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Enhanced Biomethanation: The Impact of Incorporating Fish Waste on the Co-Digestion of Pig Slurry and Orange Pomace

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Abstract: Anaerobic digestion technology can play a significant role in the transition to a low-carbon and circular economy by producing bioenergy (biomethane) and organic fertilizer (digestate). This study proposes a valorization approach for three waste streams widely produced in the Mediterranean area: fish waste (FW), pig slurry (PS), and orange pomace (OP). The FW lipid content can enhance biomethane yield as long as inhibition by long-chain fatty acids is prevented. In this study, the effect of introducing 25% and 50% FW to the anaerobic co-digestion of a reference mixture consisting of 80% PS and 20% OP pulp (OPP) was studied. Co-digestion using 50% FW presented the maximum biomethane yield (669.68 ± 8.32 mL CH_4/g VS_{added}), which corresponds to a 37% increase compared to the reference. No inhibition was detected during the anaerobic digestion assay. The kinetic study showed that the introduction of FW led to a reduction in the degradation rate constant by up to 30%. The lag phase increased as FW content increased, with 50% FW presenting a lag time approximately three times that of the reference mixture. The proposed strategy can encourage sustainable waste management practices and contribute to GHG emissions mitigation.

Keywords: bioenergy; biomethane; degradation constant; lag time; lipids content

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1. Introduction

Exponential population growth and industrialization have resulted in increased energy shortages and environmental degradation. The observed climate change has become a major concern, and greenhouse gas (GHG) emissions from anthropogenic activities, particularly CO_2, have been identified as one of the main causes of global warming [1].

In response to the observed environmental degradation, the European Commission has created several policies aimed at promoting renewable energy from endogenous sources to increase its energy independence. The "Clean Energy for all Europeans" legislative package is currently in force, where the renewable energy directive (RED I) is renewed in directive 2018/2001/EU (RED II). This package promotes a reduction of between 45 and 55% in GHG emissions compared to 2005. The incorporation of 47% of the energy produced from renewable sources and a 35% reduction in primary energy consumption will idealize an improvement in energy efficiency by 2030 [2].

Recovering energy from biomass can play an important role in energy transition, particularly if waste is used, as it also promotes the circular economy, with the possibility of establishing biorefinery approaches that contribute to rural development and sustainability.

Portugal is one of the highest consumers of fish worldwide, with an annual consumption exceeding 50 kg per capita, which is well above the world average of 20.5 kg per capita [3]. This has led to the growth of fish processing industries that have replaced conventional salting and drying conservation techniques with refrigeration and freezing [4].

According to the FAO [3], approximately 35% of the average global production from fisheries and aquaculture corresponds to waste losses. Fish waste (FW) usually consists of heads, viscera, bones, blood, and scales, but it may also contain whole fish that are considered nonsuitable for human consumption (i.e., those that do not meet all the necessary quality and safety criteria). Currently, in Portugal, FW is mainly used for the production of fish meal and oil [5] and products of low commercial value [6]; therefore, there is an opportunity to create other solutions for bioenergy production.

Fish waste is rich in lipids and proteins and therefore has a high energy content, which can result in a high biomethane yield in the anaerobic digestion process [7]. Nevertheless, few studies have reported biogas production from FW under mono- or co-digestion. According to the literature, the mono-digestion of FW can produce 445, 464.5, and 540.5 L CH_4/kg VS_{added}, respectively, with the waste consisting of heads, viscera, skin, spines, tilapia scales [8], intestines, the digestive tracts and viscera of snappers, croakers, tuna [9], and carp viscera [10].

However, the hydrolysis of protein and lipid fractions during AD can lead to the accumulation of ammonium and long-chain fatty acids (LCFAs), causing inhibition. LCFAs can attach to the surface of microbial cells and block mass transfer, leading to biomass flotation [11]. Furthermore, Tian et al. [12] verified the synergetic co-inhibition of the AD process in continuous reactors when LCFAs and ammonia concentrations were above 1.1 g Oleate/L and 4.5 g N-NH_4^+/L, respectively. According to the authors, ammonia inhibition results in an increase in volatile fatty acids (VFAs) and hydrogen concentrations, which limits the β-oxidation of LCFAs, leading to the further accumulation of LCFAs and, consequently, the higher inhibition of AD.

Anaerobic co-digestion (AcoD) is frequently adopted to avoid the inhibitions caused by lipid-rich waste, as it allows toxicity dilution, C/N ratio balancing, and higher energy recovery due to the higher theoretical biomethane yield of lipids compared to protein and carbohydrates [13]. Nevertheless, Wu and Song [14] reported a large inhibition as they increased the content of FW from 3% to 6% in a mixture with activated sewage sludge, with a decrease in biomethane production from 683.8 ± 36.4 L CH_4/kg VS_{added} to 52.6 ± 10.3 L CH_4/kg VS_{added}.

When carrying out AcoD with lipid-rich waste, it is necessary to introduce a substrate rich in carbohydrates, such as fruit waste, to adjust the C/N ratio for the microbial consortium [13].

Orange production in the EU is concentrated in the Mediterranean region, with Spain and Italy dominating it, followed by Greece and Portugal [15]. In Portugal, the Algarve region stands out, hosting 90% of overall orange production and producing 355 thousand tons in 2020 [16]. This fruit is widely used for processing orange juice, from which the resulting residue (orange pomace) represents 50% of the total mass of the fruit [17] and essentially consists of its peel.

Orange pomace (OP) has been used for the production of biogas [18]; however, there may be inhibitions due to its high biodegradability, leading to an accumulation of VFAs and, consequently, to the acidification of the medium; or due to the presence of d-limonene, which can be toxic to the microbial consortium [19]. AcoD can prevent the inhibition caused by d-limonene by diluting this component and introducing a substrate that provides alkalinity to avoid acidification due to VFA accumulation, such as pig slurry (PS) [20].

Several authors have developed studies on anaerobic co-digestion using FW, OP, or PS. FW has been co-digested with sewage sludge [14,20], the liquid fraction of the hydrothermal carbonization of bamboo residue [21], vegetable waste [22], sugar cane bagasse [23], strawberry waste [24], biowaste [25], Jerusalem artichoke [26], fruit and vegetable waste [27], and sisal pulp [28]. The combination of FW and PS in a ratio of 5:95 was tested by Regueiro et al. [29], producing 348.1 L CH_4/kg VS_{added}, whereas Alvarez et al. [30] assessed the performance of the feed mixture PS:FW:glicerine (84:5:11), achieving 321 L CH_4/kg VS_{added}.

Other studies have addressed the co-digestion of PS with slaughterhouse waste [20], pineapple peel [31], coffee grounds from soluble coffee production [32], and leftovers [33].

Regarding OP, it has been co-digested with cow manure [34,35], catering waste [36], glycerol [37], sewage sludge [38,39], the organic fraction of municipal solid waste [40], and biowaste [41].

Table 1 presents the specific biomethane production (SMP) yields from the above-mentioned studies.

Table 1. Process conditions and specific biomethane production (SMP) yields from studies on the anaerobic co-digestion of FW, OP, and PS.

Feeding	SMP ($L\ CH_4/kg\ VS_{added}$)	Reference
SS:FW (97:3)	683.8	[14]
FW:VW (50:50)	463 ± 6.73	[21]
FW:LF_{HTC} (75:25)	133	[22]
FW:Sugar cane bagasse (75:25)	277.64	[23]
StW:FW (88:12)	205	[24]
FWsilage:BrW (80:20)	482	[25]
FW:Jerusalem artichoke (50:50)	531	[26]
FVW:FW (95:5)	580	[27]
Sisal pulp:FW (67:33)	620	[28]
PS:FW (95:5)	348.1	[29]
PS:FW:Glicerine (84:5:11)	321	[30]
PS:SW (52:48)	430	[20]
PS:PP (80:20)	580	[31]
PS:ISCG (70:30)	265	[32]
PS:Leftovers	521	[33]
OP:CM (53.5:46.5)	264.5 ± 5.2	[34]
OP:CM (50:50)	331.1 ± 11.7	[35]
OP:CW (50:50)	89.6	[36]
OP:glicerol (2.3:1)	330 ± 51	[37]
SS:OP (0.4 L + 0.3 g)	458.6	[38]
SS:OP (70:30)	165	[39]
OP:OFMSW (50:50)	294.63	[40]
OP:BW (50:50)	395.6 ± 1.6	[41]

BrW, bread waste; BW, biowaste; CM, cattle slurry; CW, catering waste; FVW, fruit and vegetable waste; FW, fish waste; FWSilage, fish waste silage; ISCG, industrial spent coffee grounds; LF_{HTC}, liquid fraction of hydrothermal carbonization of bamboo residue; OFMSW, organic fraction of municipal solid waste; PP, pineapple peel; PS, pig slurry; VW, vegetable waste; SS, sewage sludge; SW, slaughterhouse waste; StW, Strawberry waste.

As shown in Table 1, the SMP values present a wide range, but it is worth mentioning the best-performing cases for each substrate or co-substrate. Concerning FW, the maximum SMP value (683.8 $L\ CH_4/kg\ VS_{added}$) was achieved in a study by Wu and Song [14] using a mixture with sewage sludge, SS:FW (97:3). Nevertheless, all studies report the relevant enhancement of the methane yield by introducing FW when compared to the reference scenarios. For pig slurry, the highest SMP was reported by Azevedo et al. [31] for the co-digestion of PS and pineapple peel (PP) in a mixture of 80:20 in a continuous stirred reactor under mesophilic conditions, producing 580 $L\ CH_4/kg\ VS_{added}$. As for OP, in a recent study by Szaja et al. [38], co-digestion with sewage sludge, SS:OP (0.4 L + 0.3 g) produced 458.6 $L\ CH_4/kg\ VS_{added}$.

This study aimed to contribute to the solution of an industry-related problem in the Algarve region, which may be benchmarked in other Mediterranean countries. A fish processing unit was willing to design a valorization route that allowed for the recovery of bioenergy from the waste produced. Hence, this study investigated the potential of combining three waste biomasses available at a local/regional scale (OP, FW, and PS) for the production of biogas by AcoD, assessing the effect of increasing the lipid content as a result of FW addition.

2. Materials and Methods

2.1. Feedstock Collection and Pretreatment

Pig Slurry (PS)

Pig slurry was collected from a fattening/finishing pig farm in the Montijo region with 2500 pigs. Pig slurry management includes solid–liquid separation, followed by storage. The sample was collected from the storage tank under stirring, and, at the lab, it was further processed to remove coarse materials (2 mm tamization) and stored at −20 °C until further use.

Orange pomace (OP)

Orange pomace, containing flavedo, albedo, membranes, and some vesicles with juice, was collected from the Instituto Superior de Agronomia bar. The OP was cut into smaller pieces and crushed in a blender with water to obtain a pulp (OPP) with 44% OP. The obtained pulps were stored at −20 °C until further use.

Fish Waste (FW)

Fish waste was supplied by a processing fish industry located in the Algarve Region. The FW consisted of whole fish from the mackerel and sardine species, which did not meet all the quality criteria for human consumption. Between January and mid-October 2021, approximately 1.52% of the processed mackerel (28,552 kg) and 0.66% of the sardines (2869 kg) were discarded as waste.

The collected FW was ground in a blender at a ratio of 1:1 (m/m) of mackerel and sardines until a homogeneous paste was obtained, which was stored at −20 °C until further use.

Inoculum

The inoculum was collected from a full-scale anaerobic digester that treats mixed sludge under mesophilic conditions at a wastewater treatment plant in the Lisbon area. As recommended by Holliger et al. [42], the inoculum was pre-incubated for five days at the test temperature to acclimatize it and reduce its contribution to biogas production.

2.2. Characterization of Biomasses and Inoculum

The different biomass, feeding mixtures, and digestate samples were characterized according to standard methods [43] for the following parameters: pH, electrical conductivity (EC), total and volatile solids (TS and VS), total and volatile dissolved solids (TDS, VDS), total and soluble chemical oxygen demand (COD_T and COD_S), total alkalinity (TA), total Kjeldahl nitrogen (TKN), total ammonium nitrogen ($N\text{-}NH_4^+$), and phosphorous (P). The oil and fat contents in the fish waste and orange pomace pulp were determined using a Soxtec HT 1046 system.

The total organic carbon (TOC) was calculated using the method described by Cuetos et al. [44]; the C/N ratio was determined by dividing TOC by TKN values. All analytical determinations were performed in triplicate using analytical-grade reagents (≥99% purity).

Regarding the inoculum, characterization included total and volatile solids to determine their contribution to the organic matter in the anaerobic digestion process.

2.3. Biodegradation Assay Set-Up

Anaerobic digestion was carried out in batches using 500 mL Schott flasks with a working volume of 350 mL. Reactors were kept under mesophilic conditions (37 ± 0.5 °C) using a water bath. Each modality was analyzed in triplicate with an organic load of 4.5 g VS and an inoculum:substrate (I:S) ratio of 2:1, as recommended by the German standard VDI 4630 [45]. Each digester was connected to a 1 L Tedlar bag for biogas accumulation.

The reference feed mixture (R, 0% FW) consisted of 80% PS and 20% OPP, according to a previous study by Carvalho et al. [46]. This feed was used to prepare mixtures with a ratio of 75:25 (*v*/*v*) (25% FW, representing a lipid content of 4.3% TS) and 50:50 (*v*/*v*) (50%

FW, representing a lipid content of 5.5% TS) to evaluate the effect of FW on the stability and efficiency of the AcoD process. Table 2 summarizes the different feed mixtures studied.

Table 2. Feed mixture composition and codes.

Code	Feed Mixture Composition
R (0% FW)	80% PS and 20% OPP
25% FW	75% R and 25% FW
50% FW	50% R and 50% FW

To monitor process performance, daily biogas production was quantified using the water displacement method. As recommended by the German VDI 4630 standard [45], the test was terminated as soon as a daily biogas production of less than 1% of the accumulated production was obtained for three consecutive days. Regarding the determination of biogas quality (CH_4, CO_2, and H_2S), the procedure consisted of homogenizing the content of the Tedlar bags with the use of a 100 mL syringe (3×) and then collecting a sample for analysis using a Geotech 5000 biogas sensor (Denver, CO, USA).

To assess the stability of the anaerobic digestion process, the following parameters were determined at the beginning and end of the trial: pH, TA, VS, VDS, CODS, and $N\text{-}NH_4{}^+$, according to standard methods [43]. The volatile fatty acid (VFA) content was determined using the titration method recommended by Kapp [47] and quantified based on Equation (1) [48].

$$Total\ VFA\left(\frac{\text{mg}}{\text{L}}\right) = \left[\frac{131,340 \times (VpH4.0 - VpH5.0) \times Nacid}{Vsample}\right] - \left[\frac{3.08 \times VpH4.3 \times Nacid}{Vsample \times 1000}\right] - 10.9 \tag{1}$$

where *VpH*4.0, 4.3, and 5.0 are the volumes of sulfuric acid used in the titration until the pH values of 4, 4.3, and 5.0 were reached; *Nacid* is the concentration of the acid (0.1 N); *Vsample* is the volume of sample (20 mL).

2.4. Kinetic Studies

The effect of the introduction of FW on process kinetics was evaluated using two mathematical models: a first-order kinetic model and a modified Gompertz model.

First-order kinetic model

The first-order kinetic model was used to evaluate the substrate degradation rate k (d^{-1}). As indicated by Angelidaki et al. [49], a linearized version (3) of the first-order model Equation (2) was used, in which the degradation constant was derived from the slope of the line formed from the experimental data.

$$B = B_\infty \times \left(1 - e^{(-k \times t)}\right) \tag{2}$$

$$\ln\left(\frac{B_\infty - B}{B_\infty}\right) = -k \times t \tag{3}$$

where B is the accumulated biomethane production during time t (mL CH_4/g VS_{added}), B_∞ is the total accumulated biomethane production (mL CH_4/g VS_{added}), and k is the degradation constant (d^{-1}).

Modified Gompertz Model

The modified Gompertz model represented in Equation (4) has already been proven to be the most accurate for predicting the potential for biomethane production (P_0), the rate of biomethane production (R_m), and the lag phase time (λ), with an R^2 of 0.99 [21].

$$P = P_0 \times \exp\left\{-exp\left[\frac{R_m \times e}{P_0}\,(\lambda - t) + 1\right]\right\} \tag{4}$$

where P is the cumulative biomethane production (mL CH_4/g VS_{added}), P_0 is the accumulated biomethane production up to time t (mL CH_4/g VS_{added}), R_m is the maximum daily production of biomethane (mL CH_4/g VS_{added}.d), and e corresponds to a value 2.72. These parameters were determined using the solver tool in Excel through the least squares method (LSE method) between the modified Gompertz model and the experimental production.

2.5. Synergistic Effect of FW Introduction

The synergistic effect of FW introduction was determined according to Akshaya and Jacob [15] by using Equation (5).

$$\propto = \frac{C}{A+B} \tag{5}$$

where C is the biomethane yield (mL CH_4/g VS_{added}) from digesting the reference mixture (R) with the incorporation of 25 or 50% FW, and A and B represent the biomethane yield (mL CH_4/g VS_{added}) from the digestion of the reference mixture (R) and FW, respectively.

The value of α indicates the nature of the interaction between the two types of waste during co-digestion; $\alpha = 1$ represents no interactive effect, whereas $\alpha > 1$ and $\alpha < 1$ indicate synergistic and antagonistic effects, respectively.

3. Results

3.1. Characterization of Biomasses and Feed Mixtures

Table 3 shows the characterization of the biomasses used, whereas Table 4 presents the physicochemical characterization of the different feed mixtures prepared.

Table 3. Physicochemical characterization of the biomass under study.

Parameters	FW	OPP	PS	Inoculum
TS (g/L)	343.15 ± 1.26	78.16 ± 2.82	61.7 ± 11.38	19.41 ± 1.17
VS (g/L)	313.45 ± 5.05	74.33 ± 0.64	39.79 ± 7.91	13.44 ± 1.14
VS/TS (%)	91.34 ± 0.52	95.20 ± 2.62	64.31 ± 0.93	69.18 ± 1.71
$Lipids^{db}$ (%)	41.45	1.49	ND	ND
TOC (g/L)	180.56	43.11	23.08	7.80
N-TKN (g N/L)	22.12 ± 0.12	0.64 ± 0.04	3.53 ± 0.03	1.51 ± 0.03
N-NH_4^+ (g N/L)	2.70 ± 0.21	ND	2.10 ± 0.04	0.82 ± 0.01
C/N	8.16	67.36	6.54	4.66
COD_T (g O_2/L)	540.54	69.30	55.00	ND
COD_S (g O_2/L)	360.36	53.00	18.50	ND
COD_S/COD_T (%)	66.67	76.48	33.64	ND
P (mg P/L)	3793.65 ± 553.85	68.39 ± 14.51	1.30 ± 0.14	ND

ND, not determined.

Table 4. Physicochemical characterization of the different feed mixtures used to prepare the batch reactors, with the increasing incorporation of FW.

Parameter	0% FW	25% FW	50% FW
TS (g/L)	51.94 ± 0.17	123.20 ± 0.13	195.50 ± 2.27
VS (g/L)	38.32 ± 0.15	105.17 ± 0.17	171.87 ± 1.16
VS/TS (%)	73.77 ± 0.04	86.02 ± 0.22	87.92 ± 0.43
TOC (g/L)	22.23	61.00	99.69
N-TKN (g N/L)	2.77 ± 0.04	7.16 ± 0.05	12.54 ± 0.1
N-NH_4^+ (g N/L)	1.74 ± 0.02	2.63 ± 0.45	2.40 ± 0.24
N-NH_4^+/N-TKN (%)	62.8	35.9	19.14
C/N	8.03	8.52	7.95
COD_T (g O_2/L)	73	161.67	334.20
COD_S (g O_2/L)	39	89	297.65
COD_S/COD_T (%)	53.42	55.05	65.85

From the analysis of Table 3, it can be seen that OPP and PS had a moisture content of above 90%. Thus, their conjugation with FW (with a TS content of 34%) is advantageous for obtaining a mixture with a TS below 10%, as is recommended for wet anaerobic digestion [45].

Regarding the organic content potentially available for biodegradation (VS/TS), it is possible to verify that FW and OPP present a percentage above 90%, which makes these biomasses promising for biological treatment.

Previous studies have reported an organic content of orange pomace identical to that observed. For instance, Bouaita et al. [40], Martín et al. [50], and Pellera and Gidarakos [51] obtained a VS/TS ratio ranging between 96 and 97%. Pellera and Gidarakos [51] also reported a C/N ratio close to that reported in this study, which was approximately 50.

As for FW, Akshaya and Jacob [15], Bouallagui et al. [27], and Mshandete et al. [28] reported a VS/TS ratio varying between 50 and 70%. Regarding the lipid content, Xu et al. [18], Mshandete et al. [28], and Wu and Song [14] present values from 12 to 15%, whereas in this study, the content was found to be 41.4%. This difference is possibly associated with the fact that in this study, whole fish were used, whereas, in the mentioned studies, only the gills, viscera, spines, and scales were used. The C/N ratio of FW was twice the value reported by Park et al. [52] in a study using dead-at-sea fish, which may be related to differences in fish species.

PS had a relatively low organic content (65%) compared with the other biomasses. Similar results were reported by González-Arias et al. [53] and Moset et al. [54], who obtained VS/TS ratios of 68 and 75%, respectively. However, this substrate plays a very important role, as it provides alkalinity and has a high buffering capacity that prevents potential acidification from the accumulation of VFAs [20]. PS has a low C/N ratio (6), which is similar to that of FW (8).

From Table 4, it can be seen that higher FW incorporation increased the COD_S/COD_T ratio, showing a higher content of soluble organic matter, which is more available for bioconversion. It was also verified that, as the FW content increased, the mineralized organic nitrogen content decreased, as shown by a reduction in $N\text{-}NH_4^+/N\text{-}TKN$ from 62.8% to 19.14 when going from the reference feed mixture (0% FW) to a feed mixture with the incorporation of 50% FW. This reduction is associated with the fact that PS has a much higher mineral nitrogen content than FW, as reported by Alvarez et al. [30].

3.2. Biodegradation Assays

Figure 1 shows the differences in biodegradation profiles with increasing FW incorporation. It is possible to observe a rapid start in the production of biomethane. In fact, the first day's production of all modalities was above 70 mL $CH_4/g\ VS_{added}$, which is much higher than the initial production already reported in the anaerobic mono- and co-digestion of FW with fruit and vegetable waste (less than 20 mL $CH_4/g\ VS_{added}$) [22,55]. The incorporation of 50% FW leads to a slower degradation; however, the accumulated production is higher than that of the other modalities.

As seen in Figure 2, the incorporation of FW contributed to higher SMP, with the median value for 25% FW and 50% FW clearly above the median achieved without FW incorporation. The specific biomethane production increased by 29% and 37% with the incorporation of 25 and 50% FW, respectively.

The higher production of biomethane with an increase in FW, and therefore of lipids, is in agreement with the increase in VS, VDS, and COD_S removal, as shown in Table 5.

The pH of the digestates showed no disturbance, which may be indicative of the absence of VFA accumulation at an inhibitory level. This was corroborated by the VFA, TA, and VFA/TA results. Despite VFA values of above 300 mg/L, as TA was between 3980 and 4270 mg/L (within the recommended range of 1500 and 5000 mg/L), the VFA/TA ratio was always below 0.35, which indicates the absence of the inhibition associated with the accumulation of VFAs [56].

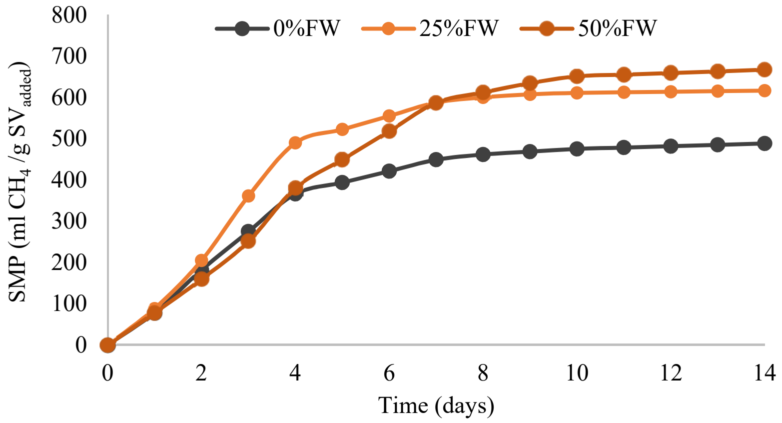

Figure 1. Cumulative specific biomethane production (SMP) production profiles for different amounts of incorporated FW (0 to 50%).

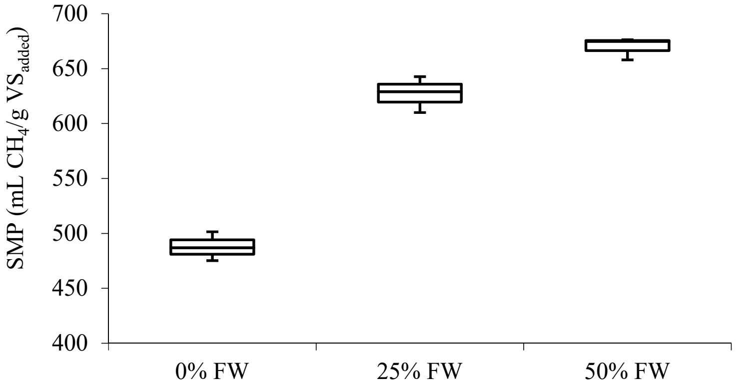

Figure 2. Box plot of the specific biomethane production (SMP) for different amounts of incorporated FW (0 to 50%).

Table 5. Performance and stability of the anaerobic digestion assay to evaluate the effect of lipid content in FW.

	0% FW	**25% FW**	**50% FW**
VS removal (%)	23.99	26.94	32.65
VDS removal (%)	56.84	67.44	70.94
COD$_S$ removal (%)	70.3	73.58	74.83
pH	7.40 ± 0.01	7.43 ± 0.02	7.46 ± 0.02
EC (mS/cm)	8.02 ± 0.05	8.93 ± 0.04	7.94 ± 0.05
N-NH$_4$$^+$ (g N/L)	0.804 ± 0.01	0.940 ± 0.01	0.856 ± 0.00
TA (mg CaCO$_3$/L)	4060	4270	3980
VFA (mg/L)	330.58	304.32	435.66
VFA/TA	0.08	0.07	0.11

3.3. Kinetic Studies

First-order kinetic model

Although there was a positive effect on the final yield of the process with the introduction of FW, it also hampered the degradation rate (Table 6). In fact, there was a reduction in the degradation rate constant of up to 30% when going from 0% to 50% FW.

Table 6. Degradation rate constant of a first-order model for studied FW incorporation.

	k (d^{-1})	R^2
0% FW	0.289	0.98
25% FW	0.277	0.96
50% FW	0.204	0.96

The reduction in the k value with the introduction of FW is associated with the high content of proteins and lipids, which are constituents that are difficult to degrade. The same effect was reported by Wu and Song [14], who verified an increase in the lag phase with the incorporation of 3% FW; however, methanogenic activity was later recovered.

Protease enzymes hydrolyze proteins into amino acids, whereas lipase enzymes transform lipids into glycerol and long-chain fatty acids (LCFAs). Since lipids tend to be adsorbed on the surface of microbial biomass, their accumulation can inhibit hydrolysis, making the interface activation of lipases [57] and mass transfer difficult. In their review research, Pavlostathis and Giraldo-Gomez [58] reported that the hydrolysis constant for lipids ranges between 0.08 and 1.7 d^{-1}.

Regarding the other constituents of the feed mixture, according to Garcia et al. [59], citrus can have a k of 0.226, PS of 0.120, and FW of 0.035.

Thus, it can be concluded that the decrease in k values with the introduction of FW is due to the presence of proteins and lipids, which make this initial phase difficult. However, the hydrolysis constants observed in this study were much higher than those previously reported.

Modified Gompertz model

The lag phase usually occurs due to possible inhibitions in the initial phase or in the fermentation phase of anaerobic digestion; therefore, it is dependent on the characteristics of the biomasses used. This tends to be higher when using substrates rich in proteins and lipids, owing to the inhibition caused by the hydrolysis products of these two elements, such as VFAs, NH_3-N, and LCFAs [60]. However, in the case of the inhibition caused by lipids after the lag phase, anaerobic bacteria are able to digest LCFAs, recovering biomethane production due to the survival of some microbial consortia [61].

Table 7 shows an increase in the lag phase as the FW content increases, as is expected, given the results obtained for the degradation rate. The 50% FW presented the highest lag time (0.58 days), which was approximately three times that of the reference mixture. Nevertheless, the above-mentioned value is quite low considering the results obtained in previous studies; for example, Fonseca et al. [8] performed a batch test with FW using an I:S ratio of 3:1, obtaining a lag phase of between 1 and 2 days. Sarker [7] reported a lag time of 7.43 days when performing the anaerobic digestion of FW, with an organic load of 8.53 gVS and an I:S ratio of 4:1. Finally, Wu and Song [14] obtained a lag time of 20 days when performing the anaerobic co-digestion of activated sludge with 3% FW, introducing a total organic load of 4.7 gVS and an I:S ratio of 1:8.

Table 7. Kinetic parameters of the Gompertz modified model.

	P_0 (mL CH_4/g VS_{added})	R_m (mL CH_4/g VS_{added}.d)	λ (d)
0% FW	480.86	99.59	0.20
25% FW	612.78	150.19	0.55
50% FW	674.96	109.31	0.58

P_0, accumulated biomethane production up to time t; R_m, maximum daily biomethane production; λ, Lag time.

These differences in the lag phase may be related to the inoculum, I:S ratio, the constitution of the substrates, and the organic load introduced into the reactor.

3.4. Synergistic Effect of FW Introduction

Table 8 presents the theoretical biomethane yields, experimental biomethane yields, and α values resulting from the incorporation of FW.

Table 8. Synergistic effects of different co-digestion modalities.

	Theoretical Biomethane Yield (mL CH_4/g VS_{added})	Experimental Biomethane Yield (mL CH_4/g VS_{added})	α
25% FW	540.32	627.23	1.16
50% FW	592.78	669.68	1.13

The experimental biomethane yields for 25% FW and 50% FW were 16% and 13% higher than the theoretical values, respectively. This indicates a synergistic effect of the introduction of FW, which was confirmed as $\alpha > 1$, reaching a maximum value of 25% FW (1.16).

This effect has already been observed in previous studies. For example, Akshaya and Jacob [22] obtained a synergistic effect identical to that observed in this study, with an α of 1.23 for the co-digestion of a mixture of 50% plant residues and 50% FW. Puig-Castellví et al. [62] analyzed the synergistic effect of introducing 25, 50, and 75% FW in co-digesting activated sludge, obtaining an $\alpha > 1$. The value of α reached a maximum with the introduction of 25% FW ($\alpha = 2.13$).

4. Conclusions

The results obtained showed that FW enhanced the biomethane yield from the anaerobic co-digestion of a reference mixture consisting of 80% PS and 20% OPP. The introduction of 25% and 50% FW increased the biomethane yield by 29% and 37%, respectively, compared to the anaerobic digestion of the reference mixture. A maximum biomethane production of 669.68 ± 8.32 mL CH_4/g VS_{added} was achieved with 50% FW.

Moreover, the results achieved suggest the stability of the process, as the digestate's pH value was around 7.5, the TA was between 3980 and 4270 mg/L (within the recommended range of 1500 and 5000 mg/L), and the VFA/TA ratio was always below 0.35, which indicates the absence of the inhibition associated with the accumulation of VFAs [56].

Nevertheless, the kinetic study showed that the introduction of FW, which increased the lipid content, negatively influenced the lag time. For example, incorporating 50% FW (which corresponds to c.a 6% of lipids on a dry basis) led to a lag time of approximately three times that of the reference mixture. This effect was also reported in previous studies [7,8,14] and was associated with an increase in proteins and lipids content. Since lipids tend to be adsorbed on the surface of microbial biomass, their accumulation can inhibit hydrolysis, making the interface activation of lipases [57] and mass transfer difficult.

The results from the kinetic study also showed that the introduction of FW reduced the degradation rate constant by up to 30%. The incorporation of 25% FW (which corresponds to c.a 4% of lipids on a dry basis) may be a compromise between increasing yield (29%) and process kinetics, as the degradation constant decreased by only 4%.

For the 25% FW mixture, about 80% of the cumulative methane volume was reached after 4–5 days of digestion, whereas 6–7 days were necessary for the 50% FW mixture. The low retention times needed have a positive impact on potential full-scale applications, as it implies a lower reactor volume with the associated economic benefits.

Therefore, the results obtained show that FW is promising for the anaerobic co-digestion process. This study provides useful information to support further research under continuous conditions, which can assess the impact of the operational parameters on process performance under conditions closer to full-scale implementation.

Another aspect that should be addressed in future studies is the impact of FW introduction on microbial community structure to identify the key micro-organisms responsible for increased process efficiency.

The proposed strategy can be useful for the management of three relevant waste streams produced in the Mediterranean region. Moreover, the energy scenario that led to the REPower EU Plan has driven the European Commission to promote the upscaling of biomethane production and consumption. This is an opportunity for member states to design a biomethane strategy at the national and regional levels. The co-digestion of different types of waste sets the grounds to establish synergies across sectors, potentially creating clusters that allow for the establishment of more sustainable biogas production units with positive economic impacts at the local scale.

Additionally, the proposed solution brings environmental benefits as it is completely aligned with the circular economy vision, recovering value from organic waste as biomethane and fertilizer (digestate) are produced. Furthermore, biomethane production from organic waste mitigates GHG emissions, encouraging sustainable waste management practices.

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Abbreviations

AcoD	anaerobic co-digestion
C/N	carbon to nitrogen ratio
COD_T	total chemical oxygen demand
COD_S	soluble chemical oxygen demand
DSs	dissolved solids
EC	electrical conductivity
FW	fish waste
GHG	greenhouse gas
I:S	inoculum to substrate ratio
LSE	least square error
LCFAs	long-chain fatty acids
OP	orange pomace
OPP	orange pomace pulp
PS	pig slurry
TA	total alkalinity
TKN	total Kjeldahl nitrogen
TOC	total organic carbon
TSs	total solids
VDSs	volatile dissolved solids
VFAs	volatile fatty acids
VSs	volatile solids

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