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A Novel Food Wastewater Treatment Approach: Developing a Sustainable Fungicide for Agricultural Use

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Abstract: Three wastewater sources, namely slaughterhouse wastewater, cheese whey, and wine lees, were used for volatile fatty acid (VFA) production with the aim of reducing polluted wastewater discharge to the water bodies and creating a useful product. Cheese whey and wine lees were proved to be good substrates to produce VFAs, obtaining maximum bioconversion percentages in g COD-VFA/g TCOD initial of 90% and 72% for cheese whey and wine lees, respectively. The composition of the VFAs produced from each wastewater stream varied, with acetic, propionic, isobutyric, and isovaleric acids being the most dominant. These VFAs were used as an environmentally friendly fungicide against *Fusarium culmorum*, resulting in a reduction of the radial mycelial growth of *Fusarium culmorum* for all the effluents tested. A thermal pretreatment of the VFAs resulted in an improved antifungal efficiency if compared to the untreated VFAs or a UV pretreatment.

Keywords: volatile fatty acids (VFAs); wine lees; cheese whey; *Fusarium culmorum*; anaerobic digestion

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1. Introduction

Food and beverage industry is one of the industries that experience extensive use of water on one hand while generating huge amounts of wastewater on the other hand. The generated wastewater has negative impacts on the environment such as destroying aquatic creatures when it is deposited to the water bodies [1]. Among the different wastewaters generated within food and beverage industry, the resultant wastewater from meat production is relevant since the EU is the third largest producer of beef in the world, contributing greatly to the gastronomy, social life, rural development, and economy of Europe [2]. Some of the dynamics that make this possible are the diversity of the breeds and animal types available in this region, which are cows, bulls, steers, heifers, and the farming systems, such as permanent or temporary pasters (intensive and extensive), mixed breeders and feeders, etc. Amidst all this, there are many challenges that this industry is faced with, such as environmental impacts, legitimacy, origin, etc. The aforementioned challenges make it imperative that the industry embarks on thorough research for the maintenance and development of its economic benefits.

One of the research areas could be to look at ways of bettering the environmental impacts posed by this industry [3]. This is line with one of the global policies that exist with the concept that economic gains must be achieved with less environmental impact, which calls for looking at eco-friendly pathways of processes to lessen the impact [4].

Essentially, the effects of this industry may have a huge influence in environmental pollution, natural resource consumption, and climate change. Environmental impacts can be investigated by looking at the product, the process, and the system depending on the research perspective. Looking at the process-based perspective, many elements can further be assessed, such as energy and water consumption, wastewater discharge, and solid waste output [5]. Wastewater in the meat production industry and slaughterhouse results from many different operations, which makes it contain a wide range of contaminants such as blood, manure, fat, dirt, meat extracts, etc. The main indicators of this wastewater differ according to the type of meat being processed and the environmental conditions [6]. In fact, when slaughterhouse wastewater is discharged to water bodies, it increases nitrogen, phosphorus, and biological oxygen demand and solids levels, which in turn affect the quality of water [7]. If this wastewater is disposed untreated, the whole ecosystem is at risk since there would be changes in the river's microflora, and pathogenic microorganisms can be spread from animal waste to humans through contact with the river water [8]. This wastewater also contains toxic metals, which can pollute groundwater and negatively impact the entire food chain if discharged untreated [9].

Dairy products make up a huge part of the food sector worldwide. Animal milk is an integral part of this industry, and about 843 M tonnes comes from cows solely [10]. Sheep milk also accounts for a pertinent amount of the milk production and products thereof. This is due to its high protein content in comparison to that of cows [11]. In the process of making dairy products such as cheese, there are several byproducts that are produced inevitably, including cheese whey. Cheese whey is characterised by its yellow-green colour and contains about 65 g total solids for every litre [12]. It can be divided into two types depending on the nutritional fractions and the pH. The main component of this substrate is lactose, which takes about 70–75% of the total solids and is accountable for the high values of BOD and COD [13]. Cheese whey is recognised as a source of protein and peptides, which are functional and bioactive compounds, but a huge amount of it is still not valorised around the world [14].

Wine lees are a sludge-like material generated from the fermentation of wine; they essentially contain yeast cells, and they are rich in mannoproteins. This is the residue attained after the progressive precipitation of yeast remains and other particles into the bottom of the wine tank after the alcoholic fermentation stops in the wine-making process [15]. Wine lees have high organic content and chemical oxygen demand, which make their disposal harmful to the environment once conducted improperly [16]. They can be used for different applications due to their protein richness, thereby valorising them. These include the extraction of phenols, ethanol, tartaric acid, and the usage of their solid fraction consisting of yeast biomass [17,18].

Volatile fatty acids (VFAs) are among the essential group of chemical building blocks, which are used in different fields such as food, pharmaceutical, wastewater treatment, and plastic production industries. These compounds are characterised by their low molecular weight, including aliphatic monocarboxylic acids with two to six carbon atoms, namely acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, and heptanoic acid. By virtue of their pKA value of 4.75, these are weak acids that dissociate partially in water into the hydrogen cation and the carboxylate anion. The production of VFAs can be achieved conventionally from fossil-based resources as well as bio-based products by following different synthetic pathways. The former is not sustainable, as the resource may be depleted due to overexploitation, and byproducts that are concerning to the environment are also produced while following this method. As the drawbacks of conventional VFA production have been highlighted earlier, it is therefore imperative to for look for alternative

routes that will align with the environmental preservation, just as the world continuously makes awareness in that regard [19].

In the search for environmentally friendly approaches to tackle water purification, solvent extraction methods have been used for the extraction of different kinds of metal [20,21]. The most prevailing option that has been explored in the last few years by researchers is the anaerobic digestion (AD) of organics for both energy harvesting and water purification. This is one of the processes that aids a facile recovery of mixed VFAs since they are part of the intermediates that are generated in the fermentation stages of the AD process [22]. The AD process can be carried out from several substrates, including waste and wastewater [23]. The use of these alternative substrates comes with multiple benefits, such as reducing reliance on fossil fuels, avoiding the usage of potential vital food substrates and/or the reduction of economic and environmental impacts embedded by the CO_2 footprint from the chemical-based industry [24–26]. These mixed VFAs produced by AD have numerous applications, such as the production of bioplastics (e.g., PHA), biofuels in the form of hydrogen and bio-butanol, microbial oil, and methane, and they can be used as a carbon source in biological nutrient removal processes [27].

Recent research indicates that VFAs can be effective as fungicides against a wide range of fungal species, including those responsible for food spoilage and plant diseases [28]. *Fusarium culmorum* is a significant plant pathogen that causes various diseases in cereal crops, particularly wheat and barley. This fungus is responsible for Fusarium head blight (FHB), crown rot, and foot rot, which can lead to substantial yield losses and reduced grain quality [29]. Economic losses attributed to *F. culmorum* infections can be severe; they can reach up to USD 46 million in affected fields, depending on environmental conditions and disease severity [30]. Moreover, the presence of mycotoxins in harvested grains can result in further economic losses due to rejected shipments and reduced market value [31]. The impact of *F. culmorum* on global food security and agricultural economics has led to increased research efforts focused on developing resistant crop varieties and improved management strategies. Effective management strategies include crop rotation, the use of resistant varieties, and the application of fungicides to mitigate the spread and impact of this pathogen [32].

The valorisation of organic wastes into VFAs for antifungal applications not only provides an environmentally friendly solution for waste management but also offers a sustainable alternative to synthetic fungicides in agriculture and food preservation. Moreover, VFAs are considered environmentally friendly and less likely to promote fungal resistance compared to conventional fungicides. This makes them an attractive option for sustainable agriculture and food safety practices [33]. The aim of the current research is to study a novel strategy for three food wastewaters valorisation, namely slaughterhouse wastewater, cheese whey, and wine lees. First, the VFA production though anaerobic fermentation was evaluated in terms of yield and composition. Then, a novel application of the produced mixed VFAs as an antifungal solution in agriculture was performed. More specifically, these VFAs were used as an environmentally friendly fungicide against *Fusarium culmorum*, which is a challenge nowadays for farmers of cereal plants in the Castilla y Leon region, which is a big cereal producer in Spain.

2. Materials and Methods

2.1. Wastewaters (SH, CW, and WL)

The substrates consisted of cattle slaughterhouse wastewater (SH) from a slaughterhouse located in Valladolid (Spain), cheese whey wastewater (CW) from a sheep milk processing factory located in Palencia (Spain), and wine lees (WL) from a wine factory located in Valladolid (Spain). All the substrates were taken for compositional analysis on

arrival and then stored in the freezer before the experiments were conducted. The anaerobic sludge (AS) used as inoculum was from the wastewater treatment plant of Valladolid (Spain) and was stored in the refrigerator until its usage. No pre-treatment was performed in all the substrates used in this study. The composition of the different substrates and inoculum is shown in Table 1.

Table 1. Substrate composition characterisation. Standard deviation between two replicated analyses are shown in parentheses.

Parameter	Unit	SH	CW	WL	AS
pH	-	7.76 (0.00)	5.94 (0.00)	3.56 (0.00)	7.73 (0.00)
Conductivity	mS cm^{-1}	2.20 (0.00)	5.73 (0.00)	2.08 (0.00)	7.01 (0.00)
TS	%	2.20 (0.84)	6.22 (0.41)	28.07 (0.57)	2.21 (0.23)
VS	%	1.40 (0.03)	5.83 (0.30)	24.73 (0.45)	1.48 (0.12)
Alkalinity	mg L^{-1} $CaCO_3$	-	950 (0.00)	-	1000 (0.00)
N-NH_4^+	mg L^{-1}	68 (0.00)	1762 (0.10)	1125 (0.02)	1206 (0.15)
TKN	mg L^{-1}	192 (0.01)	2706 (0.00)	16,540 (1.20)	2361 (0.10)
TCOD	mg^{-1}	1310	7460	17,980	22,800
Protein	mg L^{-1}	775 (0.00)	5513 (0.00)	96,340 (0.00)	7220 (0.00)

2.2. Analytical Methods

pH measurements were carried out directly on each substrate using Crison pH meter Basic 20 (Crison, Barcelona, Spain). Conductivity, total solids (TSs), volatile solids (VSs), ammonium (N-NH_4^+), total Kjeldahl nitrogen (TKN), and alkalinity were determined using standard methods for water and wastewater examination in accordance with the American Public Health Association [34]. Protein content was calculated from TKN values by multiplying a conversion factor of 6.25. Total alkalinity concentrations were conducted using titration in line with APHA methods. Total chemical oxygen demand (TCOD) was performed using commercial kits (Lovibond, Dortmund, Germany). All experiments were conducted in the laboratory in the department of agroforestry sciences at the University of Valladolid.

The concentration of the different VFAs (i.e., acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, hexanoic, and heptanoic acids) was determined using a gas chromatograph (Agilent 7890A, Santa Clara, CA, USA) equipped with a DB-FFAP column of 30 m × 250 µm × 0.25 µm i.d. followed by a flame ionisation detector (FID). The carrier gas was helium (1.74 mL min^{-1}). The temperature of the detector and of the injector was 300 °C. The temperature of the oven was set at 100 °C for 5 min, then increased by 10 °C per minute until it reached 210 °C. VFA concentrations were converted to COD concentration using the following conversion factors: 1.07 for acetic acid, 1.51 for propionic acid, 1.82 for butyric and isobutyric acid, 2.04 for valeric and isovaleric acid, 2.21 for hexanoic acid, and 2.34 for heptanoic acid. VFA bioconversion was calculated according to Equation (1):

$$\%\ \text{VFAs bioconversion} = (\text{VFAs (g COD/L)})/(\text{TCOD in (g COD/L)}) \times 100 \quad (1)$$

where VFAs and TCOD correspond to the concentration of VFAs at the end of the experiment and the initial TCOD in the correspondent wastewater, respectively.

2.3. Anaerobic Experiments

Three experiments were conducted at mesophilic conditions, namely SH, CW, and WL. Each experiment was performed in duplicates, and they consisted of 1.5 L glass jacketed reactors (reactor 1 and reactor 2) connected to a gas collecting apparatus based

on water displacement (Figure 1). A water bath for water circulation was used to keep the temperature at 37 °C ± 1. Each experiment was running for eighteen days. The substrate-to-inoculum ratio was initially adjusted at 1:1, expressed as volatile solids, according to the previous studies [35]. The inoculum volume was fixed; then, the added substrate matched the volume to keep the 1:1 ratio, and water was used to fill the 1 L mark. About 500 mL headspace was left. The reactors were put on electric magnetic stirrers at a constant stirring rate of 4000 rpm, which was allowed to rest for at least 30 min every day excluding weekends for continuous agitation and homogenisation of the contents.

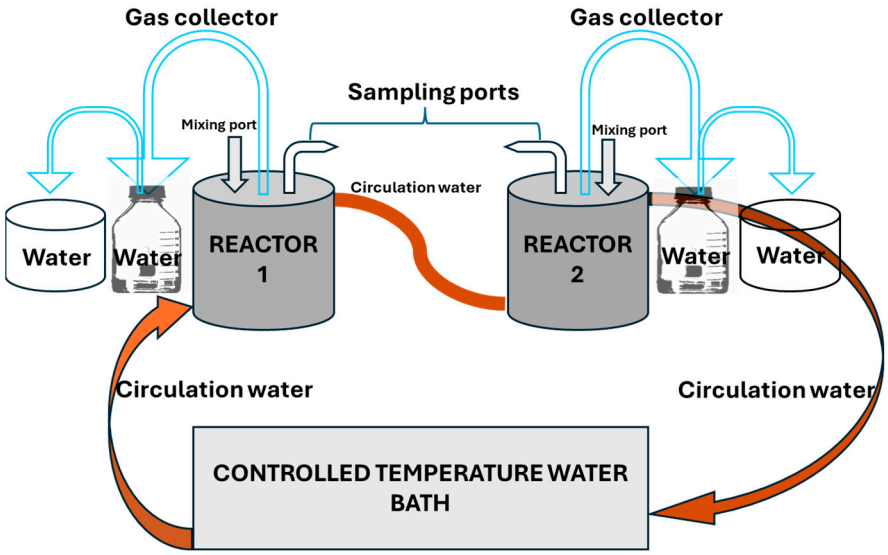

Figure 1. VFA production setup scheme used in all three wastewater streams used in the study. Each digestion was performed in duplicates under the same water bath to maintain the same conditions.

Samples were collected every day in the first week, and every second day, sampling was performed from the second week onwards. The bioreactors were tightly closed by glass caps of the same material as the reactors fitted with a rubber line to avoid glasses being stuck together. The caps had sampling ports through which pH measurements could be performed and samples for analysis could be taken. These sampling ports were ordinarily kept bolted with plastic caps and only opened during the sampling process. The contents of each bioreactor were taken in the beginning of the experiments for analysis, and a precedent of sampling followed afterwards. In all the experiments, pH was initially adjusted manually to 5.5 using NaOH and H_2SO_4 depending on whether it was acidic or alkaline. TCOD was measured at the beginning and at the end of the experiments. VFA concentration and composition were determined in the liquid fraction every two–three days. For this purpose, 10 mL of liquid sample was removed from the reactors.

2.4. Antifungal Application of VFAs

The in vitro antifungal activity of VFAs against *Fusarium culmorum* was evaluated for VFAs obtained from CW and WL through mycelial growth inhibition tests, alone or in combination with UV or heat pretreatments.

2.4.1. Fungal Isolate, Reagents

Fusarium culmorum (CECT 2148) was bought from CECT (Spanish Type Culture Collection, Valencia, Spain). Potato dextrose agar (PDA) and Potato Dextrose Agar (PDB) were purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ, USA).

2.4.2. UV and Heat Pretreatments

VFAs obtained from CW and WL anaerobic fermentations (Section 2.3) were used as a starting point. An amount of 1 L of each VFA effluent was collected for each treatment, a total of 3 L for all the substrates. One of them (1 L) was kept as it arrived (no treatment), another 1 L was subjected to a temperature of 50 °C for 30 min (heat treatment), and the last 1 L was left for 30 min under UV light (UV treatment). Subsequently, the concentration of VFAs was measured in each of the treatments used.

2.4.3. In Vitro Tests of Mycelial Growth Inhibition

For the mycelial growth inhibition assays with the 6 treatments (viz. CW no treatment, CW UV, CW Heat, WL no treatment, WL UV, and WL Heat), the methodology reported by Gutiérrez Santa Ana et al. [36] was chosen with some modifications. PDA plates with 15 concentrations of each treatment (ranging from 62.5 to 4000 μg·VFAs-COD mL^{-1}) were inoculated with 5 mm diameter plugs and incubated at 26 °C for 7 days, together with the control plate, containing only culture medium. EC50 and EC90 (50% and 90% effective concentrations, respectively) were estimated using PROBIT analysis in IBM SPSS Statistics v.25 software (IBM; Armonk, NY, USA). Growth inhibition was calculated according to Equation (2):

$$\text{Growth inhibition (\%)}: ((dc - dt)/dc) \times 100 \quad (2)$$

where dc is the average colony diameter in the control and dt is the average diameter of the treated colony.

2.4.4. Statistical Analysis

Data were subjected to analysis of variance (ANOVA) in IBM SPSS Statistics v.25 software. Tukey's HSD test at 0.05 probability level ($p < 0.05$) was used for the post hoc comparison of means.

3. Results and Discussion

3.1. Chemical Composition of the Substrates

The physicochemical characterisation data of the substrates used in the study is illustrated in Table 1. Regarding slaughterhouse wastewater (SH), it should be noted that characteristics of this substrate differ significantly depending on their geographical location, the number of animals processed, and the amount of water used for washing. In our case, the initial pH was 7.76. The TSs are somewhat comparable to what Al Smadi et al. [37] obtained in their study, while the TCOD is half of their results. Organic matter in the form of VSs or the TCOD was very low, which could affect VFA production since the SH substrate was too dilute. In the case of cheese whey (CW), the initial pH was 5.94, and this is in the same range as the value reported by Estikomah and Masykuri [38], while the TCOD is way less than theirs, although still higher than the acceptable EU standard of 125 mg/L and even general standard of 250 mg/L [39]. A high TCOD is an indicator of high organic content that is toxic to the aquatic life present in water bodies. Finally, the pH of wine lees (WL) was found to be in the same range as other values reported in the literature [40].

The amount of potential organic pollutants in these three wastewater streams was measured in the form of N-NH_4^+, TKN, protein, and the TCOD. Except for SH, the N-NH_4^+ values have been found to be at the level that needs to be dealt with, as they pose a threat to the environment [41]. It can be seen from these values that wastewater would cause adverse effects on the environment if it is discarded without treatment. The protein concentrations of the wastewater used in the current study are much higher (ranging from 775–96,340 mg/L) since they are of a food origin, which is mostly protein based. The values

were calculated from the total nitrogen content multiplying the difference between protein and nonprotein nitrogen by the 6.25 factor. After the analysis of the chemical composition of the wastewaters used in this study, it was noted that SH wastewater was very dilute, whereas wine lees had a higher protein content. Due to anaerobic fermentation occurring efficiently when there is enough organic content present in a substrate, CW and WL were expected to be more suitable as substrates for VFA production.

3.2. VFA Production and Composition

VFA production and composition are influenced greatly by a change in operating conditions, which come to play due to different groups of bacteria being present that use diverse metabolic routes [35]. pH was then monitored throughout the anaerobic digestion period to investigate the effects on the as-produced VFAs. Figure 2 shows the variations in pH during the experimental time. As stated before, the pH was initially adjusted to 5.5 in all the experiments. Looking at Figure 2, it can be observed that the pH in SH fermentation did not vary much compared to the other two wastewater streams, and the VFA production only occurred during the first two days. In the case of CW, a pH drop from 5.5 to 3.65 $\pm$ 0.50 was observed during the first 24 h of the experiment. It was reasoned that this might mean a production of lactic acid, which was not the desired product for our research [42]. According to Lagoa-Costa et al. [43], it was detected that lactase can be the first fermentation product that reveals the presence of lactic acid bacteria during the fermentation of cheese whey. After lactic acid reached its maximum peak (when lactose is fully consumed), it would then be converted into VFAs. However, in the current study, this pH was adjusted to 5.5 for both reactors. On the third day (48 h later), the pH of both reactors was checked and found to be 5.72 and 5.68 for R1 and R2, respectively, and was left at those values. Then, the pH remained stable until the end of the fermentation experiment without adjustment. The pH profile of the WL had more fluctuations, suggesting a presence of different bacteria and hence different VFAs being produced. This led to a need to control the pH closely, as it would easily lead to undesired products (such as methane) if left uncontrolled. After 2 days of digestion, the pH of the reactors increased, and it was observed to regulate itself after a few hours. The pH of the reactors was found to be 5.13 $\pm$ 0.20 after 3 days and again was left without any adjustments until day 4, where a drop in the pH to 4.85 $\pm$ 0.05 was observed. At this point there was an adjustment to 5.5 to aid the formation of VFAs, being on day 9 where there was a maximum production of VFAs. After that day, the pH increased above 7, and 1730 mL of methane gas was produced on day 15.

Table 2 presents the initial and final TCOD and VFA concentrations in the fermentation media (g COD L^{-1}) and bioconversion percentage for each wastewater. In the case of SH and CW, the concentration of the TCOD was stable during the experiments, and a variable proportion of that TCOD was converted to VFAs. However, in the case of WL, the TCOD was reduced by 53.4%, meaning that almost half of the organic matter was converted into other products than VFAs. More specifically and as previously stated, the pH increased (Figure 2), and 1730 mL of methane was produced at day 15 of the experiment, so then, TCOD was most probably converted to methane. The maximum VFA concentrations accounted for 70 $\pm$ 25, 7858 $\pm$ 376, and 8939 $\pm$ 425 mg COD L^{-1} for the fermentation experiments of SH, CW, and WL, respectively. VFA maximum bioconversion efficiencies for the experiments were in the order of SH < WL < CW, as seen in Table 2.

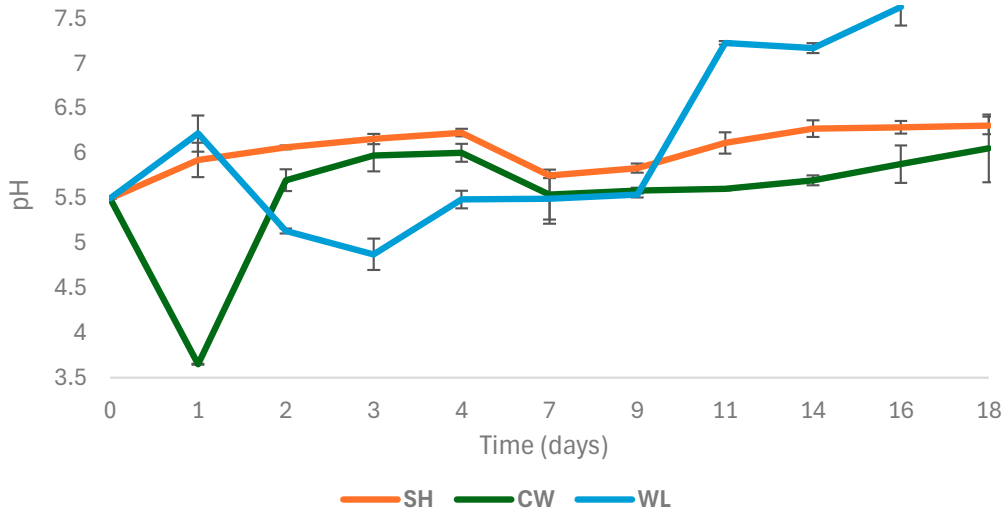

Figure 2. pH variations from the beginning until the last experimental day.

Table 2. The TCOD, VFA, and bioconversion percentage for the studied assays.

Experiment	TCOD Initial (mg L^{-1})	TCOD Final (mg L^{-1})
SH	696 (255)	608 (88)
CW	8725 (248)	6525 (1789)

Figure 3 shows the composition of the produced VFAs. SH did not produce much VFAs, probably due to the high dilution of the original wastewater (Figure 3A). The VFAs originally present in the wastewater were consumed until day 11. However, the composition of the VFAs varied with time, with the presence of four VFAs on day 4, only acetic acid on day 7, and propionic acid on day 11. This behaviour could suggest that SH substrate could be a good source for single VFA production, but further studies should be performed to prove this conclusion.

In the case of CW (Figure 3B), acidogenesis was dominant where the production favoured acetic, butyric, and propionic, as reported by Bengtsson et al. [44]. However, valeric, caproic, and heptanoic acids were also fairly noticeable, which can be attributed to the chain elongation of the dominant VFAs, while isovaleric, isobutyric, and isocaproic were in much smaller volumes. Precisely, after day 9, acetic acid production started to decrease gradually, while propionic acid followed the same trend only after day 11. Moreover, a constant production of valeric acid was witnessed from day 9 until day 18. At this point, it is worth mentioning that on day 9, the VFA production yield was at its maximum, and this has been taken as the optimal point for the next part of the experiment. This observation is contrary to that of Bengtsson et al. [44], where they used the same substrate within the same parameters. VFA production and composition are greatly affected by pH changes and different metabolic routes being at play as results of various bacterial presence; this has been seen in previous studies. In this case, it is observed that VFA production is in the following order: acetic > butyric > propionic > caproic > valeric > heptanoic > isovaleric > isocaproic > isobutyric acid. The main products from this fermentation are acetic, propionic, and butyric acids, which is a similar observation to Lagoa-Costa et al.'s [43] in a study of VFA production from CW working in acidic conditions in a sequencing batch reactor. Their results are like the current results obtained, where two major constituents of these mixed VFAs are acetic and butyric acids with compositional percentages of 14–33 and 10–20%, respectively.

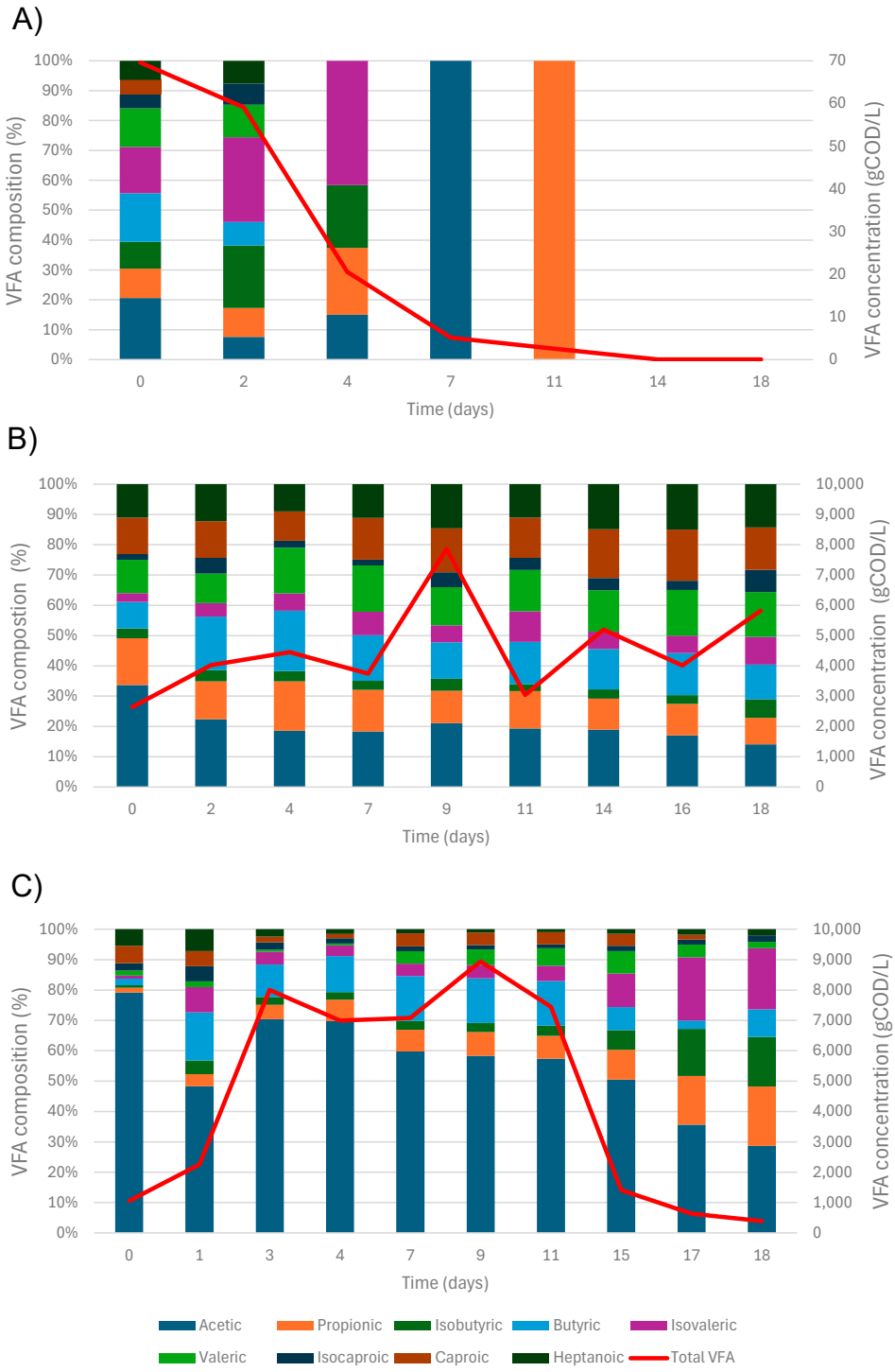

Figure 3. The concentration (red line) and composition of the VFAs produced from (**A**) SH, (**B**) CW, and (**C**) WL.

Looking through the fermentation of WL (Figure 3C), it can be observed that acetic acid production is greatly favoured throughout the experiment period, taking about 29% to 80% of the VFAs produced. The fact that VFAs were readily produced even on the first day of the experiment shows that the yeast and nutrients present in the substrate were the active drivers and sources of microorganisms when combined with the activated sludge [45]. Out of the VFAs produced from this substrate, acetic, butyric, and propionic have been in significant proportion, which is like Villegas-Rodríguez et al.'s [46] results under comparable conditions with a few exceptions. It could be clearly seen that the total

VFA production was decreasing after day 9 until day 18, where caproic acid was no longer produced, which could be due to the consumption of caproic acid by the active bacteria in that pH range. Conversely, in the same period, propionic, isobutyric, and isovaleric acid production started to increase as the pH increased. At this point, it is worth mentioning that on day 9, the VFA production yield was at its maximum, and this has been taken as the optimal point for the next part of the experiment. The main product obtained from wine lees is acetic acid, with an appearance of propionic, butyric, and valeric acids towards the end of the experiment. This could be attributed to the chain elongation due to pH changes and different bacteria presence in the substrate.

Lastly, from Figure 3B,C, it can be seen that the two substrates that performed comparably had a peak on day 9. In both cases, all VFAs could be detected, and these are the products used further in the antifungal assessment, as they were deemed better results. The SH substrate could not be used for antifungal analysis since it was too diluted, and for that reason, the conversion was not good.

3.3. Antifungal Application of VFAs

3.3.1. Influence of Pretreatment on the VFA Reduction and Contamination

Table 3 shows chemical characterisation of VFA effluents before and after heat and UV treatments. In the two substances tested, CW-VFA effluent and WL-VFA effluent, the application of the treatments led to a reduction in the total amount of VFAs, being greater in both cases when the UV treatment was applied. Heat treatment reduced by 0.30% the amount of VFAs in CW and by 23.27% in WL, while UV reduced by 11.11% the amount in CW and by 29.69% in WL.

Table 3. Chemical characterisation of VFA effluents before and after heat and UV treatments. Units are expressed in mg COD·L^{-1} CW and WL, which stand for cheese whey and wine lees, respectively.

	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric	Isocaproic	Caproic	Heptanoic	Total
CW_original	1248	1263	617	11,267	1878	229	169	251	169	17,092
CW_heat	1716	1365	566	11,099	1726	199	121	209	46	17,047
CW_UV	1124	1089	531	10,241	1675	156	123	215	39	15,194
WL-original	2257	608	258	2493	494	212	133	305	37	6797
WL-heat	1580	464	225	1879	428	181	130	290	39	5216
WL-UV	1498	415	207	1723	385	161	105	251	36	4779

In the context of utilising volatile fatty acids (VFAs) as antifungal agents, the comparative effectiveness of heat treatment versus ultraviolet (UV) treatment has significant implications for microbial inactivation. Our findings indicate that heat treatment consistently outperforms UV treatment in achieving higher levels of fungal inactivation, which can be attributed to several key factors, including the mechanisms of action and the limitations of UV treatment.

Heat pretreatment has been shown to significantly reduce bacterial and fungal contamination in effluents containing VFAs. This reduction is primarily attributed to the elimination of heat-sensitive microorganisms, creating a more selective environment for VFA-producing microbes [47]. Moreover, heat treatment effectively disrupts the cellular integrity of microorganisms through the denaturation of proteins and the destabilisation of cellular membranes. This thermal disruption leads to increased permeability of the microorganisms' cell wall, facilitating the penetration of VFAs and enhancing their antimicrobial efficacy [48]. The effectiveness of heat pretreatment varies depending on the time–temperature combination applied. For instance, Shelomi [47] found that 10 min at 60 °C was sufficient to eliminate deliberately inoculated *Salmonella* and *Staphylococcus au-*

reus from certain waste substrates [47], whereas Gutiérrez-Santa Ana et al. [36] have shown that heat treatment at 75 °C for 150 min can achieve over 5 log reductions in *Salmonella* populations, highlighting its potent inactivation capabilities [36]. In contrast, UV treatment primarily targets nucleic acids, inducing DNA damage that can lead to cell death. However, the effectiveness of UV treatment is often limited to surface exposure, as UV light does not penetrate deeply into materials or tissues, which can restrict its overall efficacy against embedded microbial pathogens [49]. While UV pretreatment has shown promise in reducing microbial contamination, its application to effluents containing VFAs requires careful consideration. VFAs are organic compounds that can potentially absorb UV radiation, which might affect the overall efficiency of the treatment. However, this absorption could also lead to photochemical reactions that may further contribute to the breakdown of organic compounds and potentially enhance the overall treatment process. The limitations of UV treatment are further underscored by its reliance on direct exposure to light. Microbes that are shielded by organic matter or biofilms may evade effective inactivation, as UV light cannot penetrate these barriers effectively. In studies comparing UV and heat treatments, UV alone often resulted in minimal reductions in microbial populations, with reported log reductions of only 1.3 to 3.8 logs for various pathogens [48]. Additionally, the potential for DNA repair mechanisms in microorganisms can mitigate the effects of UV damage, allowing some cells to recover and proliferate after treatment [50]. Future research should continue to explore the optimal conditions for these treatments, including their combinations, to maximise the antimicrobial efficacy. The use of mild heat in conjunction with UV treatment has been shown to enhance microbial inactivation beyond what either method could achieve alone [51]. This synergistic approach leverages the strengths of both methods, potentially leading to more effective control of *Fusarium* and other fungal pathogens in agricultural settings. As far as contamination is concerned, in the case of the tests with the original solutions, the presence of other microorganisms did not prevent the reading of the petri dishes, but the results would not be conclusive since it is not known whether the reduction in mycelial growth is due to the action of the VFAs or to competition between microorganisms.

3.3.2. Antifungal Activity: In Vitro Growth Inhibition Tests

The results of the mycelial growth inhibition tests are summarised in Figure 4. The radial mycelial growth of *Fusarium culmorum* was reduced for all the effluents tested. More specifically, when *Fusarium culmorum* was tested with the original solutions (no treatment), both WL and CW reduced mycelial growth but did not reach complete inhibition at the highest assayed concentration. In the case of UV pretreatment, full inhibition was reached at 4000 $\mu g \cdot mL^{-1}$ for the CW and at 3500 $\mu g \cdot mL^{-1}$ for the WL, whereas the efficacy of the heat was 2500 $\mu g \cdot mL^{-1}$ (WL) and 2000 $\mu g \cdot mL^{-1}$ (CW). Also, in both substances tested, in those broths in which no treatment was applied, fungal contamination was observed. When the treatments were applied at low concentrations (62.5 $\mu g \cdot mL^{-1}$–93.75 $\mu g \cdot mL^{-1}$), contamination was observed for both treatments. At 125 $\mu g \cdot mL^{-1}$ and above, no contamination was observed.

Upon comparison of the effective concentrations (Table 4), differences in the efficacy of the treatments could be observed more clearly. The highest efficacy (i.e., the lowest EC_{50} and EC_{90} values) corresponded to the WL heat followed by the CW heat, WL UV, CW UV, WL no treatment, and CW no treatment.

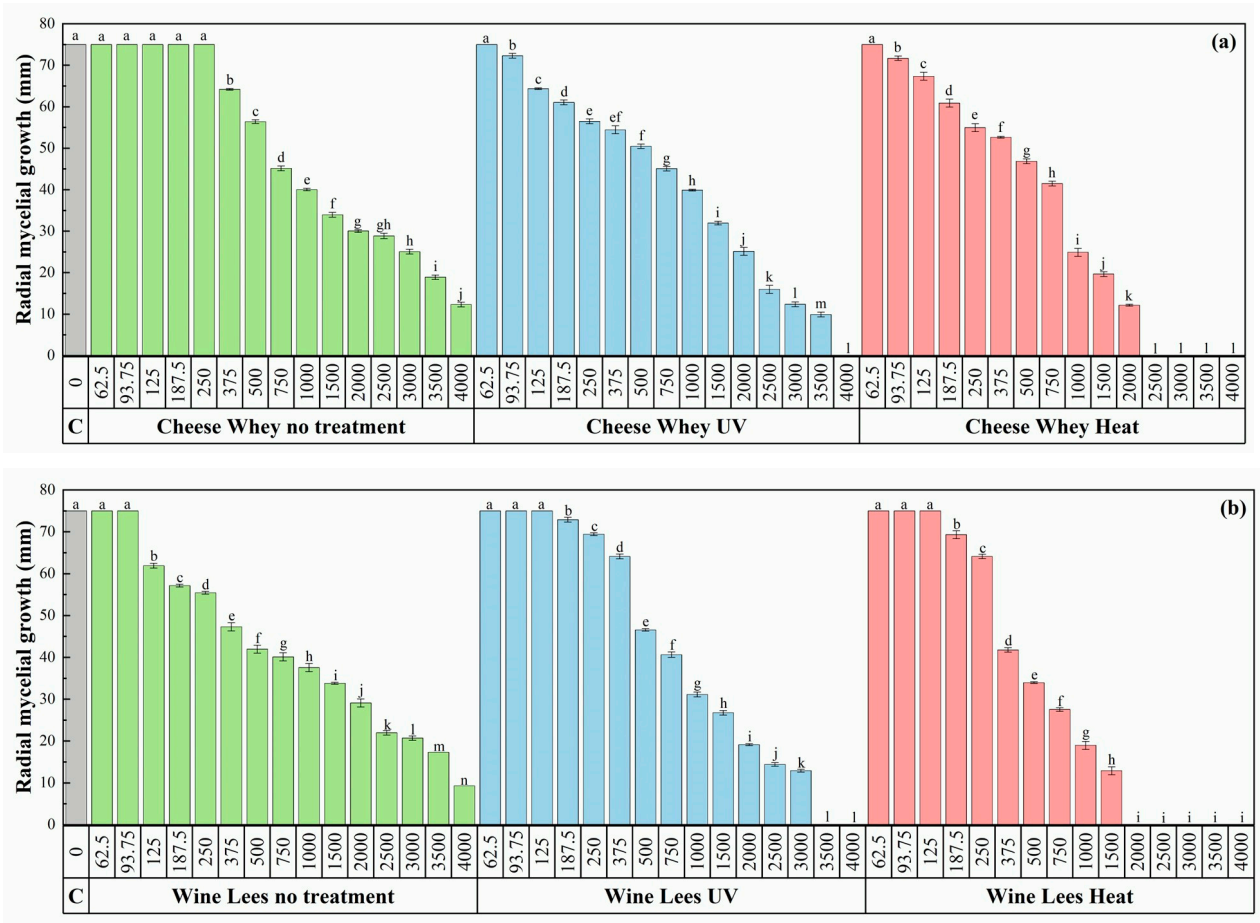

Figure 4. Radial growth values of *F. culmorum* in the presence of the different concentrations (in μg·mL^{-1}) for (**a**) cheese whey and (**b**) wine lees. Concentrations labelled with the same lowercase letters are not significantly different at $p < 0.05$ by Tukey's test. All values are presented as the average of three repetitions. Error bars represent the standard deviation across three replicates.

Table 4. EC_{50} and EC_{90} effective concentrations against *F. culmorum*, expressed in μg·mL^{-1}.

Effective Concentration	CW No Treatment	CW UV	CW Heat	WL No Treatment	WL UV	WL Heat
EC_{50}	2000	696	780	1036	685	682
EC_{90}	5780	3832	2202	4936	3145	1880

The antifungal activity of volatile fatty acids (VFAs) against various fungal pathogens, including *Fusarium* species, has been a subject of increasing interest in recent years. Our study demonstrates the efficacy of several VFAs in inhibiting fungal growth, with notable variations in their minimum inhibitory concentrations (MICs) depending on the pretreatment used. In this study, the best result was wine lees at a MIC of 2000 μg/mL. While not specifically for VFAs, Cárdenas-Laverde et al. [52] tested 44 plant end-products against *F. oxysporum* and found that the mycelial growth inhibition covered was ranging from 12% to 95%. This implies that no compound was able to achieve total growth inhibition.

In contrast, the study by Buzón-Durán, et al. [53] mentions that gallic acid showed antifungal activity against *Fusarium culmorum* at a concentration of 384 μg/mL. Brito et al. [54] indicates that Volatile Organic Compounds can effectively inhibit the growth of *Fusarium* pathogens. For example, VOCs has been shown to reduce the mycelial growth of *Fusarium verticillioides*, with concentrations similar to our study for Cis-2-hexen-1-ol

(1920 µg/mL) and Cis-3-hexen-1-ol (1820 µg/mL), demonstrating significant antifungal activity [54]. Furthermore, Xu and Chen [48] studied five different fatty acids to know their antifungal effects. They found that when analysing FA separately, there are differences in MICS between compounds. For *A. niger*, the lowest MIC was found in 13-HOE, with a value of 250 µg/mL, while the highest MIC was found in linoleic acid, with a value of 4000 µg/mL. In contrast, for *P. roqueforti*, coriolic acid was found with 260 µg/mL and 5330 µg/mL for linoleic acid. These values are in the range of values obtained in this study. They show that by using the compounds separately, better MICs can be obtained than by using the total VFAs [48]. In addition, environmental factors such as pH, temperature, and the presence of organic matter can significantly influence the efficacy of VFAs. For example, the activity of VFAs is often enhanced in acidic conditions, which may not always be present in field applications [50]. Understanding these interactions will be crucial for optimising the use of VFAs in agricultural settings. Moreover, the relatively low MICs observed for VFAs against *Fusarium* species compared to some synthetic fungicides (Table 5), which often require higher concentrations, suggest that VFAs could be a more environmentally friendly and a potentially cost-effective alternative for controlling fungal pathogens in agriculture and food preservation.

Table 5. A comparative analysis of the minimum fungicidal concentration (MFC) and minimum inhibitory concentration (MIC) data for fatty acids and common fungicides belonging to the strobilurin and azole classes, expressed in $\mu g \cdot mL^{-1}$.

Compound Class	Specific Compound	Target Fungus	MFC	MIC	MFC/MIC Ratio	Reference
Fatty Acids						
FAME extract	Mixed fatty acids	*Aspergillus fumigatus*	16,000	8000	2.0	[55]
FAME extract	Mixed fatty acids	*Aspergillus niger*	16,000	8000	2.0	[55]
Hydroxy fatty acids	Various	Multiple fungi	10–100	-	-	[28]
Strobilurins						
Azoxystrobin	Pure compound	*Passalora fulva* (sensitive)	-	0.031–0.5	-	[56]
Azoxystrobin	Pure compound	*Passalora fulva* (resistant)	-	8–32	-	[56]
Azoxystrobin	Pure compound	*Rhizoctonia solani*	-	10 (100% inhibition)	-	[57]
Tebuconazole + Trifloxystrobin	Combined formulation	*Rhizoctonia solani*	-	10 (100% inhibition)	-	[57]
Azoxystrobin + Difenoconazole	Combined formulation	*Rhizoctonia solani*	-	14 (100% inhibition)	-	[57]
Azoles						
PC1244	Novel triazole	*Aspergillus fumigatus*	0.14	0.064	2.2	[58]
Posaconazole	Triazole	*Aspergillus fumigatus*	0.42	0.125	3.4	[58]
Voriconazole	Triazole	*Aspergillus fumigatus*	>32	1.67	>19	[58]
Clotrimazole	Imidazole	*Microsporum gallinae*	1.00	0.50	2.0	[59]
Ketoconazole	Imidazole	*Microsporum gallinae*	1.00	0.50	2.0	[59]
Miconazole	Imidazole	*Microsporum gallinae*	1.00	0.50	2.0	[59]

FAME: Fatty Acid Methyl Esters.

The comparative data reveal several important patterns in antifungal effectiveness across compound classes. Azole fungicides generally demonstrate the highest potency in terms of MFC values, with some compounds effective at concentrations as low as 0.01 µg/mL. Strobilurin fungicides show good effectiveness against plant pathogenic fungi, with complete inhibition achieved at concentrations of 10–14 µg/mL for specific formu-

lations. Fatty acids demonstrate more variable effectiveness, with generally higher MFC values than synthetic fungicides. In all cases presented in Table 5, the minimum inhibitory concentrations (MICs) (8000 μg/mL) correspond to twice, at least, of the MICs obtained in these assays. For UV pretreatment, complete inhibition was achieved at 4000 $\mu g \cdot mL^{-1}$ for CW and 3500 $\mu g \cdot mL^{-1}$ for WL. In contrast, the efficacy of heat pretreatment resulted in MICs of 2500 $\mu g \cdot mL^{-1}$ for WL and 2000 $\mu g \cdot mL^{-1}$ for CW. These results indicate that their natural origin and potential for synergistic combinations with other compounds make them interesting candidates for integrated pest management approaches and applications where synthetic fungicides face resistance issues.

4. Conclusions

Volatile fatty acid production from food wastewater was successfully obtained with cheese whey and wine lees wastewater, performing better than the slaughterhouse wastewater. Since the slaughterhouse wastewater was highly diluted, pre-concentration can be performed to improve its interaction with the digestive bacteria. This was facilitated by pH control throughout the anaerobic digestion process, resulting in bioconversion maximum yields of up to 90 g and 72 g VFAs/100 g TCOD for cheese whey and wine lees, respectively. Volatile fatty acids offer a promising natural alternative for fungal control in agricultural applications. The radial mycelial growth of *Fusarium culmorum* was reduced for all the effluents tested. The efficacy of the treatments varied, with heat pretreatment showing the highest effectiveness for CW, achieving complete inhibition at 2000 $\mu g \cdot mL^{-1}$. This was followed by heat pretreatment for WL at 2500 $\mu g \cdot mL^{-1}$. UV pretreatment demonstrated lower efficacy, with complete inhibition achieved at 3500 $\mu g \cdot mL^{-1}$ for WL and 4000 $\mu g \cdot mL^{-1}$ for CW, respectively. Their effectiveness, combined with their relatively safe profile, makes them an attractive option for sustainable antifungal strategies. Future research should aim to optimise the application of VFAs through synergistic combinations and a better understanding of environmental influences to enhance their efficacy in sustainable agricultural practices.

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Abbreviations

The following abbreviations are used in this manuscript:

CW	Cheese whey
WL	Wine lees
SH	Slaughterhouse

VFAs	Volatile fatty acids
TCOD	Total chemical oxygen demand
COD	Chemical oxygen demand
pH	Potential hydrogen
AD	Anaerobic digestion
TSs	Total solids
VSs	Volatile solids
TKN	Total Kjeldahl nitrogen
$N\text{-}NH_4^+$	Ammonium
R1	Reactor 1
R2	Reactor 2
ANOVA	Analysis of variance
DNA	Deoxyribonucleic acid

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