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Paolo S. Calabrò[1], Filippo Fazzino[1], Rossana Sidari[2], Demetrio Antonio Zema[2]

[1] *Mediterranean University of Reggio Calabria, Department DICEAM, Via Graziella, loc. Feo di Vito, I-89122 Reggio Calabria, Italy.*

[2] *Mediterranean University of Reggio Calabria, Department Agraria, loc. Feo di Vito, I-89122 Reggio Calabria, Italy.*

Abstract

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

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

Highlights:

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

1. Introduction

Orange peel waste (OPW), the residue of orange juice production, is about 50-60% of the processed fruit (in weight). The specific physical and chemical properties of OPW (the high amount and seasonal nature of the production, the low pH, the high water content, the presence of essential oils - 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 compounds) and biofuels (e.g. biogas from anaerobic digestion - AD) [3–9]. However, the biomethane yield of OPW is curbed due to its high content of d-Limonene [6], which is highly toxic to microorganisms [2,10]. Three alternatives are available to overcome this limitation: (i) the preliminary removal of d-Limonene [5,7,11–13]; (ii) the co-digestion with other substrates [14–18]; (iii) the digestion of OPW alone adopting moderate organic loading rates (OLRs) and/or using additives [6,8,9].

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

2. Materials and methods

2.1. OPW sampling

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

2.2. OPW ensiling

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

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.

2.3. Treatments on ensiled OPW

After ensiling, the OPW was extracted from the batches and subjected to two treatments, 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 used as 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.

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).

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

2.4. Physico-chemical measurements

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 tests, the analysis is difficult for OPW, since the concentration is strongly influenced by the extraction conditions and the degradation level of the substrate. Moreover, the complexity increases 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 breaking of the small flavedo sacs which contain it. Following previous tests [9], which used a "mild" EO extraction only the d-Limonene that was available immediately after substrate feeding was determined .

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, MO, USA), which was used as internal standard, for two hours. This blend was then injected into a gas chromatograph (Agilent 6890) equipped with a wide-bore capillary column and a flame ionization detector (FID), the latter set at 250 °C. The capillary column (J&W DB-WAXetr 50 m x 320 mm x 1 mm) used nitrogen as gas carrier with a flow rate of 10 mL/min. The temperature, initially kept 50 °C for 8 min, was then raised to 230 °C (at 5 °C/min) for 2 min and finally set at 240 °C for 4 min during the post run.

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 μm) 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.

The amount of organic acids extracted was determined with a gas chromatograph (Agilent 6890) equipped with a wide-bore capillary column (CPWAX52CB, 50 m, i.d. ¼ 0.53 mm) and a flame ionization detector (FID). The injector was settled at 250°C. The temperature program started at 50°C, held for 5 min, the temperature was raised to 230°C at 5°C/min, held for 8 min, raised to 240°C and held for 2 min during the post run.

2.5. Biochemical methane potential (BMP) tests

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

1. BMP1: ENS; WET; DRY.
2. BMP2: ENS+CEN; WET+CEN; DRY+CEN.
3. BMP3: ENS+CHEM; WET+CHEM; DRY+CHEM.

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 non-specific biogas production (i.e. the production of the inoculum itself). The TS of the inoculum of the three BMP tests was 5.5±0.2%, the VS 69.5±2.4% 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±0.5 °C).

In each batch the substrate was mixed with 200 mL of inoculum at a ratio (on a VS basis) equal to 0.3, this value being in the range suggested by UNI/TS 11703:2018. According to the same regulation three nutrient solutions were also added, to supply nutrients and micronutrients for the bacterial metabolism. The three solutions (indicated as A, B and C) contained KH2PO4, Na2HPO4･12H2O, NH4Cl (A, 5% final volume), CaCl2･2H2O, MgCl2･6H2O, FeCl2･4H2O (B, 5% of final volume) and MnCl2･4H2O, H3BO3, ZnCl2, CuCl2, Na2MoO4･2H2O, CoCl2･6H2O, NiCl2･6H2O, Na2SeO3 (C, 1% of final volume). Finally, water was added to the batch, in order to reach final volume (600 mL) and to keep the TS content at about 35 gTS/L, which is consistent with 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 lower by 1% than the cumulated volume from the process start.

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 dioxide in the biogas was absorbed by an alkali solution (NaOH 3M), while the methane bubbles, 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 cumulated value since test start. The net specific methane production (that is, the methane volume, normalised to standard conditions, per unit of VS depurated by the blank production) was calculated as follows:

$$BMP = \frac{\left(V_{CH_{4},s} - V_{CH_{4},blank}\right)}{VS_{s} \cdot V_{s}} \qquad [1]$$

where:

- $V_{CH4,s}$ = final cumulated methane production (NmL_{CH4})

- $V_{CH4,blank}$ = final cumulated methane production of the blank (NmL_{CH4})

- VS_s =initial VS concentration of the substrate ($g_{VS}\cdot L^{-1}$)

- V_s =total volume of the batch (L)

According to the aforementioned Italian standard procedure, the test was accepted, if the batch fed with cellulose in the same BMP series produced 335 $NmL\cdot g_{VS}^{-1}$ ± 25%.

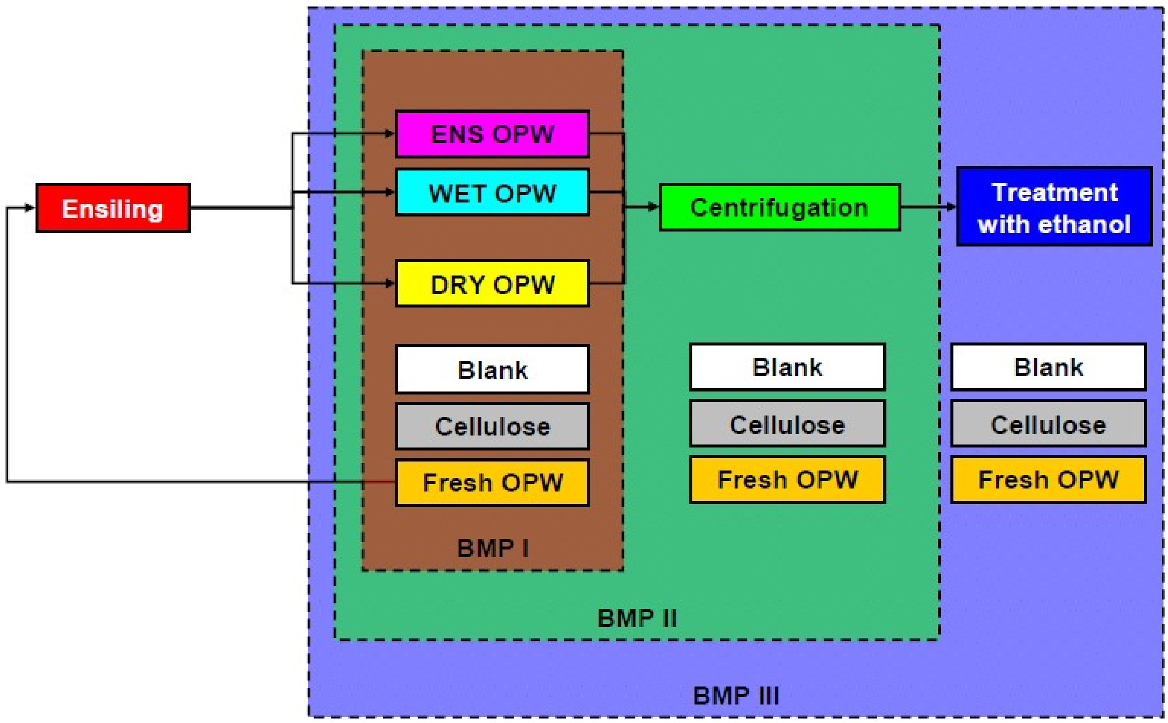


Figure 1. Experimental scheme of the BMP (I, II and III) tests carried out after OPW (orange peel waste) ensiling subjected to different ensiling conditions and treatments (ENS - naturally ensiled OPW; WET - OPW ensiled in wet conditions; DRY - OPW ensiled in dry conditions).

2.6. *BMP kinetic modelling*

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:

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

where:

- B ($NmL \cdot g_{VS}^{-1}$) = specific methane production at time t (d)
- P ($NmL \cdot g_{VS}^{-1}$) = specific methane production at $t = \infty$
- R_m ($NmL \cdot (g_{VS} \cdot d)^{-1}$) = maximum methane production rate
- λ (d) = lag phase duration.

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

2.7. *Statistical analyses*

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.

2.8. Microbiological analyses and strains isolation

The microbiota associated with raw and ensiled OPW (ENS, WET and DRY modes) was analysed at day 0 (raw OPW), 7, 14 and 28 of the ensiling period, the leachate collected during DRY ensiling was also considered. To this end, each type of solid sample was firstly homogenized to allow the microorganisms release from the solid matrix; more specifically, 10 g of each solid OPW sample was homogenized in a solution of 0.9% NaCl. Then, the obtained homogenates and the leachate of 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); (ii) de Man–Rogosa–Sharpe (MRS) agar (VWR International srl, Italy), supplemented with 15 mg/L cycloheximide (Oxoid), to count lactic acid bacteria (LAB); and (iii) Yeast Peptone Dextrose (YPD) agar (VWR, International srl, Italy), supplemented with 100 mg/L chloramphenicol (Liofilchem Diagnostici, Italy), to count yeasts. All the plates were incubated at 30 °C for two days under aerobic conditions for yeasts and TMC, and under anaerobic conditions for LAB. At day 0, 7, 14, 21, and 28 during ensilage, the colonies grown on YPD and MRS agar were randomly picked from the highest dilution sample [35]; then, the isolates were purified by streaking on the corresponding isolation medium and stored as glycerol stock at – 80 °C until use. The isolated bacteria were tested for catalase and for Gram by KOH method [36].

2.9. Restriction analyses and sequencing

DNA from overnight grown yeasts (101 isolates) and bacteria (97 isolates), isolated throughout the ensilage and from the different treatments, was extracted by InstaGene Matrix (Bio-Rad Laboratories, USA), according to the manufacturer's instructions. Then, yeasts were analyzed by PCR of the 5.8S-ITS regions using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and amplification conditions, according to [37], and bacteria were analysed by PCR of the 16S rRNA gene, using the Y1 (5′-TGGCTCAGAACGAACGCTGGCGGC-3′) and Y2 (5′-CCCACTGCTGCCTCCCGTAGGAGT-3′) primers, according to [38]. Firstly, yeasts and bacteria were grouped by Restriction Fragment Length Polymorphism (RFLP) of the 5.8S ITS rRNA region (HaeIII and HinfI restriction enzymes) 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 profile were chosen to sequence the 26S D1/D2 rRNA region (NL1 and NL4 primers) and 16S 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 sequences were analyzed and compared with the sequences of the National Center for Biotechnology Information (NCBI) using BLASTN [40]. To differentiate the genotypically closely related *Lactobacillus plantarum*, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum*, the multiplex PCR of *recA* gene was carried out, according to [41].

3. Results

3.1. Ensiling and subsequent treatments

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

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

OPW	**Loss (% on fresh weight)**	**TS (% on fresh weight)**		**VS**		
		initial	*final*	*initial*	*final*	
				(% on TS)	(% on TS)	(% on raw biomass)
RAW (non-ensiled)	-	17.8 ± 0.53	-	96.9 ± 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 ± 0.40*	9.2 ± 0.20 a	96.9 ± 0.01	94.6 ± 0.00 a	8.7 ± 0.19 b
DRY	2.72 ± 0.81 a	17.8 ± 0.53	13.1 ± 0.1 b	96.9 ± 0.01	94.6 ± 0.01 a	12.4 ± 0.09 c

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 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].

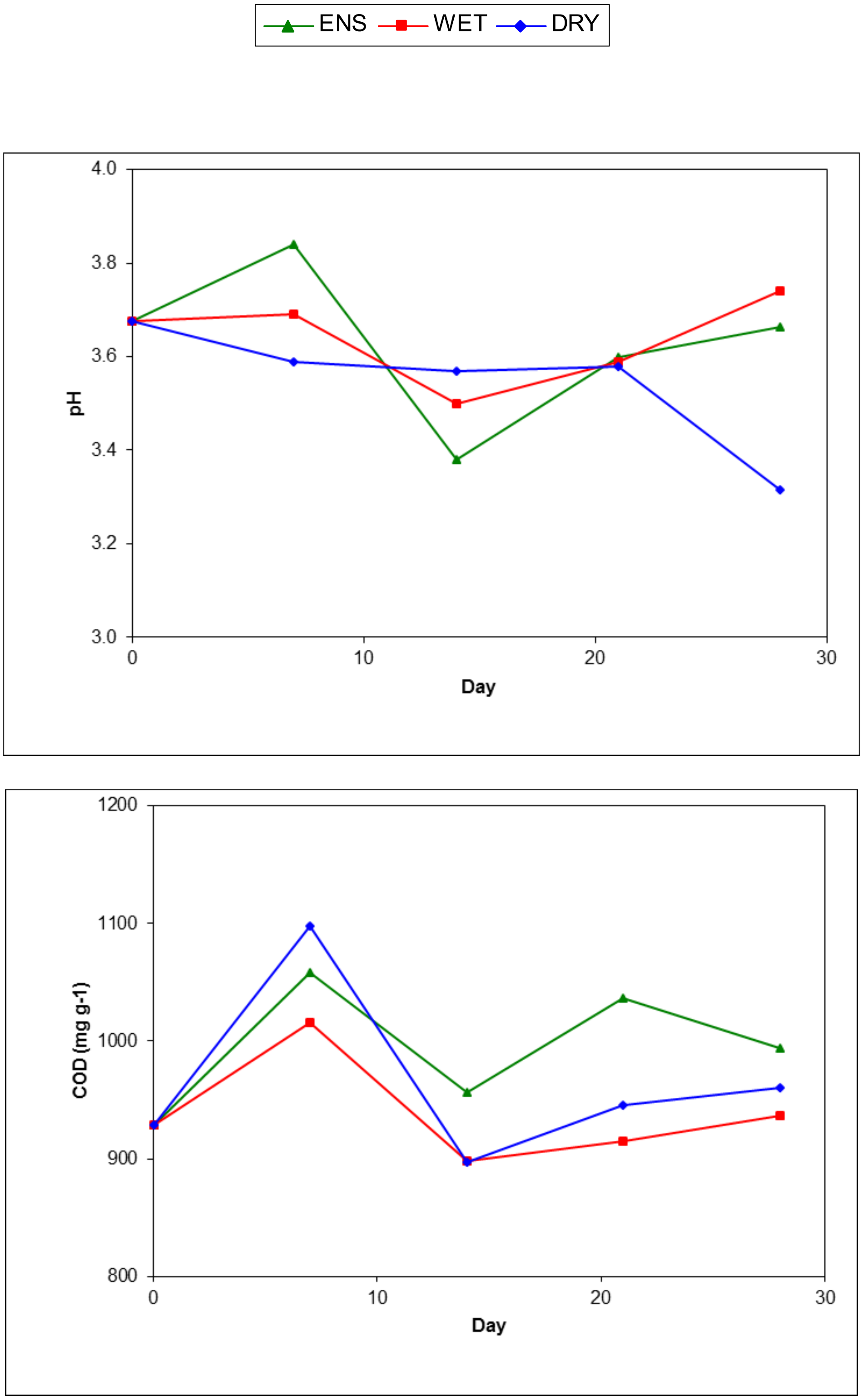
The highest reduction in TS was measured for ENS samples (-37.4% ± 3.44%) and the lowest for DRY (-26.2% ± 1.79%). VS reduced on average by only 2.4% ± 0.02% (WET and DRY) – 2.6% ± 0.05% (ENS) (Table 2). Also for TS and VS the parameters, the decrease was faster at the start of ensiling and then tended to slow (Figure 2). This is in agreement with biogas production that was quantitative only in the first days (Figure 2).

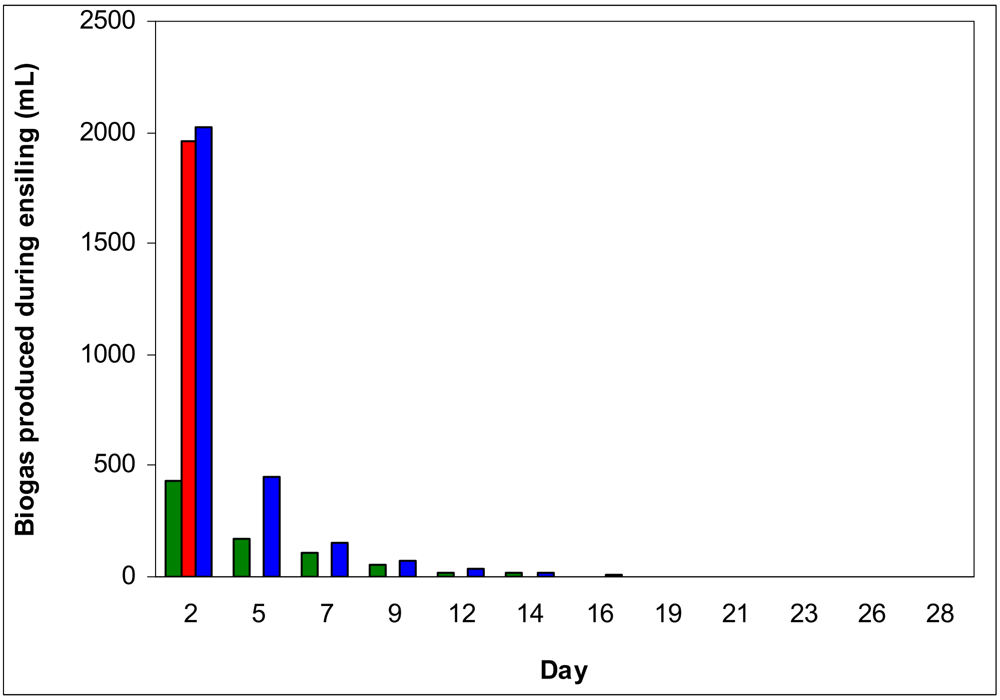
The initial COD of the OPW (928 ± 158 $mg \cdot g^{-1}$) did not noticeably change for the tested conditions and treatments, with a maximum value (994 ± 95 $mg \cdot g^{-1}$) measured for ENS and a minimum of 936 ± 41 $mg \cdot g^{-1}$ for WET (Figure 2).The pH (initially 3.7 ± 0.0) was stable for ENS 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).

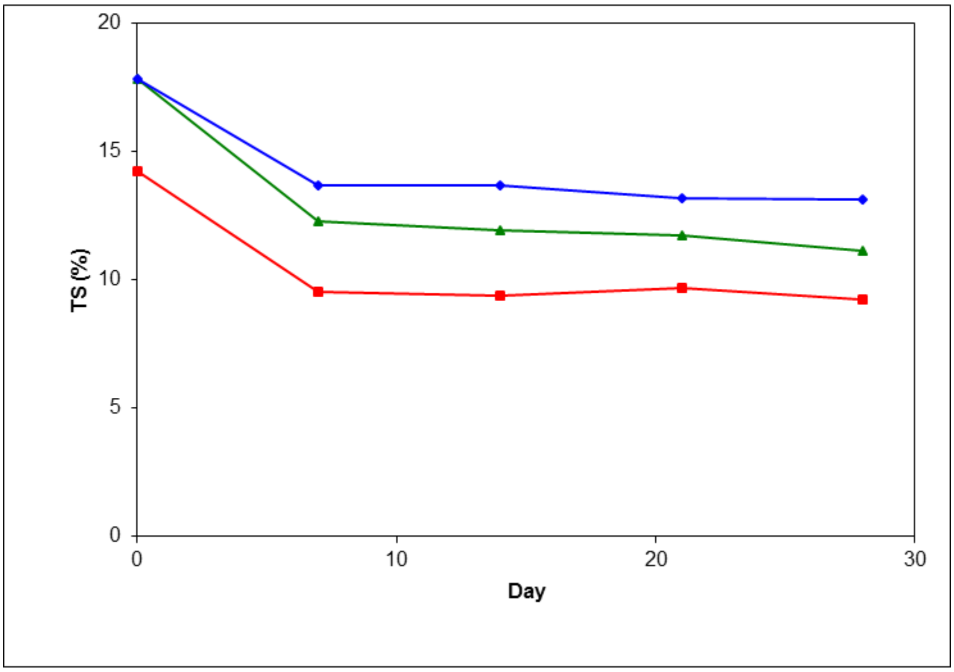
The liquid recovered by centrifugation from ENS and WET samples or collected at the reactor bottom for DRY samples (passive drainage) contained amounts of lactic acid, while butyric, and propionic acid have not been measured due to their low concentrations. Table 3 reports the lactic acid concentrations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

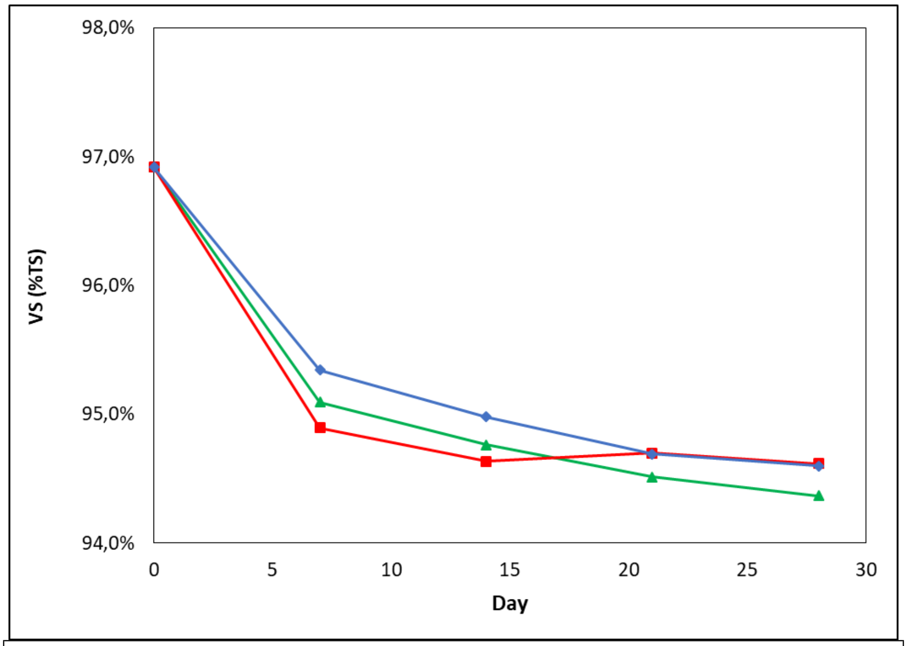
Table 3. Lactic acid concentration in the liquid phase separated by centrifugation (ENS and WET ensiling modes) or by passive drainage (DRY ensiling mode).

	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


ENS
WET
DRY
pH
4.0
3.8
3.6
3.4
3.2
3.0
0
10
20
30
Day
COD (mg g-1)
1200
1100
1000
900
800
0
10
20
30
Day


Biogas produced during ensiling (mL)
2500
2000
1500
1000
500
0
2
5
7
9
12
14
16
19
21
23
26
28
Day


TS (%)
20
15
10
5
0
0
10
20
30
Day



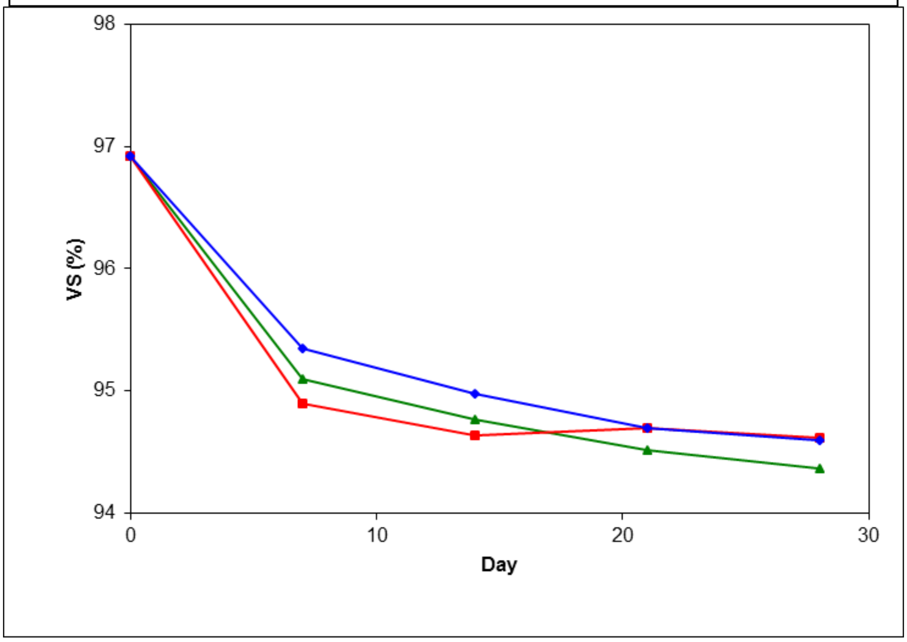


Figure 2. Temporal evolution of the main physico-chemical properties of the OPW (orange peel waste) subjected to different ensiling conditions and treatments (ENS - naturally ensiled OPW; WET - OPW ensiled in wet conditions; DRY - OPW ensiled in dry conditions)

Table 4. Values of d-Limonene and residual content of VS in OPW subjected to subjected to 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

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.

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

The reduction in VS content was in the range -41% (ENS) to -63% (WET and CHEM) with an average gradient CHEM (-54%) > CEN (-51%) > ENS/WET/DRY OPW (-45%) (Table 4).

3.2 Microbiological changes

The microbial loads refer to raw OPW analysed before the start of ensiling and to OPW treated by ENS, WET (solid material), and DRY methods (solid material and leachate). As regards the ENS and WET OPW, the aerobic TMC loads gradually increased up to the maximum values at the 14th day of ensilage. Then, the population decreased down to 7.50 and 7.71 Log CFU/mL, respectively, after 28 days. The decrease was more marked in WET OPW compared to ENS samples. On the contrary, the TMC loads of the leachate of DRY OPW always increased until 9.53 Log CFU/mL at the end, but the rate of increase was higher until the 14th day and lower thereafter (Figure 3).
Acetic acid bacteria (AAB) were only counted in raw OPW, therefore at day 0. Then, the bacteria detected were LAB. At the first stages of ensiling, 0 and 7 days, for all samples the load of yeasts was higher compared to LAB Subsequently, LAB were present in greater quantity than yeasts and 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) yeast counts were lower (4.72 - 4.51 Log CFU/mL) than in ENS OPW (6.11 Log CFU/mL) while 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).

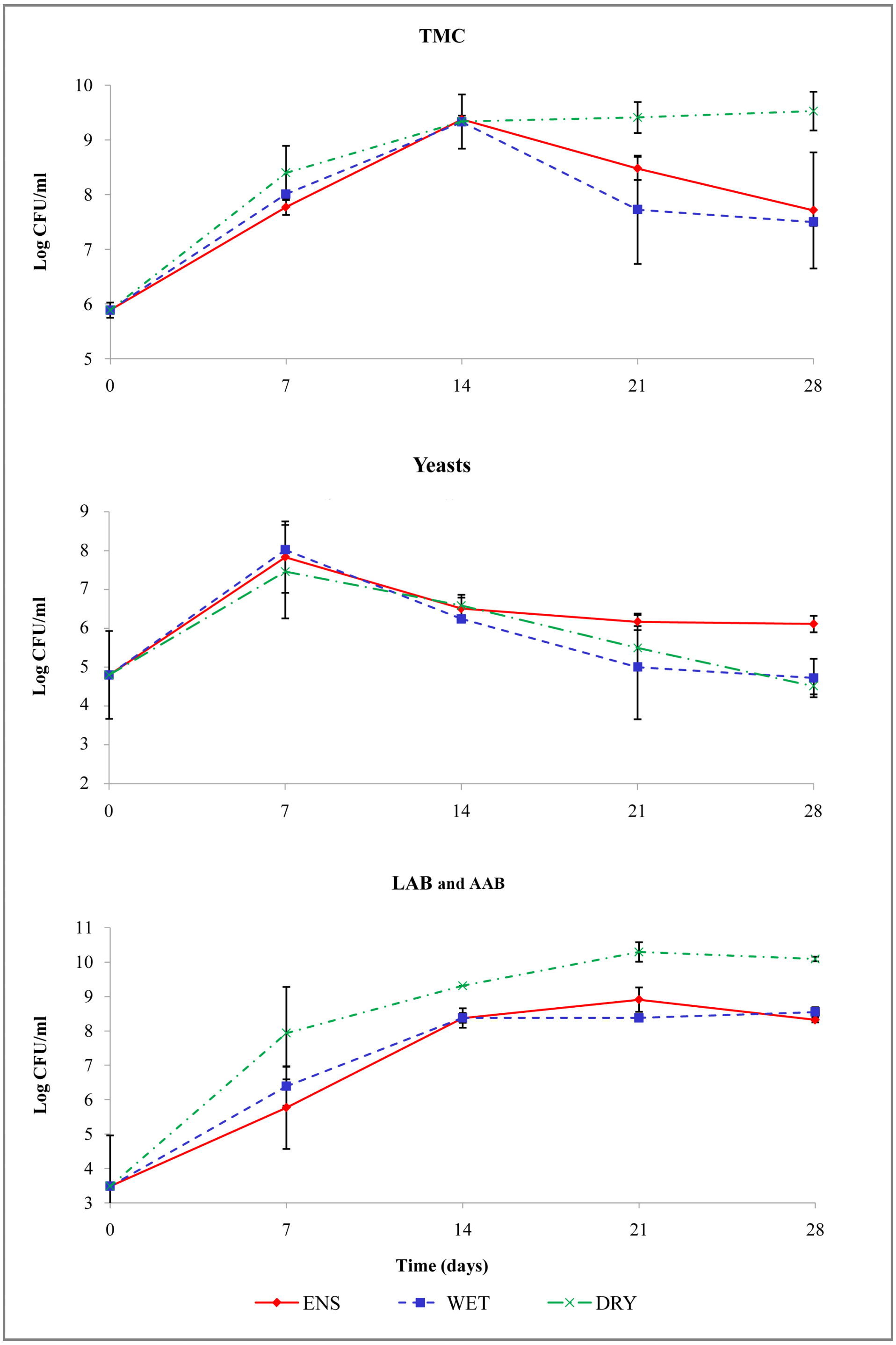


Figure 3. Total microbial count (TMC), yeasts, as well as lactic (LAB) and acetic acid (AAB) bacteria counts of raw OPW (day 0) and OPW subjected to different ensiling conditions (ENS: naturally ensiled OPW; WET: OPW ensiled in wet conditions; DRY: leachage of OPW ensiled in dry conditions).

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.

3.3 Bacteria and yeast's identification

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

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

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].

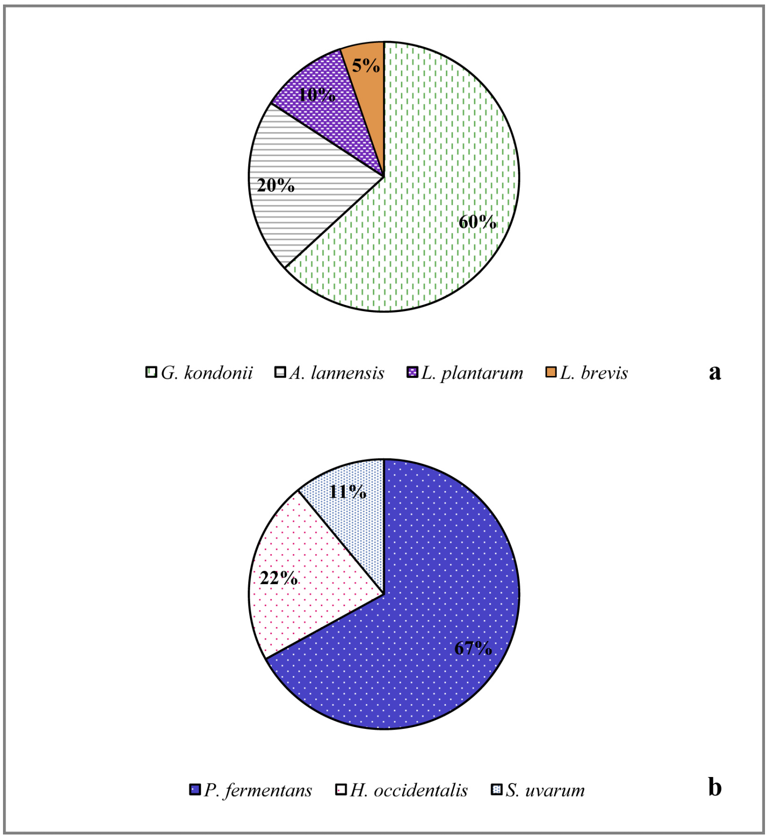


Figure 4. Number (in % on the total) of LAB and AAB (a) and yeast (b) species recovered from raw orange peel waste (OPW) (day 0).

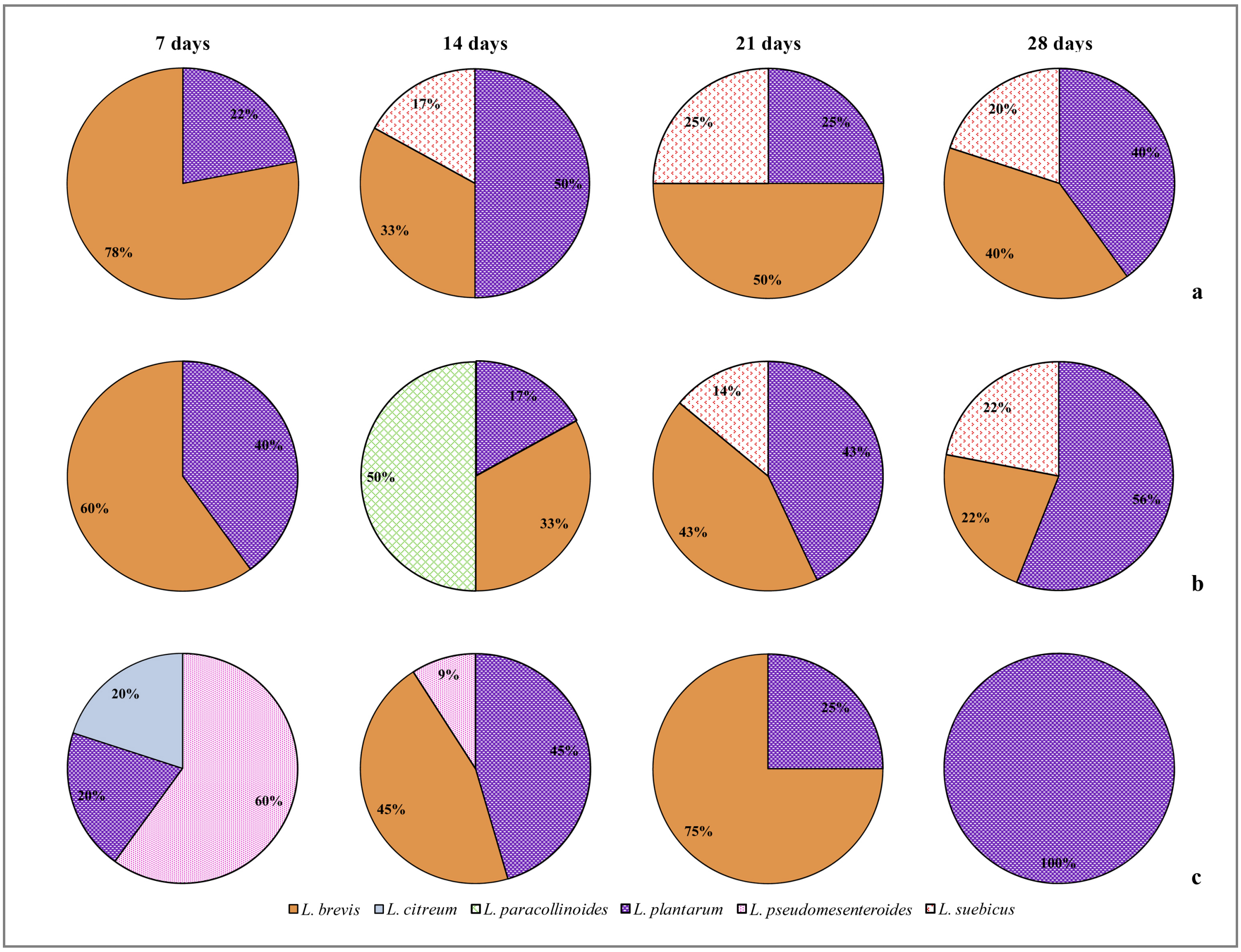


Figure 5. Number (in % on the total) of bacteria recovered from ENS (a), WET (b), and leachate of DRY (c) orange peel waste (OPW) throughout ensilage.

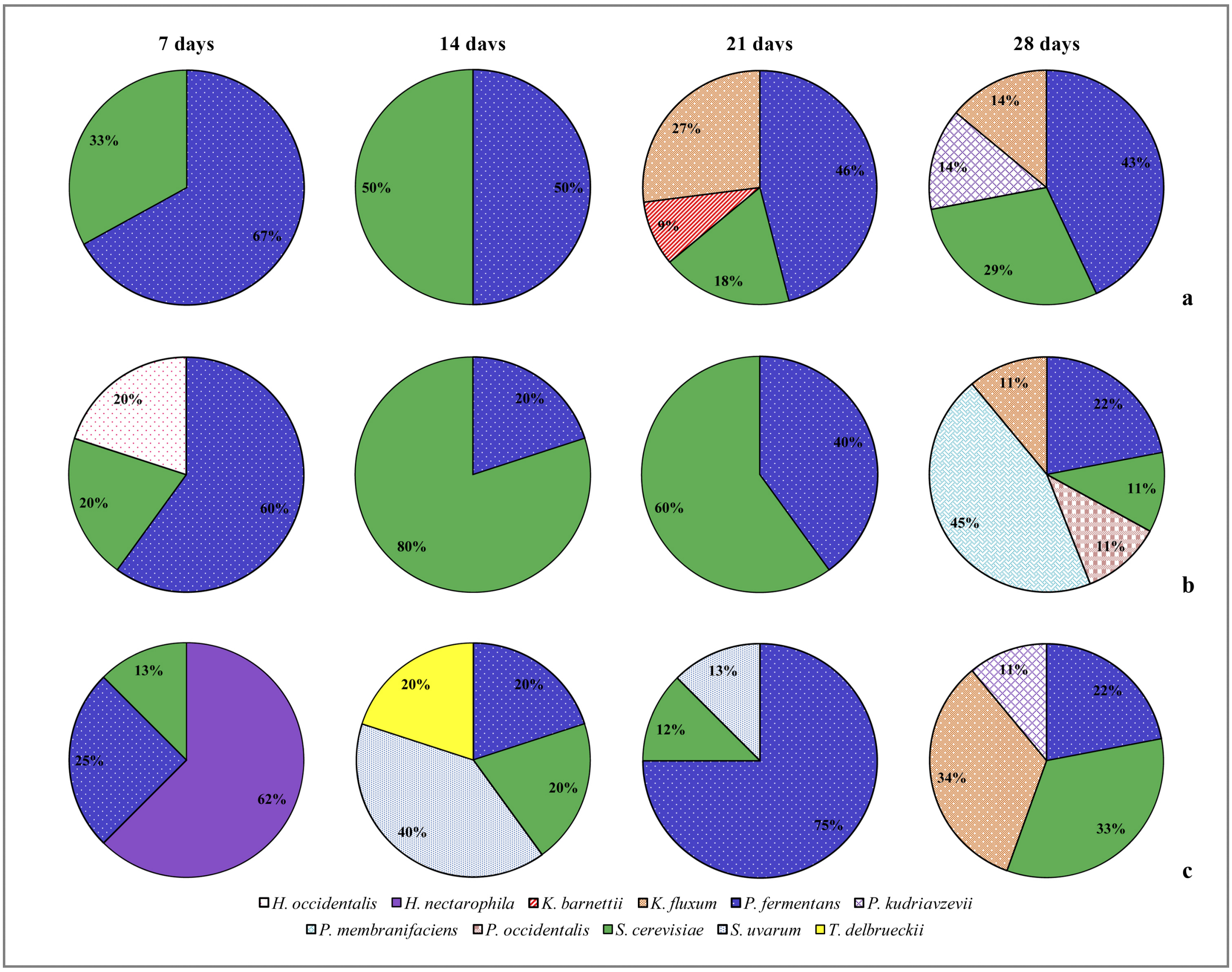


Figure 6. Number (in % of the total) of yeasts recovered from ENS (a), WET (b), and leachate of DRY (c) orange peel waste (OPW) throughout ensilage.

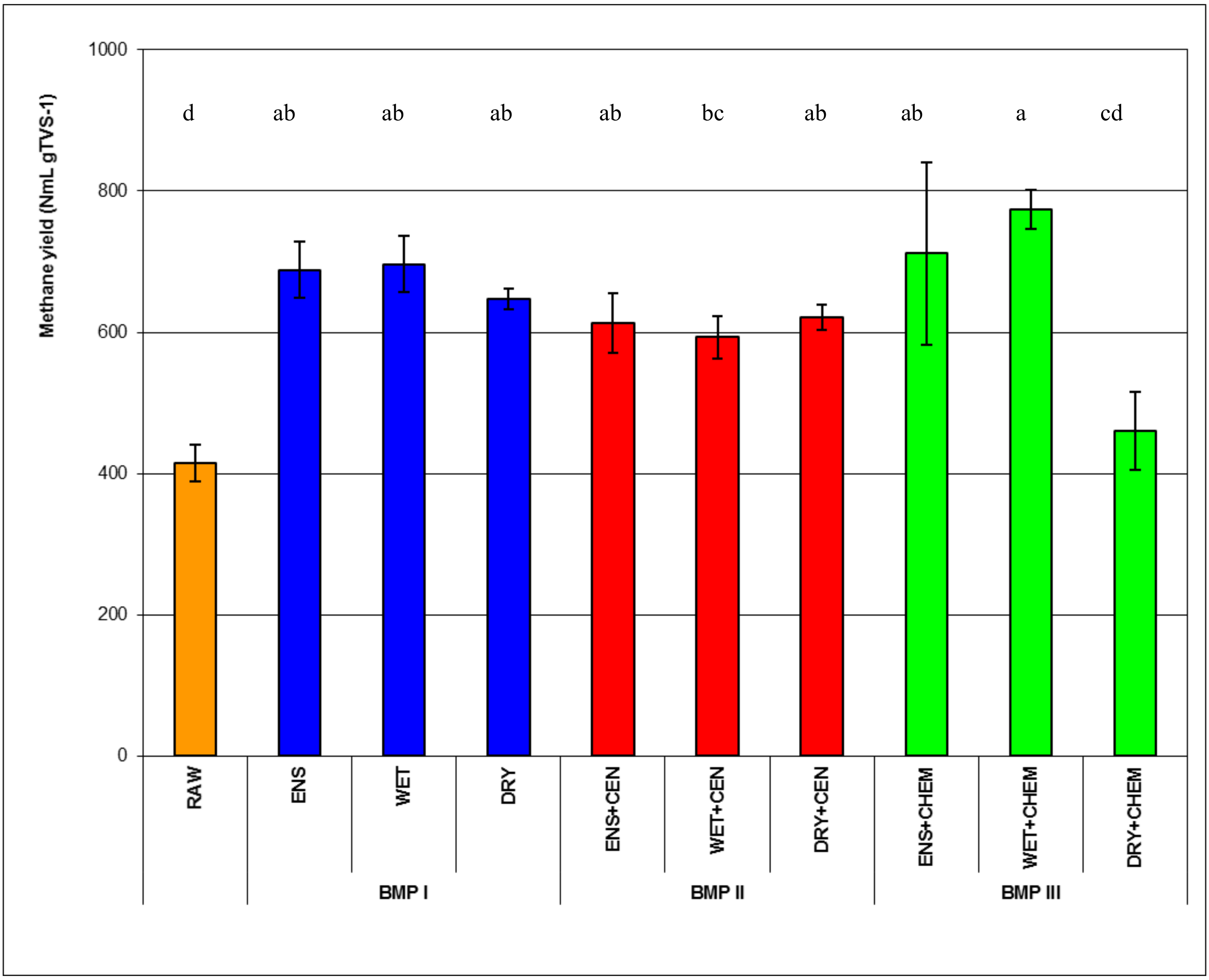
3.4 BMP test results

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 ± 26.4 $NmL \cdot g_{VS}^{-1}$). 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.

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

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.

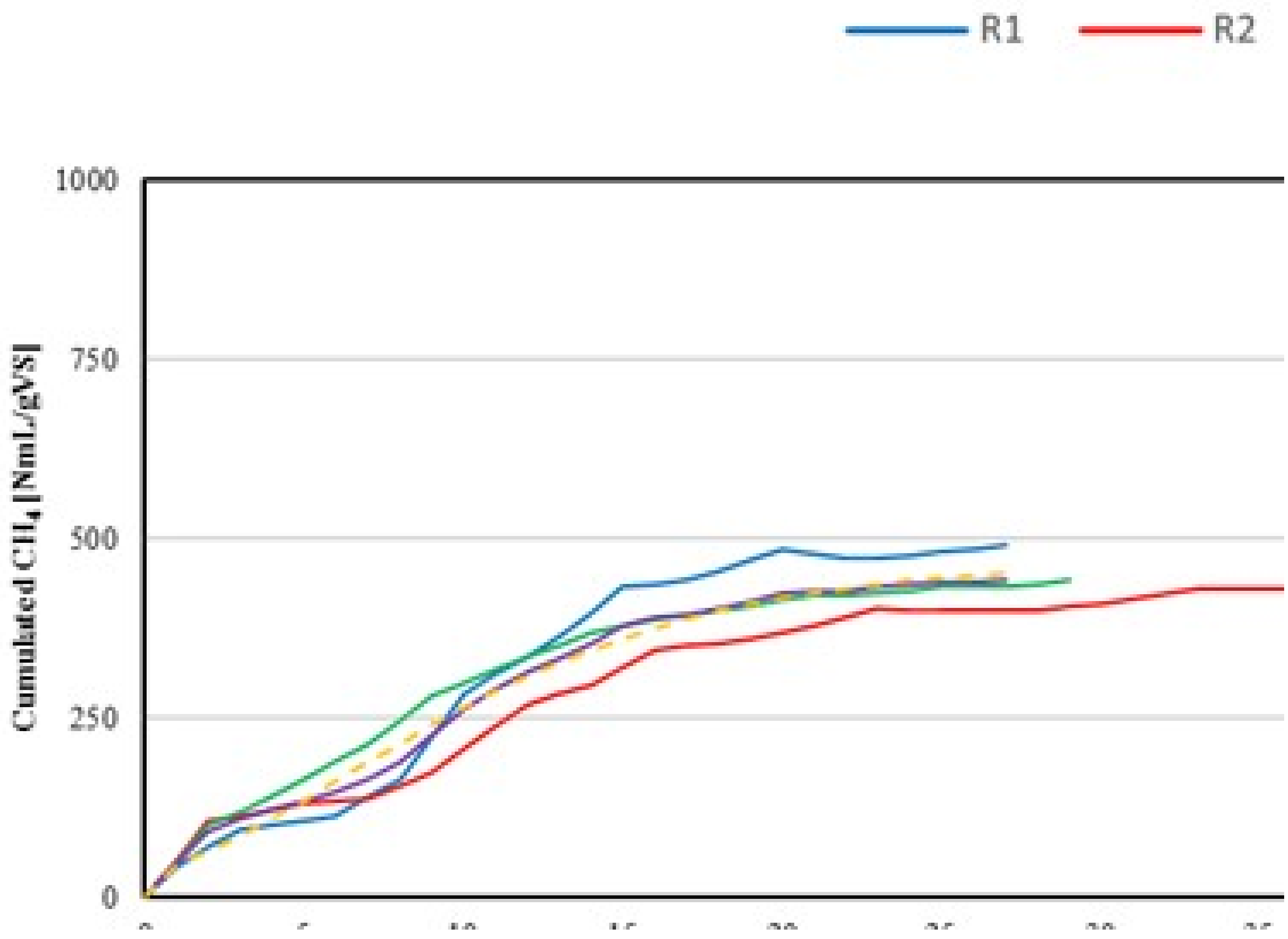
	Strains	**Species**	**Similarity (%, accession no. of the closest relative by Blast)**
Yeasts	PY 1	*Saccharomyces uvarum*	100% - KY109468.1
	PY 12	*Saccharomyces cerevisiae*	100% - NG_042623.1
	PY 21	*Hanseniaspora nectarophila*	97% - NG_055397.1
	PY 2	*Pichia fermentans*	99.82% - KY108804.1
	PY 82	*Pichia 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

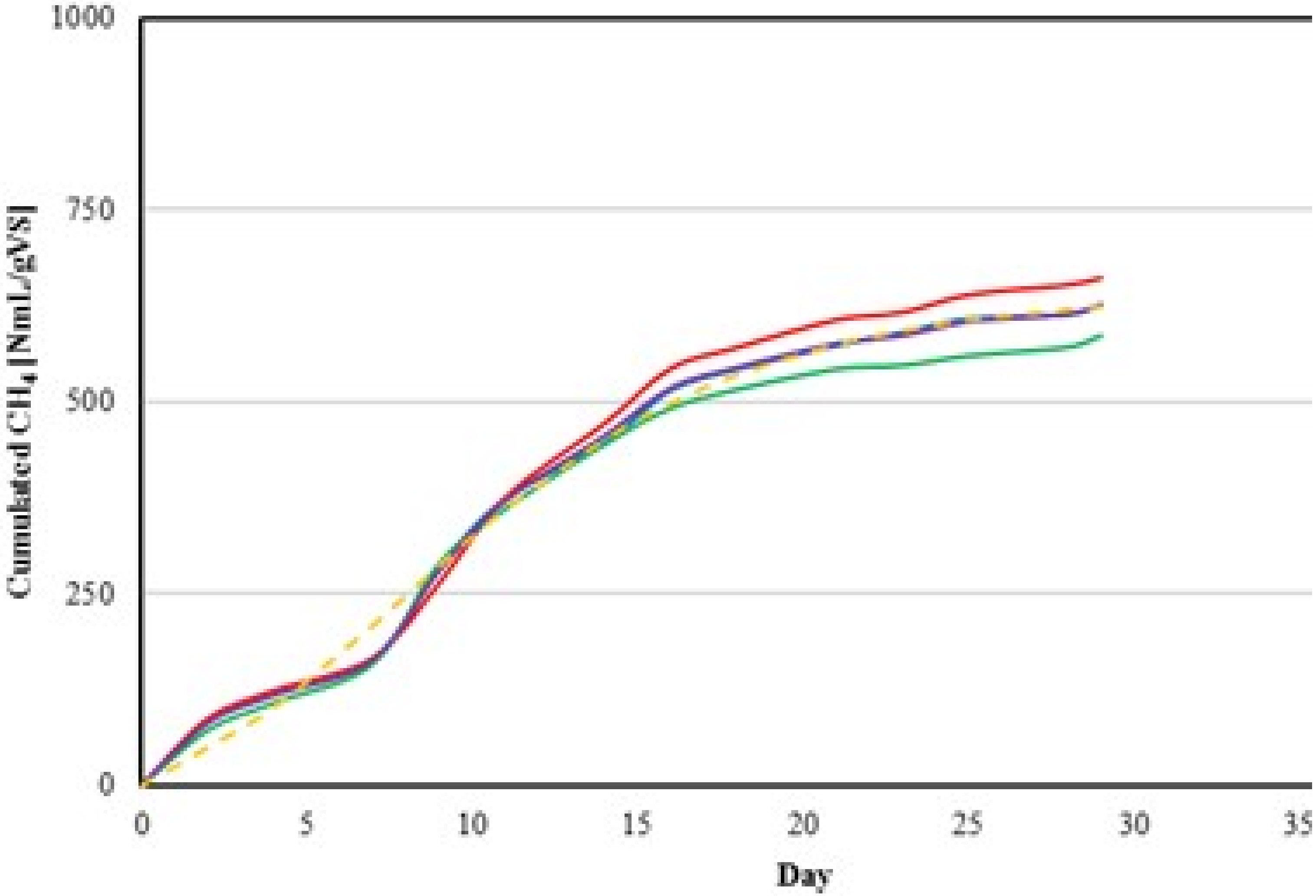


Note: different lowercase letters indicate significant differences (at $p < 0.05$).

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

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 digestion of OPW). This theoretical production can not be achieved because of the reasons already explained.


R1
R2
Cumulated CH_4 [NmL/gVS]
1000
750
500
250
0



501

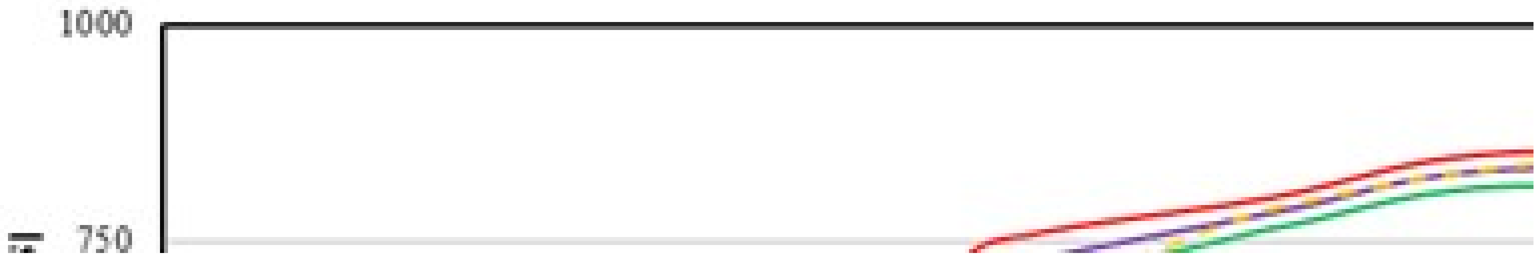


Figure 8. Cumulated net specific methane yields and interpolating Gompertz equation for the orange peel waste (OPW) under the tested conditions and treatments: a. raw OPW; b. ENS OPW; c. WET OPW; d. DRY OPW; e. ENS+CEN OPW; f. WET+CEN OPW; g. DRY+CEN OPW; h. ENS+CHEM OPW; i. WET+CHEM OPW; l. DRY+CHEM OPW).

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.

OPW	**P** ($NmL \cdot g_{VS}^{-1}$)	**λ (d)**	$\mathbf{R_m}$ ($NmL \cdot (g_{VS} \cdot d)^{-1}$)	$\mathbf{r^2}$	$\mathbf{CH_4/CH_{4raw\ OPW}}$
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

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

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.

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

Presumably, during ensiling, OPW decomposition allows the d-Limonene-containing sacs in the flavedo to rupture, and the simulaneous biogas production enhances its stripping. This process is confirmed at a the sensorial level by the strong orange smell during biogas venting.

The treatment with ethanol did not increase the d-Limonene removal rate compared to the untreated biomass (on average by 70% against 72%), whereas simple centrifugation reduced this rate by only 47%. These results are obviouly influenced by a number of factors (specific cultivar and ripening stage of oranges, type of processing, ensiling conditions), in order to confirm these results, experiments at a larger scale would be beneficial.

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.

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

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

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 homogenization and fermentation during ensiling and/or the subsequent treatments significantly improve the specific (that is, the methane production per unit VS added) efficiency of the OPW energy conversion by AD. As a matter of fact, higher methane yields were measured for ensiled OPW (close to upper limit of the literature range [13,47]), compared to the raw substrate, which is close to the literature average [1,3,12]. In the case of the OPW subjected to the chemical treatment (ENS+CHEM, WET+CHEM), the biodegradation of residual ethanol [48] presumably enhanced the methane yield, since ethanol can be an additional carbon source for microorganisms. In the other cases (ENS, WET, DRY, 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 anaerobic digester, where fresh and ensiled OPW is routinely used as co-substrate during the orange processing season. For this reason, the inoculum is adapted to the tested substrate, increasing methane yields [28].

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

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).

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

5. Conclusions

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 CH_4 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-Limonene removal from ensiled OPW and to suggest additional valorisation opportunities for the leachate released from DRY ensiling.

Acknowledgement

We thank the journal Editor and three anonymous Reviewers, whose comments helped improve this manuscript.

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SUPPLEMENTARY INFORMATION

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