26 a
WET	2.79 ± 0.09 a	$14.2 \pm 0.40*$	9.2 ± 0.20 a	96.9 ± 0.01	94.6 ± 0.00 a	$8.7\pm0.19~\text{b}$
DRY	2.72 ± 0.81 a	17.8 ± 0.53	13.1 ± 0.1 b	96.9 ± 0.01	$94.6\pm0.01~\text{a}$	$12.4\pm0.09~\mathrm{c}$

330 Notes: different letters indicate significant differences according to t-test (at p < 0.05); TS = Total Solids; VS = Volatile Solids; ENS: naturally ensiled OPW; WET: OPW ensiled

331 in wet conditions; DRY: OPW ensiled in dry conditions; *water addition.

As expected, biogas (> 95% CO_2) was produced only in the first days of ensiling (Figure 1 - SI) In fact, in this period, aerobic bacteria and yeasts were dominant, producing CO_2 through their metabolism, mainly due to the air entrapped in the OPW pores, in accordance to [36,40].

The highest reduction in TS was measured for ENS samples (-37.4% \pm 3.44%) and the lowest for

336 DRY (-26.2% \pm 1.79%). VS reduced on average by only 2.4% \pm 0.02% (WET and DRY) – 2.6% \pm 337 0.05% (ENS) (Table 2). Also for TS and VS the parameters, the decrease was faster at the start of 338 ensiling and then tended to slow (Figure 2). This is in agreement with biogas production that was 339 quantitative only in the first days (Figure 2).

The initial COD of the OPW (928 $\pm 158 \text{ mg} \cdot \text{g}^{-1}$) did not noticeably change for the tested conditions and treatments, with a maximum value (994 $\pm 95 \text{ mg} \cdot \text{g}^{-1}$) measured for ENS and a minimum of 936 $\pm 41 \text{ mg} \cdot \text{g}^{-1}$ for WET (Figure 2).The pH (initially 3.7 ± 0.0) was stable for ENS and WET and lowered for DRY (3.3 ± 0.0) (Table 2). Generally, the pH evolution was not monotonic, but fluctuated around the initial value with a slightly more noticeable variability detected for ENS ensiling mode (Figure 2).

The liquid recovered by centrifugation from ENS and WET samples or collected at the reactor bottom for DRY samples (passive drainage) contained amounts of lactic acid, while butyric, and propionic acid have not been measured due to their low concentrations. Table 3 reports the lactic acid concentrations in the liquid phase after 15 and 30 days of ensiling, respectively.

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- 353
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Table 3. Lactic acid concentration in the liquid phase separated by centrifugation (ENS and WET
ensiling modes) or by passive drainage (DRY ensiling mode).

	Lactic acid (g/L)
ENS _{15 days}	1.8
WET _{15 days}	1.5
DRY _{15 days}	1.1
ENS _{30 days}	2.6
WET _{30 days}	1.8
DRY _{30 days}	1.6

















Figure 2. Temporal evolution of the main physico-chemical properties of the OPW (orange peel waste) subjected to different ensiling conditions and treatments (ENS - naturally ensiled OPW; WET - OPW ensiled in wet conditions; DRY - OPW ensiled in dry conditions)

374	Table 4. Values of d-Limonene and residual content of VS in OPW subjected to subjected to
375	different ensiling conditions and treatments

OPW	d-Limonene(mg/g)	VS/VS of raw OPW
RAW	0.55	1.00
ENS	0.14	0.59
WET	0.14	0.49
DRY	0.18	0.58
ENS+CEN	0.34	0.53
WET+CEN	0.25	0.41
DRY+CEN	0.28	0.54
ENS+CHEM	0.14	0.51
WET+CHEM	0.19	0.37
DRY+CHEM	0.16	0.50

377

Notes: VS = Volatile Solids; ENS: naturally ensiled OPW; WET: OPW ensiled in wet conditions; DRY: OPW ensiled
in dry conditions; CEN = OPW subjected to centrifugation; CHEM = OPW subjected to centrifugation and chemical
treatment with ethanol.

381

382 Compared to the raw biomass, ensiling reduced d-Limonene content of OPW by 67 (DRY) to 75%

383 (WET and ENS conditions). The chemical treatment of ensiled OPW gave slightly lower d-

Limonene contents only in for DRY+CHEM (-71% respect to -67% for DRY), while centrifugation

- of the ensiled OPW achieved the lowest decreases.
- 386 The reduction in VS content was in the range -41% (ENS) to -63% (WET and CHEM) with an

387 average gradient CHEM (-54%) > CEN (-51%) > ENS/WET/DRY OPW (-45%) (Table 4).

- 388
- 389

The microbial loads refer to raw OPW analysed before the start of ensiling and to OPW treated by 392 ENS, WET (solid material), and DRY methods (solid material and leachate). As regards the ENS 393 and WET OPW, the aerobic TMC loads gradually increased up to the maximum values at the 14th 394 day of ensilage. Then, the population decreased down to 7.50 and 7.71 Log CFU/mL, respectively, 395 after 28 days. The decrease was more marked in WET OPW compared to ENS samples. On the 396 contrary, the TMC loads of the leachate of DRY OPW always increased until 9.53 Log CFU/mL at 397 the end, but the rate of increase was higher until the 14th day and lower thereafter (Figure 3). 398 Acetic acid bacteria (AAB) were only counted in raw OPW, therefore at day 0. Then, the bacteria 399 detected were LAB. At the first stages of ensiling, 0 and 7 days, for all samples the load of yeasts 400 was higher compared to LAB Subsequently, LAB were present in greater quantity than yeasts and 401 402 evolved by similar rates in all the tested ensiled OPWs (Figure 3). In WET OPW and leachate of DRY OPW, yeasts evolved with similar trend throughout the process and, at the end (after 28 days) 403 404 yeast counts were lower (4.72 - 4.51 Log CFU/mL) than in ENS OPW (6.11 Log CFU/mL) while 405 LAB counts were higher in the leachate of DRY OPW and lower in ENS and WET samples (10.09 Log CFU/mL against 8.32 - 8.54 Log CFU/mL) (Figures 3). 406



Figure 3. Total microbial count (TMC), yeasts, as well as lactic (LAB) and acetic acid (AAB) bacteria counts of raw OPW (day 0) and OPW subjected to different ensiling conditions (ENS: naturally ensiled OPW; WET: OPW ensiled in wet conditions; DRY: leachage of OPW ensiled in dry conditions).

Concerning the solid fraction of the DRY ensiling, the microbial population observed was negligible except for the yeasts at the 7th day (3.70 Log CFU/mL) (data not shown). This could be due to a progressive loss of humidity as the liquid part flowed into the lower part of the fermenter.

418

419 *3.3 Bacteria and yeast's identification*

420

87% of the total population of the bacteria isolated was catalase-negative and Gram positive, while 421 the remaining 13% was catalase-positive and Gram negative. Eight patterns of ARDRA profiles 422 were observed. Bacteria were identified as L. plantarum, Lactobacillus brevis, Gluconobacter 423 kondonii. 424 Lactobacillus suebicus. Leuconostoc pseudomesenteroides, Lactobacillus paracollinoides, Leuconostoc citreum, and Asaia lannensis. LAB species of L. plantarum and L. 425 brevis were present in the OPW at day 0 together with AAB species of G. kondonii and A. lannensis 426 (Figure 4). These AAB dominated the matrix at this stage, consistent with the presence of oxygen. 427 The AAB were not recovered from all the samples throughout the ensilage. 428

Figure 5 reports the species distribution detected in the samples. At the 7th day, all the samples 429 harboured L. plantarum. The leachate of DRY OPW and the WET OPW favoured the growth of L. 430 citreum, L. pseudomesenteroides characterised the leachate of DRY OPW. After 14 days, L. brevis 431 was detected in all the samples, while L. suebicus, L. pseudomesenteroides, and L. paracollinoides 432 were present in ENS, leachate of DRY OPW and WET OPW, respectively. After 21 days, ENS and 433 WET samples of OPW were characterised by L. plantarum, L. brevis, and L. suebicus, while the 434 leachate of DRY OPW contained L. brevis and L. plantarum. At the end of the ensilage process, 435 ENS and WET OPW showed LAB composition similar to the population on the 21st day, while the 436 leachate of DRY OPW was dominated by L. plantarum. 437

Eleven patterns of RFLP profiles were observed. Yeasts were identified as *Pichia fermentans*, *Saccharomyces cerevisiae*, *Kregervanrija fluxum*, *Saccharomyces uvarum*, *Pichia membranifaciens*, *Hanseniaspora occidentalis*, *Pichia kudriavzevii*, *Pichia occidentalis*,

Hanseniaspora nectarophila, Kazachstania barnettii, and Torulaspora delbrueckii. P. fermentans,
H. occidentalis, and S. uvarum were detected in OPW at day 0 (Figure 4). P. fermentans and S.
cerevisiae were isolated from all the samples throughout the ensilage. On the 7th day, H.
occidentalis and H. nectarophila were found in WET OPW and leachate of DRY OPW,
respectively. In the middle stage of ensilage, ENS OPW was characterised also by K. barnetii and
K. fluxum, while the leachate of DRY OPW by Saccharomyces sp. and T. delbrueckii. At the end of
the ensilage, all the samples contained K. fluxum and Pichia spp. (Figure 6).

As regards the representative strains of LABs and yeasts sequenced, the percentage of similarity, and the accession numbers of the closest relative by BLAST, reported in Table 5, the sequences with a percentage homology of 97% or higher were considered to belong to the same species, according to [42].

452



453

454 Figure 4. Number (in % on the total) of LAB and AAB (a) and yeast (b) species recovered from

455 raw orange peel waste (OPW) (day 0).





Figure 5. Number (in % on the total) of bacteria recovered from ENS (a), WET (b), and leachate of

459 DRY (c) orange peel waste (OPW) throughout ensilage.



461 Figure 6. Number (in % of the total) of yeasts recovered from ENS (a), WET (b), and leachate of
462 DRY (c) orange peel waste (OPW) throughout ensilage.

464	3.4	BMP	test	result
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In general, all the values of the net specific methane production for OPW under the tested conditions and treatments were significantly higher compared to the value measured for the raw biomass (415 \pm 26.4 NmL·g_{VS}⁻¹). In more detail, the lowest increases in the methane yield was measured for the centrifuged OPW (on average +47%), while the highest production was detected for WET OPW subjected to the chemical treatment with ethanol and then centrifuged (+86% compared to raw OPW) (Figure 7). The differences were significant both for the conditions and the treatments and the same was for the interaction condition x treatment.

473	AD process regularly evolved in time in all the BMPs, as shown by the monotone cumulated
474	methane production. The chemical treatments with ethanol - specifically the tests carried out on
475	ENS+CHEM, WET+CHEM and. DRY+CHEM OPW - were the exceptions. In these tests, the AD
476	process were slower at the earlier stages (until the 10th-20th day) (Figure 8, h, i, l). Other slight
477	evidences of AD inhibition were detected for one reactor fed with ENS OPW (Figure 8b) and for
478	the reactors fed with ENS+CEN, WET+CEN and DRY+CEN OPW (Figure 8e, f, g).
479	

Table 5. Representative strains of LABs and yeasts sequenced together with the percentage of
similarity and the accession numbers of the closest relative by BLAST.

	Strains	Species	Similarity (%, accession no. of the		
	Strams		closest relative by Blast)		
Yeasts	PY 1	Saccharomyces uvarum	100% - KY109468.1		
	PY 12	Saccharomyces cerevisiae	100% - NG_042623.1		
	PY 21	Hanseniaspora nectarophila	97% - NG_055397.1		
	PY 2	Pichia fermentans	99.82% - KY108804.1		
	PY 82	Pichia occidentalis	100% - KY108912.1		
	PY 76	Pichia kudriavzevii	100% - KY108786.1		
	PY 80	Pichia membranifaciens	100% - KY108889.1		
	PY 24	Kregervanrija fluxum	100% - KY108172.1		
	PY 4	Hanseniaspora occidentalis	100% - NG_055416.1		
	PY 56	Kazachstania barnetii	100% - KY107903.1		
	PY 44	Torulaspora delbrueckii	100% - NG_058413.1		
Bacteria	PB 22	Lactobacillus plantartum	100% - NR_115605.1		
	PB 31	Lactobacillus brevis	100% - NR_116238.1		
	PB 47	Lactobacillus suebicus	100% - NR_114977.1		
	PB 30	Lactobacillus paracollinoides	100% - NR_042322.1		
	PB 12	Leuconostoc citreum	100% - NR_041727.1		
	PB 24	Leuconostoc pseudomesenteroides	99.89% - NR_109004.1		
	PB 4	Asaia lannensis	100% - NR_114144.1		
	PB 5	Gluconobacter kondonii	100% - NR_104680.1		



486



488 Figure 7. Cumulated net specific methane yields of the BMP I, II and III batch tests.

490 Gompertz equation fitted well the experimental data of all BMP tests ($r^2 > 0.99$) (Table 6).

Table 6 reports a comparison between two options: (i) the digestion of a given amount of raw OPW without pre-treatments; and (ii) ensiling (with or without other treatments, such as ethanol addition and centrifugation) before digestion. In more detail, the ratio $CH_4/CH_{4raw OPW}$, which considers the VS losses occurring during the different treatments before AD, shows that the VS losses are not balanced by a corresponding increase of the specific methane production combining all the processes (ensiling, chemical treatment and centrifugation); in fact, the methane production is between 55 and 89% of the methane production without any pre-treatment (that is, by the direct 498 digestion of OPW). This theoretical production can not be achieved because of the reasons already499 explained.









Table 6. Methane net specific production compared to the theoretical value yielded by the anaerobic digestion of raw orange peel waste (OPW) together with the parameters of the interpolating Gompertz equation.

509

OPW	$\frac{\mathbf{P}}{(\mathrm{NmL} \cdot \mathrm{g}_{\mathrm{VS}}^{-1})}$	λ (d)	$\frac{\mathbf{R}_{\mathbf{m}}}{(\mathrm{NmL} \cdot (\mathrm{g}_{\mathrm{VS}} \cdot \mathrm{d})^{-1})}$	\mathbf{r}^2	CH ₄ /CH _{4raw OPW}	
RAW	0.47	0.00	0.027	0.996	1.00	
ENS	0.69	0.00	0.051	0.997	0.89	
WET	0.68	0.00	0.067	0.998	0.75	
DRY	0.64	0.00	0.057	0.999	0.82	
ENS+CEN	0.64	1.62	0.039	0.997	0.73	
WET+CEN	0.61	0.83	0.041	0.999	0.55	
DRY+CEN	0.63	0.74	0.041	0.999	0.76	
ENS+CHEM	0.72	0.00	0.058	0.994	0.84	
WET+CHEM	0.88	5.34	0.044	0.992	0.67	
DRY+CHEM	0.93	12.57	0.032	0.990	0.80	

510 Notes: P, R_m and λ = parameters of Gompertz equation.

512 4. Discussions

The changes in the OPW (i.e. weight loss, variations of TS and VS, and biogas production), mainly occurring in the first week of the process, were quite similar among the three ensiling conditions and the differences were not significant at all, with the exception of TS variations. The latter was determined by the very different conditions between WET and DRY ensiling mode in terms of water management. Weight loss and biogas production are coherent as observed in previous literature [20,27]. Also the pH, noticeably acid as usually recorded for raw OPW [1,19], was kept basically stable during the process.

520 The removal of d-Limonene was efficient under all the tested ensiling conditions, which 521 confirms the viability of its removal before OPW anaerobic digestion to increase the methane 522 yield of this process.

Presumably, during ensiling, OPW decomposition allows the d-Limonene-containing sacs in 523 the flavedo to rupture, and the simulaneous biogas production enhances its stripping. This 524 process is confirmed at a the sensorial level by the strong orange smell during biogas venting. 525 The treatment with ethanol did not increase the d-Limonene removal rate compared to the 526 untreated biomass (on average by 70% against 72%), whereas simple centrifugation reduced 527 this rate by only 47%. These results are obviouly influenced by a number of factors (specific 528 cultivar and ripening stage of oranges, type of processing, ensiling conditions), in order to 529 confirm these results, experiments at a larger scale would be beneficial. 530

The low efficacy of the OPW chemical treatment may be explained by the scarce suitability of ethanol for d-Limonene leaching (despite its biodegradability, which suggested its use for this scope) for the chemical treatment and the low solubility of d-Limonene in water and the high affinity of the solid compounds of OPW for centrifugation. With regards to the latter, in fact, d-Limonene concentration was higher in the centrifuged OPW compared to the simply ensiled biomass. In terms of residual VS content after ensiling and treatments, natural ensiling, allowing the minimum removal (on average 45% of the initial content against 51% of chemical treatments and 54% of the centrifugation), assures the lowest loss of VS and thus, potentially, a more efficient preservation of the bio-methane potential production of OPW.

Therefore, this study suggests using natural ensiling to decrease the d-Limonene loads in the substrate without further treatments, since this choice maximises the removal and minimizes complexity and cost of the processing. Wet conditions are not advised, because a higher reduction of VS content is achieved, which may determine lower bio-methane yields. For dry conditions the overall balance of the ensiling process would be more favourable if a valorisation option (e.g. for bio-ethanol production or as an additive to wastewater treatment plants for denitrification) is found for the leachate extracted from OPW.

548 Centrifugation is not advisable since it causes an additional loss of substrate (e.g. soluble 549 sugars, lactic acid) through the discarded liquid and does not improve the efficiency of d-550 Limonene removal.

Under the microbiological approach, LAB population increases throughout the process, as 551 expected considering the type of fermentation characterizing the ensiling. This increase 552 corresponded to a decrease in yeast population, observed with a more noticeable trend in 553 leachate of ensiling under dry conditions than in the others. As facultative anaerobes, yeasts 554 were not suppressed during ensilage. Despite the presence of EO in the matrix, both yeasts 555 and LABs grew and persisted to the end of the ensilage. Most likely, the autochthonous 556 microorganisms are accustomed to the OPW environment confirming a certain adaptation as 557 reported for the treatment of citrus processing wastewater in aerated ponds [43,44]. 558

The analysis of the organic acids confirmed that LAB population was dominant, since butyric acid produced by Clostridia was absent [25]; the very low initial pH presumably helped to prevent their presence in the reactors.

The study confirms that the d-Limonene removal, the particle size reduction, and the biomass 562 homogenization and fermentation during ensiling and/or the subsequent treatments 563 significantly improve the specific (that is, the methane production per unit VS added) 564 efficiency of the OPW energy conversion by AD. As a matter of fact, higher methane yields 565 were measured for ensiled OPW (close to upper limit of the literature range [13,47]), 566 compared to the raw substrate, which is close to the literature average [1,3,12]. In the case of 567 the OPW subjected to the chemical treatment (ENS+CHEM, WET+CHEM), the 568 biodegradation of residual ethanol [48] presumably enhanced the methane yield, since ethanol 569 can be an additional carbon source for microorganisms. In the other cases (ENS, WET, DRY, 570 571 ENS+CEN, WET+CEN, DRY+CEN), it is possible that the high methane yields can be ascribed to the peculiar characteristics of the inoculum. The latter was taken from a full-scale 572 anaerobic digester, where fresh and ensiled OPW is routinely used as co-substrate during the 573 orange processing season. For this reason, the inoculum is adapted to the tested substrate, 574 increasing methane yields [28]. 575

The increase in methane yield partially compensated for the reduction in VS during ensiling. 576 In general, the process regularly evolved, that is, no evidence of partial inhibition was 577 observed, except for the reactors fed with centrifuged OPW and, especially, for the reactors 578 with chemically treated OPW. In the first case the slight inhibition was presumably due to the 579 higher residual d-Limonene content (over the inhibition limit of the anaerobic process) of 580 centrifuged OPW compared to the other treatments, Table 6). However, this partial inhibition 581 played a lower effect on methane yields compared to other BMPs of literature [7,18]. For the 582 substrates treated with ethanol the inhibition was more evident; it was due to the adaptation of 583 the microbial consortium to this compound [48]. 584

Among the tested BMPs, the treatment with ethanol gave the highest methane yields but also caused an irregular AD process, while the simple centrifugation of OPW was not efficient

compared to the other techniques. The analysis of the parameters estimated for Gompertz equation confirms that ensiling significantly improves the net specific methane yield and in many cases also the degradation rates, as shown by the highest R_m estimated for the naturally ensiled OPW and the biomass subjected to chemical treatment after natural ensiling).

591 The best performing treatment is ENS, which reduces methane production by 11% compared

592 to to the AD of the raw OPW, ENS+CHEM (-16%) and DRY (-18%).

The methane production of OPW digested after ensiling (natural or subjected to the treatments of centrifugation or solvent extraction) is 55 - 89% of the production of the same quantity of raw OPW in AD under the same process conditions (Table 6).

596

597

598 **5. Conclusions**

599

The possibility to increase the viability of the anaerobic digestion of OPW through ensiling and subsequent treatments has been explored in this study. The laboratory tests have confirmed that biomass storage allows a high (over 70%) d-Limonene removal but with heavy significant reductions (41 - 63% compared to the raw OPW) of the content in volatile solids (to be degraded during the energy conversion process). ENS and DRY ensiling modes without subsequent treatments appear to be the most suitable techniques since they minimize the reduction in CH₄ production of the overall process.

LAB and yeast species associated with ensiled OPW were assessed for the first time. The microbiological population showed high biodiversity that can be further explored with the aim of applying specific microbial strains as ensiling inocula to try to further accelerate the process with a subsequent better preservation of the methane potential.

Further research is needed to select more efficient biodegradable solvents for improving d-611 Limonene removal from ensiled OPW and to suggest additional valorisation opportunities for 612 the leachate released from DRY ensiling. 613 614 Acknowledgement 615 616 We thank the journal Editor and three anonymous Reviewers, whose comments helped 617 improve this manuscript. 618 619 References 620 D.A. Zema, P.S. Calabrò, A. Folino, V. Tamburino, G. Zappia, S.M. Zimbone, [1] 621 Valorisation of citrus processing waste: A review, Waste Manag. 80 (2018) 252-273. 622 doi:10.1016/J.WASMAN.2018.09.024. 623 [2] B. Ruiz, X. Flotats, Citrus essential oils and their influence on the anaerobic digestion 624 process: overview, 625 An Waste Manag. 34 (2014)2063-2079. doi:10.1016/j.wasman.2014.06.026. 626 V.N. Gunaseelan, Biochemical methane potential of fruits and vegetable solid waste [3] 627 (2004)feedstocks, Biomass and Bioenergy. 26 389-399. 628 doi:10.1016/j.biombioe.2003.08.006. 629 [4] M.A. Martín, J.A. Siles, A.F. Chica, A. Martín, Biomethanization of orange peel waste, 630 Bioresour. Technol. 101 (2010) 8993-8999. doi:10.1016/j.biortech.2010.06.133. 631 [5] R. Wikandari, H. Nguyen, R. Millati, C. Niklasson, M.J. Taherzadeh, Improvement of 632

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SUPPLEMENTARY INFORMATION



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772	Figure 1 - SI – Images of Orange Peel Waste (OPW) ensiled in different conditions
773	in the laboratory tests.