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Microbial ecology in food waste bioconversion systems containing antimicrobial compounds

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Microbial ecology in food waste bioconversion systems containing antimicrobial compounds

By

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ABSTRACT

One-third of food waste (FW), or 1.3 billion tons produced globally every year imposes a heavy burden on the future of sustainability and food safety. As a result, global political programs and policies aim to ascertain food security and improve food waste management. This dissertation first reviews and summarizes different Food Waste valorization schemes and describes challenges associated with compounds that have microbial activity, and then highlights the benefits of bioconverting food wastes. The following parts analyze challenges associated with anaerobic digestion of wastewater with high levels of F-29 sanitizer, anaerobic digestion of orange peel with high levels of limonene and biosolarization using amendments composed of different bioactive compound matrices. The biosolarization chapter also describes the effectiveness of this method against pathogens, highlighting the potential to replace chemical treatment of soil. Our findings reveal the efficiency of this green method and can be used to guide future research.

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“Many would be cowards if they had courage enough.”— Thomas Fuller

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ABSTRACT

One-third of the food wasted (FW), or 1.3 billion tons globally produced every year imposes a heavy burden on the future of sustainability and food safety. As a result, global political programs and policies aim to ascertain food security and improve food waste management. This chapter reviews and summarizes different Food Waste valorization schemes with a focus on the re-utilization of food waste and bioconversion into energy. Methodological aspects, such as challenges and advantages regarding biomass composition and technical parameters are outlined. FW bioconversion, as an important method of Food Waste valorization without deleterious effects to humans, such as those seen in incineration and landfilling, contributes a beneficial impact on the environment and is worth further inquiry"

INTRODUCTION

According to the Food and Agricultural Organization of the United Nations (FAO)(1), approximately 1.3 billion tons of food is wasted along the food supply chain every year. This equates to roughly one-third of food produced globally by weight or one in every four kilocalories produced, and is inclusive of fresh vegetables, fruit, meat, baked goods, and dairy products(2). Wasted food products result in a loss of embedded resources, hinder food security, and raise social and economic problems(3). As consequence several governmental regulations have been created, the United Nations(4) has defined Target 12.3, within their Sustainable Development Goals (SDG) on food waste, which declares that the world needs "by 2030, to halve per capita global food waste (FW) at the retail and consumer levels and reduce food losses

along production and supply chains, including post-harvest losses". Waste reduction recognition programs are also governmental tools to address food waste. In reviewed by Chen and Chen(5), the authors highlighted policies within the Food Waste Challenge (FWC), the EPA Food Recovery Challenge (FRC) associated with food waste management, food reduction, and food donation set to allow and incentivize reduction in food waste. Still according to the authors, these programs follow the Food Recovery Hierarchy from the FDA where reducing the production of FW should be the main management strategy followed by sustainable techniques (e.g compost) and non-sustainable disposal (e.g landfilling) the last form of management. With the need for greener techniques in mind, this chapter aims to introduce and provide an update on different FW valorization schemes that have been or are currently being studied.

ORIGINS OF FOOD WASTE

Several distinctions are made in the literature for the organic residues from the food chain. Terms such as food losses and food waste, edible and inedible food waste, and avoidable and nonavoidable food waste are often used. For example food waste and food loss can be defined as "the decrease in quantity or quality of food" and food "left to spoil or expire as a result of negligence by the actor (predominantly, but not exclusively, the final consumer) respectively"(1,6). In turn, the methods reviewed in this paper are not restricted to certain types of residues and the use of multiple terms won`t benefit the discussion; thus, in this paper, we use Food waste (FW) to refer to the combined amount and types of food loss, waste and residues generated during all stages of processing, distribution, retail, final consumption, and post-consumption stages of the food life cycle.

A summary of processes that can generate residues and examples of associated FW are depicted in Figure 1.

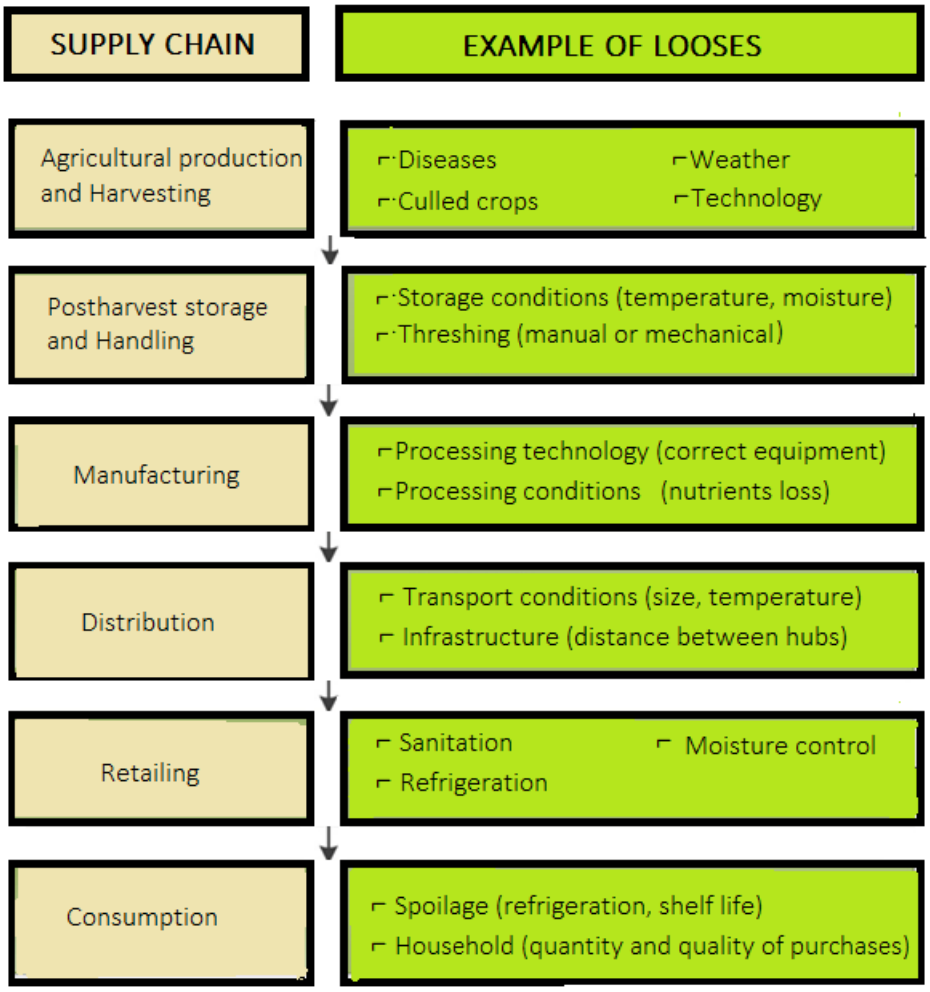

Figure 1: Summary of food waste stages throughout the food supply chain and example of loses from the subprocesses. This figure was adapted from Xue et al.(7).

Examples of these processes are difficult to quantify(8,9). This is due in part because more FW originates from farming practices in low-income countries when compared with high-income countries as their access to advanced technologies and infrastructure tends to be more restricted. For example, Xue *et al.*(7) reported that agricultural waste accounts for 13% of the total FW in North America and 26% in South Africa. According to the same review, this

variability also holds true for post-harvest handling and processing. Over 18 kg per capita of cereals and cereal products can be lost during postharvest handling and storage, whereas for fruits, meat, and fish products and eggs 33%, 0.3%, and 6%, of the total yields are lost during post-harvest handling and storage, respectively. This variability was reiterated in a review by Spang *et al.*(9). The authors synthesized the existing knowledge regarding FW measurements, and as a result, verified that there is a significant gap in publications regarding food type, stage of supply chain, and region, especially for developing countries.

The literature on FW is more robust for developed countries. Vidyarthi and Simmons(10) thoroughly reported on waste generation in California's industrial tomato processing practices with a market value of approximately US$1 billion. Whole loads of tomatoes can become waste if rejected by the processor (in the processing plant), and this occurs if they are determined to have defects, green fruits, or the quantity of other non-tomato objects is high. siThis water alongside water from other processing stages (e.g., cleaning the decanter tanks, tomato juice evaporative condensation, and surface cleaning and sanitation) is usually discharged in a comingled stream and needs to be monitored as it includes nitrogen, organic matter, and high salinity. As discussed above, the origin of FW is essential for its efficient bioconversion to biomass.

The domestic production of FW waste is of particular interest, as, in part, there are more practical opportunities here for interventions and reduction than with other sources (e.g., educational programs), but its variability is still greatly present. As expected, adults waste more than children, and larger families waste less per person than smaller families. A single person tends to throw away more food per capita, and families with children tend to waste more than

families without children. However, in this case, FW production in kg/cap does not seem to be related to income, as both low and high income families show similar trends(11).

FW variability is influenced by different regions and seasons, as well as agricultural, processing, and consumption practices. According to a review by Kiran(12), FW is composed of a highly complex matrix of components, mainly carbohydrates (simple sugars, starch, cellulose, and hemicelluloses), lignin, proteins, lipids, organic acids, and inorganic compounds, which have two main sources: plant (such as cereals, tubers, oil crops and pulses and animal (meat products, fish and seafood and dairy products(13).

A review by Otles *et al.*(14) reported other challenges when dealing with food waste. For example, inadequate biological stability and the existence of pathogens can cause an unwanted increase in microbial activity and the associated emissions of greenhouse gases and nuisance odors along with the accumulation of fermentation byproducts and foodborne pathogens. Furthermore, the high water content in FW has a huge impact on transport fuel usage and costs.

FOOD WASTE MANAGEMENT METHODS

Overview Summary

Figure 2 introduce and provide an overview of the main methods to treat FW and the main products that are generate. In-Situ degradation returns farming waste (e.g. rice straws) to soil aiming to release nutrients during further decomposition by native soil microorganisms mostly under non-controlled conditions(15), for this reason, this technique will not be further discussed.

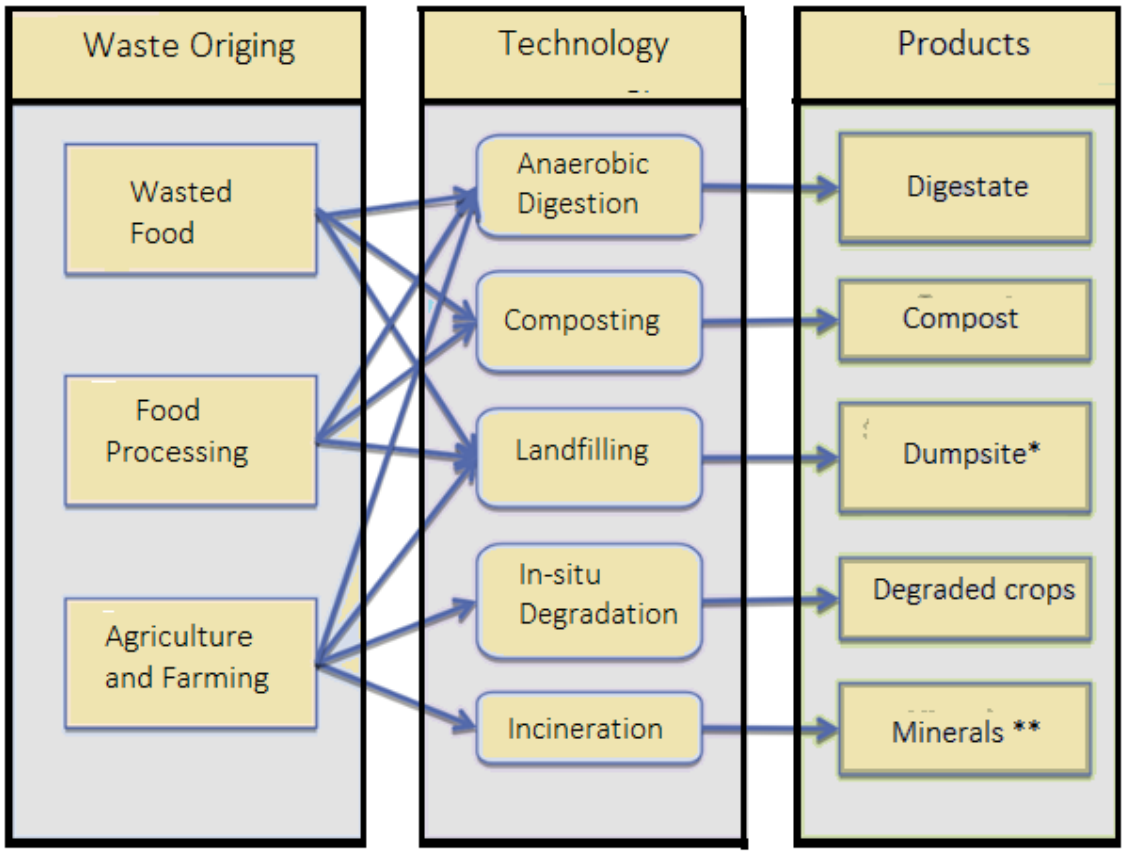

Figure 2: Summary of the main FW management technologies currently in use and the respective main products. The figure was adapted from Du *et al* (15), The product marked with * do not refer to a product but to the physical dumping site, ** Smoke and other deleterious products will be addressed in the next subsections.

Non-Sustainable management methods

Incineration and landfilling are traditional organic waste recycling technologies. However, these management methods to convert FW-to-energy have several environmental trade-offs and are not sustainable or environmentally friendly(16). In some countries, FW is added as a component to municipal solid waste, being incinerated or dumped in open areas, which may cause severe health and environmental issues due to the release of dioxins(17). These processes contribute significantly to the carbon footprint of FW and increase greenhouse gas (GHG) emissions into the atmosphere by approximately 3.3 billion tons of CO2 per year(17). In

contrast, studies on the upcycling of FW have demonstrated that greener and sustainable techniques can help to mitigate this environmental damage; tomato pomace, for example, when used as a soil amendment during biosolarization contributed in total to savings of 7.7 M kg CO2-e and 203,000 GJ annually(18). Other studies using almond processing residues recycled into the soil produce a high diversity of bioactive degradation compounds that act as alternatives to chemical fumigants, with low potential risks to humans(19). Based on these advantages over other deleterious methods such as incineration and landfilling, the rest of this review will focus on sustainable practices such as composting, anaerobic digestion, and biosolarization.

Alternative agricultural uses of FW

Insect farming is considered a promising method as insects can be farmed in high densities with low space requirements, furthermore, they have a high bioconversion ratio and can be reared on several waste streams(20). Black soldier fly larvae (BSF), *Hermetia illucens* L. (Diptera: Stratiomyidae) is a polyphagous larva known to feed on a wide range of sources, such as kitchen waste and manure. The larvae are higher in fat than other fly larvae, making it an alternative source of protein and fat for animal feed(20,21).

Mulching is a method used to manage and manipulate the soil microclimate and is classically defined as covering the soil to create “a shallow layer that appears at the soil/air interface, with properties that differ from the original soil surface layer”(22).

Mulching soil with biodegradable cotton creates conditions that are capable of suppressing weeds(23), while straw mulching can reduce superficial runoff, recycle organic matter, and enhance microorganism activity(24), and the physical barrier created can also prevent pathogens (i.e., Salmonella) from contaminating plants in infested soils(25).

As will be further described in the next section, the bioconversion methods explored in this review usually results in sustainable residues that can have beneficial impact on the environment being used, for example as soil organic fertilizers to replace synthetic chemical (15).

Composting

According to the literature(26–29), composting is the biologically "hygienic" conversion of organic wastes into mineral material (homogeneous and plant available) or its stabilization as humic substances. When added to the soil, compost conditions it by increasing the carbon stock, supplying nutrients to the plants and improving other physical (water holding capacity), chemical, and microbiological properties. Moreover, the Organic Regulations from the USDA(30) developed composting parameters aimed at improving the safety of the final compost. For example, these guidelines establish the temperature range to be maintained between 55ºC-77ºC for 3 days in anaerobic composting systems (statics pile) or for 15 days in aerated composting (windrow system) and the pile must be turned a minimum of five times within this time period. The impact of composting have been reviewed in terms of sustainability and food and nutrition security in a study by Domínguez-Hernández(31). The author concluded that composting is an economic, environmental, and socially sustainable option to improve vegetable and nutrient yields that could be recommended for horticulture interventions.

Composting is the sum of complex metabolic processes performed by different microorganisms (bacteria and fungi), which utilize oxygen (O_2), nitrogen (N), and carbon (C) to produce their own biomass, and this is converted into a stable material with the emission of carbon dioxide (CO_2). N is also mineralized into NH_4^+. The nutrient mineralization pattern during composting depends on the cellulose and pectin contents of the recycled products(26,28).

The first active stage of composting is an exothermic process governed by aerobic decomposition reactions. Typically, the efficiency of the composting process depends on the proper aeration of the material, under which the degradation rates can be maximized. These conditions yield high rates of heat emission, which can eventually lead to the thermal inactivation of pathogens, such as *Salmonella infantis*(32). Composting materials can create pockets of anaerobiosis, and even under good management, composting conditions can emit methane (CH_4) and nitrous oxide, which can trap 34 and 298 times more heat than CO_2(28). In addition to methane, NO_2 can also be produced in these anaerobic pockets, but simply combining composting with other techniques, such as biofiltration and/or combustion, effectively helps to decrease GHG emissions throughout the process(33).

Although the process is normally completed in 4–8 months, rapid composting techniques such as frequent turnings and shredding of the feedstock, and the use of effective microorganisms, chemical nitrogen activators, worms, natural minerals, and various additives and amendments are often used to decrease this time(34). The situation is even more critical in static piles, where a minimal management regime is commonly applied. Indeed, several studies have demonstrated the persistence of zoonotic pathogens in the finished compost at different levels of maturity and in compost-amended soils(32). Adding immature compost to the soil can also increase the adaptation and initial period of net immobilization of N to 70 days versus 3 when using mature compost. Longer periods of N immobilization can result in N deficiency(35).

Anaerobic digestion (AD)

In addition to being environmentally friendly (as discussed previously), another advantage of AD is that in most plants, biogas and compost can both be produced. The use of anaerobic digestion (AD) to produce biogas from waste streams was developed in the 1980s and the early 1990s and has been receiving increasing attention worldwide(36). A report from 2019(37) showed that, in Colorado alone, 32% of the facilities that are compatible with AD, but do not currently practice it, could readily go greener (with AD) if enough grant and loan programs were available.

Methane, the biogas from AD, is a renewable form of energy in emerging markets that can be transformed into electric energy and/or heat energy. In terms of the energetic balance, FW is consumed during the process and the energy generated is higher than the input; therefore, this biological treatment makes sense(26). During AD, a complex consortium of microorganisms produces biogas through the decomposition of organic matter (OM) in the absence of oxygen by the synergistic action of various bacterial species(26,38). The production of biomethane or biohydrogen has four sequential phases: sequential hydrolysis of complex nutrients in the OM, acidogenesis, acetogenesis, and methanogenesis(39,40). In the first step, hydrolysis, complex organic substances are decomposed into soluble monomers, followed by acidogenic fermentation. In this second step, bacteria convert these soluble monomers into smaller products such as volatile fatty acids (VFAs). In the third step, acetogenesis, acids such as lactic acid and pyruvic acid are converted into acetic acid and hydrogen. Finally, the products obtained from the previous steps (e.g., acetic and formic acids, CO_2/H_2, etc.) are metabolized into methane by strictly anaerobic methanogens.

In addition to the energy from methane, the resulting digestate can be used as a soil amendment. For example, amended soil has been reported to have increased enzymatic activity when compared to non-amended soil, and these enzymes are indicators of soil fertility and microbial oxidative activity(39).

Biosolarization (BSB) and anaerobic soil digestion (ASD)

Other processes of FW bioconversion, such as biosolarization (BSB) and anaerobic soil digestion, offer greener and more sustainable techniques that can efficiently replace chemical fumigation (e.g., methyl bromide) to disinfest soil prior to planting(19,41–44). These methods are an evolution of mulching with a plastic tarp added to help the in-soil anaerobic digestion of added FW. The synergistic combination between the mulching, and the physical and chemical conditions from fermentation are lethal to agricultural pests such as unwanted seeds, weeds, fungi, and nematodes in as little as 2 weeks(44–46). Anaerobic conditions were created by adding OM to the moist soil and covering with a plastic tarp. BSB differs from ASD as the process occurs during periods of sustained elevated temperatures, increasing the thermal inactivation of pests. VFAs such as propionic acid, acetic acid, and butyric acid are produced and accumulate during both BSB and ASD, acidifying the soil. The drop in pH of soil works synergistically with anaerobioses and changes in temperature to promote pest inactivation(19,42,47–49). Finally, the phytotoxicity and other negative effects of BSB and ASD can be overcome rapidly after removing the tarps(42,46,50). Recent studies have shown promising results when using this method to upcycle wine waste while still disinfecting the soil from human pathogens(51,52).

PROPERTIES RELEVANT TO BIOCONVERSION

As previously mentioned, some of the main methods used for FW management involve bioconversion, where a consortium of microorganisms degrades organic matter (OM) into value-added bioproducts, including methane, hydrogen, and ethanol(12,48).

According to Azim(26), there are various FW biodegrading parameters in the literature, such as the carbon–nitrogen ratio (C/N), microbial activity, total nitrogen (TN), cation exchange capacity (CEC), and humic substance content.

Nitrogen is an essential component of microbial activity, as it forms proteins involved in all living systems. For example, proteins in the form of enzymes are involved in waste hydrolysis. Even though the origins of FW are highly diverse, the quantity of nitrogen is typically low in many ecosystems and waste streams. For this reason, the carbon to nitrogen ratio (C/N) is an important factor affecting degradation, and C/N ratios of 25–30 are usually considered optimum for processes such as composting(53,54). If the C/N ratio is low, nitrogen can be volatilized into the atmosphere as NH_3. In contrast, if the initial C/N ratio is above 35, microorganism succession will prioritize the oxidation of excess carbon(26).

According to the literature(12,40), the form of carbon is also important. For example, enzymes involved in the hydrolysis of carbohydrates in FW are necessary to break glycoside bonds and release compounds amenable to fermentation. However, lignocellulosic biomass consists primarily of cellulose, hemicellulose, and lignin, whose interactions create a highly resistant and recalcitrant biomass structure. In short, carbohydrate polymers in lignocellulosic biomass are not accessible during the initial hydrolysis step. Besides accessibility, the enzymes or microorganisms should be easily adsorbed on the polymer chains, but after hydrolysis they

should be desorbed to start the hydrolysis of other chains. Consequently, the lignocellulose content of the FW and hydrolysis are often the rate-limiting steps during FW bioconversion.

Moisture content is also an important factor that has a direct impact on microbial activity, as it solubilizes the nutrients required by microorganisms, and as a result, most of the decomposition occurs in the liquid film formed on the surface of FW organic solids. Although it depends on the process and the feedstock, for example, an initial moisture content of 50%–70% is generally considered ideal for bioconversion methods such as composting(53). Conversely, with biosolarization, the water content can exceed 80% of the water-holding capacity of the soil(46).

As seen in all complex biochemical metabolisms, the bioconversion of FW is also greatly influenced by pH variations in the system. Considering the example of anaerobic digestion and the conversion of FW into methane, pH plays an important role in determining the type of anaerobic fermentation pathway in acidogenic processes. In the pH ranges of 4.0–4.5, 4.5–5.0, and 5.0–6.0, we have respectively the formation of butyrate, ethanol, and propionate. Propionate fermentation is easily carried out by either hydrogen-producing bacteria or other microbial populations. However, pH maintained in the neutral range is optimal for the next step of methanogenesis(55).

Another factor is the production of bioactive compounds during bioconversion. Microbes use the energy from the breaking of carbon chains in the FW to grow; however, metabolic networks have evolved to maintain metabolic homeostasis(56). As a result and according to Ngbede *et al.*(57) other metabolites, known as secondary metabolites, usually accumulate during the later stage of microbial growth known as the “Idiophase.” The compounds produced in this stage have no direct relationship with the synthesis of the cell material and normal growth of the

microorganisms(56,58). Similarly, plants, which are an important part of FW, produce secondary metabolites that are not directly involved in normal growth, development, or reproduction, but are crucial for the interaction between the plant and its environment(59). These secondary metabolites are carried over to food, and many have beneficial effects in humans. Many also have toxic and antimicrobial effects, and in both cases are referred to as bioactive(60). Examples of secondary metabolites from microbes are antibiotics, steroids, and alkaloids(58), whereas from plants, they are organic compounds divided into three groups: terpenes, nitrogen-containing metabolites, and phenolic compounds(59).

For this reason, bioconversion occurs in a complex matrix, where the bioconversion of the substrate usually occurs in the presence of multiple bioactive molecules(60). For example, phenolics present in grape skins and seeds are extracted over the course of fermentation(61) while at the same time, yeasts convert sugars into ethanol. As a result, both increased concentrations of ethanol and the presence of phenolic compounds can modulate the growth and viability of microorganisms(56). Considering the importance of bioactives, this topic will be further explored in the subchapter called “Bioactives.”

Finally, the microbiota available for initiating and carrying out this process are also important. As mentioned previously, biosolarization elevates soil temperatures to thermophilic conditions(62). Studies(48,63) have shown that using inoculum from digestate contributes to soil fermentation during SBS and affects organic matter turnover and soil microbiota. Soils amended with digestate from thermophilic digestors had the greatest microbial divergence from the initial soil state, whereas the microbiomes from mesophilic digested treated soils were more similar to the non-amended soil(48). The same study has shown that many of the most abundant Operational Taxonomic Units (OTUs) present after amending the soil with thermophilic

inoculum were nonexistent or of very low abundance in the original non-amended soil. For example, *Acinetobacter* strains that are siderophore-producing bacteria have shown *in vitro* inhibition of *Fusarium oxysporum*. Enrichment of *Cellvibrio, Pseudomonas,* and *Bacillus* spp. is also important, as the microbes are known as potential biofertilizers through nitrogen fixation and nutrient solubility in soils.

Finally, the microbiota directly impacts other organisms through competition. For example, BSB conditions can enhance the growth and survival of beneficial fungi that inhibit pathogenic fungi, which will later cause microbial degradation of weed seed coats(48).This symbiosis is an important challenge when creating a state of the art microbial consortia that can efficiently act as a biological tool for FW bioconversion, as antagonistic relationships between microorganisms can lead to the instability of the consortium and the expected functions may not be obtained(64). Therefore, the growth, survival, and activity of any one species or strain, or a desirable biocontrol will, in most cases, be determined by the presence of other microorganisms and intra-and inter-microbial interactions(65).

COMPOUNDS WITH BIOLOGICAL ACTIVITY

In recent decades, the concept of functional foods has grown as it offers a new and practical approach to achieving optimal health through the use of natural products with physiological benefits(66). As a result, the bioactive matrix in the food waste can become very complex and can include antibiotics, steroids, and alkaloids from microbial secondary metabolites(58), terpenes, nitrogen-containing metabolites, and phenolic compounds from plant-derived waste(59). This matrix can also include animal sources that can be combined with bioactive peptides during the stage of processing fortification, such as peptides that are inactive

in food itself but are released as a result of fermentation, enzyme treatment, or during digestion(67).

Phenolic compounds (PP)

The term “phenolic compounds” generally describes compounds having a phenol moiety stabilized by a resonance structure that allows the molecule to donate a H-atom, giving the compound antioxidant properties through a radical scavenging mechanism. PP**s** are the most abundant compounds in food wastes of plant origin. These compounds can vary in size from a simple structure with a single phenol ring and low molecular weight to highly complex structures such as lignins and tannins. PPs can be subdivided into phenolic acids, flavonoids, coumarins, isoflavonoids, stilbenes, lignans, and phenolic polymers(59,68).

Considering the complexity of these compounds, different phenolic compounds exhibit different effects on the bacterial species. A review by Xia *et al.*(69) reported that *Staphylococcus* is very sensitive to PPs commonly found in wine extracts, followed by *Escherichia coli*, although they showed no effects against *Salmonella* sp., while stilbenes, such as resveratrol, have potent antifungal activities.

Many mechanisms have been proposed to explain these activities. One study(70) demonstrated that combinations of flavonoids and/or nonflavonoids, even at low concentrations, are synergistic with an important antibacterial effect. For example, the combination of gallic and caffeic acids inactivates all cells of *Escherichia coli* ATCC 35218, even at low temperatures(4 °C). Other researchers(52,71) have shown that phenolic compounds from grape pomace have no antimicrobial effect against an *E. coli* O157:H7 surrogate when used as a soil amendment and under mesophilic conditions and/or anaerobioses, but when applied under biosolarization

conditions (50 °C and anaerobioses), no live cells could be detected. Flavonoids and non-flavonoids can hinder heat shock responses by regulating genes related to bacterial metabolic state, modulating the formation of persister cells, efflux pumps(72), and genes related to biofilms, swarming, and motility(73). Finally, phenolic acid-rich extracts (24–49 μg phenolics/mL) from peanut by products showed antimicrobial effects comparable to those of ampicillin (10 μg/mL)(74).

Kumar *et al.*(59) summarized the groups of phenolic compounds and provided examples (Table 1, adapted).

Table 1 : Classification of phenolic compounds with example of respective sub-groups adapted from Kumar et al.

MAIN GROUP OF PHENOLIC COMPOUNDS	SUB-GROUP OF PHENOLiC COMPOUNDS	EXAMPLE OF SUB-GROUP OF PHENOLIC COMPOUNDS
Phenolic acids	Hydroxy benzoic acid	p-hydroxybenzoic acid, protocatechuic acid, gallic acid, vanillic acid, syringic acid
	Trans-cinnamic acid	p-coumaric caffeic and ferulic acids
Coumarins	Coumarin derivatives	Umbellirone, aesculetin, herniarin, psoralen, imperatorin
Flavonoids	Flavones	Chrysin, apigenin, tangeritin luteolin, acacetin
	Flavonols	Quercetin, quercetin, myricetin, 3-hyroxyflavone, kaempferol
	Flavanones	Naringenin, eriodictyol, hesperitin
	Flavanonols	Taxifolin, romadedrin, engeletin
	Flavanols	(+)-Catechin, (−)-epicatechin, epigallocatechin
	Anthocyanidins	Cyanidin, delphinidin, pelargonidin,
Isoflavonoids	Isoflavones	Daidzein, genestein, glycetin
	Coumestans	Coumestrol
Stilbenes	Stilbenes derivatives	Resveratrol, piceatannol, pterostilbene
Polyphenols	Condensed tannins	Procyanidin, prodelphinidin propelargonidin
	Hydrolyzable tannins	Gallotannins, ellagitannins

Essential oils

Essential oils (EOs) are complex compounds extracted from plants. These oils contain a wide variety of terpenes and oxygenated compounds, such as alcohols, aldehydes, and esters(75). Among the 300 commercially important EOs, limonene is one of the most important because of its citrus-like odor, its abundance in nature, and its antimicrobial power. This compound will be further discussed in Chapter 3, but in short, the main antimicrobial mechanism is cytotoxicity, in a manner similar to many antibiotics. These compounds can induce membrane instability (cytotoxicity) and increase reactive oxygen species (ROS). The compounds can be used in combined treatments; for example, nanoemulsions with limonene have been proven to increase antimicrobial potency and retain organoleptic attributes.

Other sources of active compounds

A source of compounds that can modulate bioconversion is exogenous. Quaternary ammonium compounds (QACs) are sanitizers frequently used in food facilities due to their efficacy as disinfectants(76). However, because of their substantial use, they are often released into the environment, leading to the contamination of wastewaters as industrial plants that treat wastewater act as a major ‘receiver’ of QACs(77). According to a review by Voumard et al.(78), concentrations of QACs can greatly vary based on the source; municipal and hospital wastewaters, for example, can be measured in the μg l^{-1} and mg l^{-1} range respectively. This is important as anaerobic digestion is the technique usually applied to biologically convert wastewater organic matter to renewable gaseous biofuel, and the bacteria in digester sludge are responsible for the bioconversion. A laboratory study have shown that increasing the sanitizer

concentration negatively impacts the methane production rate and biogas quality(76). Finally, QACs can also be found in relatively high concentrations in soils and surface waters (78).

Additionally, waste streams can contain pharmaceuticals and personal care products(79) or exotic compounds, such as phenolics, cerberosides, and triterpene glycosides (saponins) from seafood(80). Finally, the effects of manmade compounds, such as artificial sweeteners, are yet to be explored. These compounds are consumed in large quantities because of their low-calorie content; however, they are not metabolized by humans and, as a result, are found pervasively in water bodies.

CONCLUSIONS AND OUTLOOK

This review on FW bioconversion has highlighted benefits that can be explored as well as several challenges that need to be overcome in order to guarantee a sustainable growth. This chapter offers a reliable initial support for decision-making in relation to FW reduction, valorization policies and possible intervention programs.

Food loses are intrinsic to the food chain and can only be reduced but not totally eliminated, hence this chapter confirms the importance of techniques that add value to food wastes. FW bioconversion methods such as anaerobic digestion, mulching and incorporation methods reutilize FW streams and produce energy while generating stable, non-hazardous products that can be used as soil fertilizers or disinfesting methods and decrease or replace the need for chemical alternatives.

FW is highly variable and influenced by several factors such as regions, processing technology, and consumption practices. This chapter explores the complex biochemical matrix where multiple microorganisms bio-converts organic matter into energy and highlight the role of bioactives and other food contaminants (disinfectants) in the process.

Overall, this work is an introductory guide that informs and updates the reader about sustainable methods of FW reutilization and raise awareness of their importance and relevance to a sustainable future.

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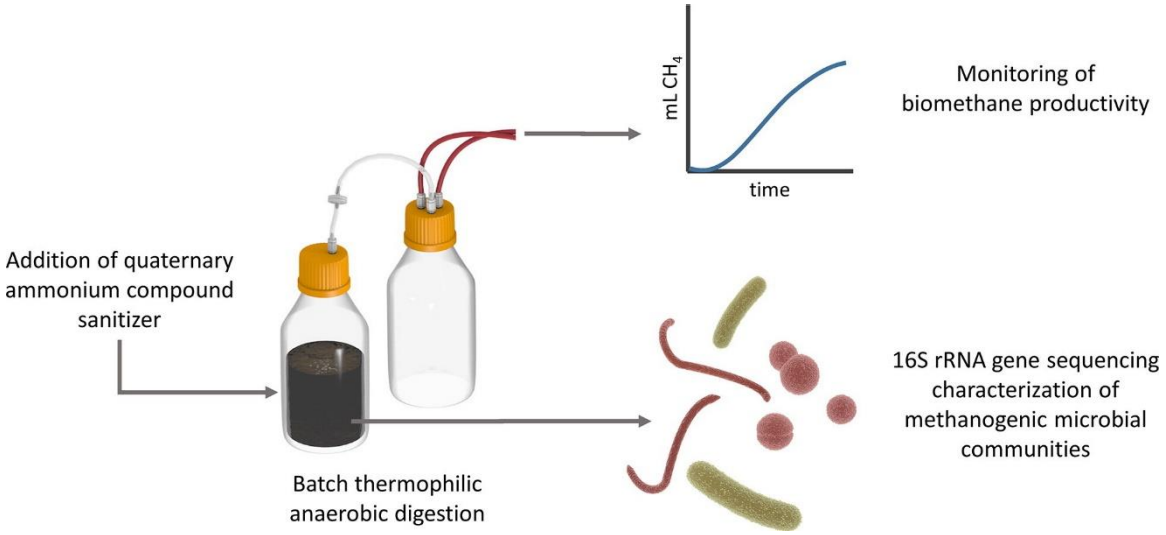
CHAPTER 2 - STRUCTURE AND ACTIVITY OF THERMOPHILIC METHANOGENIC MICROBIAL COMMUNITIES EXPOSED TO QUATERNARY AMMONIUM SANITIZER

ABSTRACT

Food processing facilities often use antimicrobial quaternary ammonium compound (QAC) sanitizers to maintain cleanliness. These QAC can end into wastewater used as feedstock for anaerobic digestion. The aim of this study was to measure the effect of QAC contamination on biogas production and structure of microbial communities in thermophilic digester sludge. Methane production and biogas quality data were analyzed in batch anaerobic digesters containing QAC at 0, 15, 50, 100 and 150 mg/L. Increasing sanitizer concentration in the bioreactors negatively impacted methane production rate and biogas quality. Microbial community composition data was obtained through 16S rRNA gene sequencing from the QAC-contaminated sludges. Sequencing data showed no significant restructuring of the bacterial communities. However, significant restructuring was observed within the archaeal communities as QAC concentration increased. QACs in thermophilic anaerobic digester sludge impacted the level and quality of biogas production, which corresponded to a significant changes in the sludge archaea. Further studies to confirm these effects on a larger scale and with a longer retention time are necessary.

Keywords: anaerobic digestion; antimicrobials; biofuels; microbial ecology; waste management

GRAPHICAL ABSTRACT



INTRODUCTION

Anaerobic digestion

According to the literature, (Khalid et al., 2011; McCarty et al., 2011) anaerobic digestion is a waste management technology that can biologically convert wastewater organic matter to renewable gaseous biofuel, or biogas. Bacteria in digester sludge are responsible for the hydrolysis, acidogenesis and acetogenesis of complex organic compounds to acetic acid, carbon dioxide and hydrogen gas. These products serve as the substrates for the methanogenic archaea that ultimately convert them to methane and carbon dioxide, the principal components of biogas. A research (De Vrieze et al. 2018) evaluated the archaeal and bacterial community via transcriptome and 16S rRNA (gene) amplicon sequencing. Operational taxonomic unit (OTUs) showed the predominance of Firmicutes, followed by Bacteroidetes, Proteobacteria, Thermotogae and Spirochaetae phyla. The same study showed that Archaea bacteria are associated with the metabolisms of volatile fatty acids (VFAs) and Acetate, while bacteria are associated with changes AD parameter such as pH, temperature, total ammonia nitrogen and conductivity

Quaternary ammonium compounds (QACs)

Quaternary ammonium compounds (QACs) are frequently used in food facilities due to their efficacy as a sanitizer (Gerba, 2015). QACs are composed of anions with the general formula NR4 + where the “R” group are commonly a branched hydrocarbon, a linear alkyl chain or an aryl group(Yu et al. 2018). but their inactivation efficacies are usually diminished by organic materials contamination or at low temperature(ALAM et al. 2018). QACS interact with the cytoplasmic membrane of bacteria, the plasma membrane of yeast and because of their hydrophobic, they are also effective against lipid-containing viruses. QACs also interact with intracellular targets and bind to DNA. At low concentrations (0.5 to 5 mg/liter) they prevent the growth of microorganisms. At concentrations of 10 to 50 mg/liter, they st microbicidal (Gerba, 2015).

After disposal, QACs are persistent in the environment, including estuary sediments, soils, and receiving waters, especially when associated with solid phases and total concentrations are much greater than other conventional organic contaminants ranging from less than 1 μg/L to approximately 60 μg/L in surface water and up to 10 times these levels in wastewater(Yu et al. 2018; Li and Brownawell 2010; Hora et al. 2020).

Previous studies have shown that QAC contamination can affect methane production in mesophilic digesters operating at 35 ˚C (Tezel et al., 2006, 2007). However, thermophilic digesters employing temperatures of 50-60 ˚C are often used as well, which can have considerable differences in sludge microbial community structure (Chachkhiani et al., 2004; Shi et al., 2013) that can lead to more rapid cellulose degradation (Shi et al., 2013) and elevated methane production rates (Hashimoto, 1983). However, these benefits may come at the cost of decreased community stability (Dinsdale et al., 1996; Kim et al., 2002). To date, the

susceptibility of thermophilic sludges to QACs has not been determined nor has the phylogenetic composition of QAC-contaminated sludge microbial communities been linked to biogas production data. In this study, the structure of thermophilic sludge microbial communities exposed to varying levels of QACs was determined via 16S rRNA gene sequencing to determine correlations between the bacterial community dynamics and biogas production.

1. EXPERIMENTAL

1.1. Anaerobic digestion

Batch anaerobic digesters were comprised of 250-mL glass media bottles fitted with modified caps containing a port connected to tubing and an in-line check valve (catalog #80103, Qosina, Ronkonkoma, NY). The methanogenic sludge used for these digesters was obtained from a thermophilic anaerobic digester located near the University of California. The sludge used in the biodigester is adapted and acclimated to digest Davis campus`s waste composed mainly of food and organic residues (food scraps, spoiled packaged food, manure, yard waste, and paper waste). It is worth mentioning that yard waste fed into the digesters did not have been treated with antimicrobials and the paper waste mainly contain napkins and compostable package (opposingly to printing paper for example), for this reason, the sludge did not contain nor had been exposed to chemical compounds that might modulate the initial microbiome within the sludhe. Moreover, according to the operational staff, the digester did not process any rinse water from facilities using QAC sanitizers and thus the background QAC level in the sludge was assumed to be negligible.

The sludge had a total solids content of 3.5%. The commercial QAC sanitizer F-29 (Rochester Midland Corporation, Rochester, NY, USA) consisted of 4% (*W/W*) alkyl (C12-16)

dimethylbenzylammonium chloride, 3% decyldimethyloctylammonium chloride, 1.5% didecyldimethylammonium chloride, and 1.5% dioctyldimethylammonium chloride). According to the label (Figure S. 1), the recommended dilution can vary from 1200 mg/L to 400 mg/L and 150 mg/L to sanitize respective food plants with fogging, by contact on food plants and by contact on non-food plants. Based on this values and subsequent dilutions (rinsing, mixing) throughout the sanitizing steps, F-29 was to the sludge at varying levels (0, 15, 50, 100 or 150 mg QAC/L). Finally according to the guidelines where this paper was previously published, it was adopted mg/L, but for reference, 1 mg/L represents one part per million (ppm).

To establish methanogenic cultures, sludge was initially incubated for 2 days at 55°C to exhaust most residual methane production. Each digester was then loaded with 100 ml of sludge and 0.5 mg of finely-milled tomato pomace to simulate organic matter that may be found in food processing wastewater. Varying volumes of F-29 sanitizer were loaded into digesters to achieve 0, 15, 50, 100 or 150 mg QAC/L. Reactor headspace was flushed with nitrogen gas. Reactors were incubated at 55°C for 4.5 to 7.25 days to elucidate differences in methane production between treatments without the confounding ecological effects of substrate exhaustion. Methane, carbon dioxide, and hydrogen content in biogas was measured via a MicroOxymax respirometry system (Columbus Instruments, Columbus, OH). More details on the setup can be found in previous works by Simmons`s Lab (Achmon et al. 2019; Fernandez-Bayo et al. 2017; Achmon et al. 2020). The pH of the sludge was measured for two reactors from each treatment at the end of the incubation.

1.2. DNA isolation and 16S rRNA gene sequencing

Before sampling, the reactors were homogenized and the genomic DNA was purified directly from non-diluted liquid sludge microbial communities according to the PowerSoil DNA Isolation Kit guidelines (MO BIO Laboratories Inc and The V4 region of the 16S rRNA gene was amplified and sequenced according to previously described methods (Simmons et al., 2014) with one alteration. In short, primer used was 515f as the forward with a 5' Illumina adapter amended via pad and linker sequences, The reverse primer was 806r the reverse primer with Illumina adapter compliment, barcode, pad and linker sequences amended to the 5'end. Amplicons were sequenced on anIllumina MiSeq system as described by the manufacturer's proto-col with PhiX Control v3 library (Illumina, San Diego, CA) as the sequencing control. Under the altered protocol, a qPCR library quantification kit (KAPA Biosystems) was used to determine the concentration of V4 amplicons capable of being sequenced ahead of sequencing.

1.3. Data processing and analysis

Still according ing to Simmons et al., (2014), DNA sequencing reads were filtered to remove Illumina adapter, PhiX sequences and to remove unpaired reads from the data set. The filtered data was trimmed to 165 bp to be compatible with FLASH assembly software. Following assembly with FLASH, reads were once again scanned but this time for primer sequences and were removed. The mean Phred quality scores (unitless) of the first 20 bp from each end were determined and used to trim contiguous sequences (aimed quality score greater than or equal to 30). The last quality filtering removed sequences containing more than five ambiguous base calls or 10 base calls with quality scores less than 15. Reads with at least 97% similarity were

clustered into groups using an in-house clustering algorithm developed by the Joint Genome Institute. The taxonomy of each operational taxonomic unit (OTU) group was determined using QIIME (http://www.qiime.org). Finally, singleton and doublet OTUs were removed from the data set to reduce noise.

Ecological analyses were performed using RStudio (version 0.98.1103) with the vegan and entropart packages. Prior to analysis, singletons were removed from operating taxonomic unit (OTU) read count data to reduce noise. Linear regression analyses of community diversity, dissimilarity, OTU abundance, and biogas production data were performed using JMP software (version 12.0.1, SAS). For comparison of bacterial OTU changes in response to QAC concentration, critical P-values were adjusted for multiple comparisons to achieve a familywise error rate of 0.05 using the Bonferroni method (Bland and Altman, 1995).

Bray-Curtis dissimilarity (BC) was calculated based on the relative abundance data and Equation 1, where X_i and X_J are the relative abundance for each OTUs.

$$BC_{ij} = 1 - \sum min(X_{i,}X_j) \qquad \text{Equation 1}$$

2. RESULTS AND DISCUSSION

2.1. Biomethane production

According to Figure 3a**,** a significant negative trend was observed between QAC level and methane production over the culture period (P = 0.002). Differences in cumulative methane production between treatments related to changes in methane production rates (Figure 3**b**).

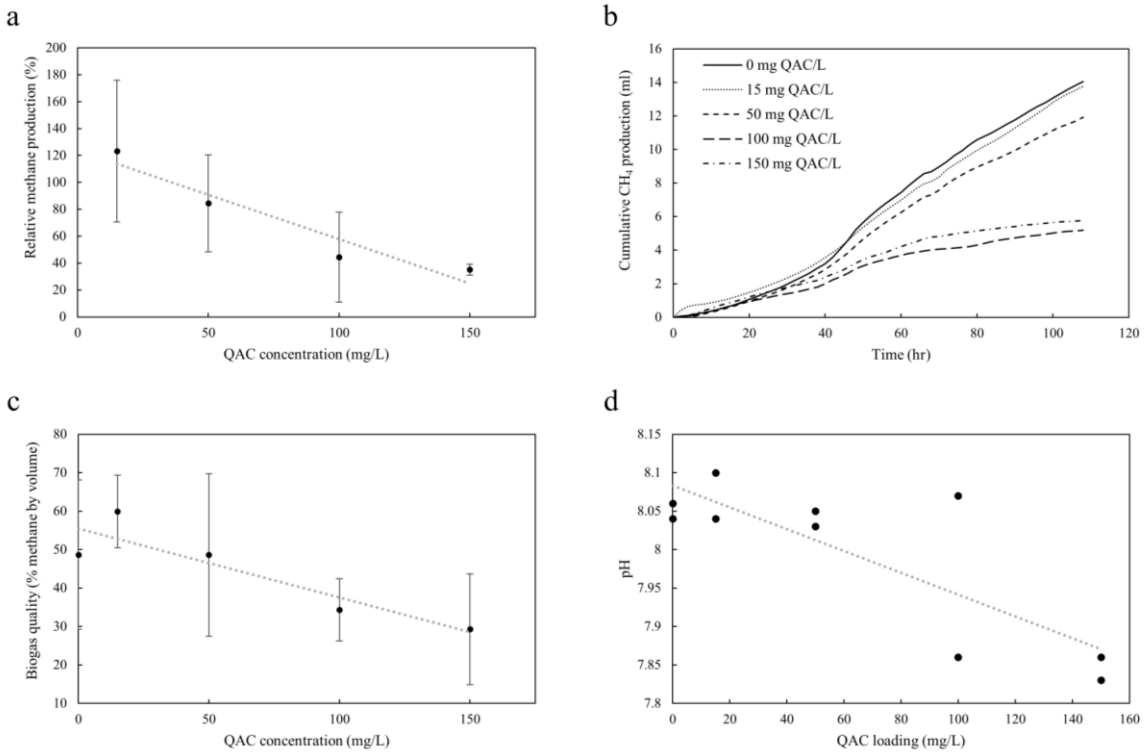


Figure 3: Biomethane production from sludge containing varying levels of quaternary ammonium compounds (QAC) after 4.5 to 7 days of incubation. (a) Final cumulative methane production expressed as a percentage of that observed in control reactors lacking QAC. (b) Average of cumulative methane production over the first 4.5 days of culture. (c) Biogas quality estimated from cumulative production of methane and carbon dioxide over the culture period. (d) Sludge pH at the conclusion of the incubation. Dotted lines indicate the line of best fit for the data. Error bars represent one standard deviation. n=4 for a-c, n=2 for d

Sludges containing 0, 15, or 50 mg QAC/L appeared to maintain more similar methane generation rates compared to sludges with 100 or 150 mg QAC/L. Specifically, sludges with at least 100 mg QAC/L showed a marked decrease in methane production rate 48 hr post-QAC addition compared to those with lower QAC levels. These data suggest a critical QAC level between 50 and 100 mg QAC/L for the thermophilic sludge. Previous studies observed inhibitory effects above 25 mg QAC/L for mesophilic methanogenic communities (Tezel et al., 2006, 2007). QAC concentration also affected the quality of biogas produced by sludge (Figure 3c). The methane content of the biogas produced by sludge significantly decreased as QAC concentration increased (P = 0.008). These data indicate that methanogenesis in the thermophilic

sludge was more sensitive to the concentration of QAC compared to upstream metabolic processes that produce carbon dioxide. A similar response was previously observed for mesophilic sludge (Tezel et al., 2006). Although some of these upstream processes, such as the production of acetate from other organic acids, produce gaseous hydrogen in tandem with carbon dioxide, no accumulation of hydrogen gas was detected for any treatment (data not shown). A significant negative correlation was observed between the final sludge pH and the QAC level ($P = 0.004$, Figure 3d). However, the lowest pH measured (7.85) was still well within the tolerable range for anaerobic digestion (Cioabla et al., 2012).

2.2. Microbial community composition

Calculation of Good's coverage values predicted that over 99.9% of OTUs were accounted for in the sequencing data for each microbial community analyzed. At the whole community level, diversity index (H') did not show any significant difference in response to QAC level (Table 2).

Table 2: Sequencing coverage and community diversity indicators for sludge communities incubated with varying levels of QAC. Values are presented as mean ± standard deviation (n = 4).

QAC level (mg/L)	Good's coverage	Shannon (*H*')
0	$0.9997 \pm 5 \times 10^{-5}$	2.07±0.07
15	$0.9996 \pm 5 \times 10^{-5}$	2.11±0.09
50	$0.99975 \pm 5 \times 10^{-5}$	2.13±0.05
100	$0.9996 \pm 5 \times 10^{-5}$	2.09±0.11
150	$0.9997 \pm 5 \times 10^{-5}$	2.13±0.11

Separate analysis of archaea and bacteria within the communities revealed differing trends between these sub-communities in response to QAC contamination (Figure 4 and Table 3).

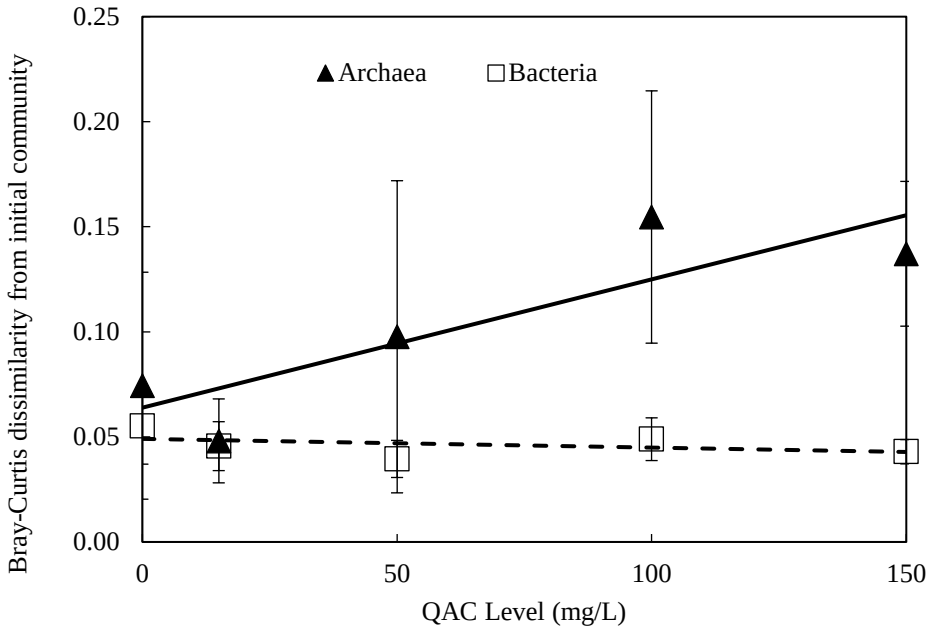


Figure 4: Bray-Curtis dissimilarity of bacteria and archaea within sludge communities Incubated with QACs relative to the initial sludge community. Solid and dotted lines represent lines of best fit for archaeal and bacterial sub-communities, respectively. Error bars indicate one standard deviation. n = 4.

Alternately and still according to Figure 4 and Table 3, the level of all archaea was not impacted by exposure to QAC, however there was a significant alteration to the composition of archaeal sub-communities due to increasing QAC concentration"

Table 3: Bray-Curtis dissimilarity of varying levels of QAC from the initial community. Values are presented as mean ± standard deviation (n = 4).

QAC level (mg/L)	Bray-Curtis dissimilarity from initial community	
	Bacteria	Archaea
0	0.0611± 0.0169	0.0169±0.0744
15	0.0525±0.0117	0.0482±0.0197
50	0.0462±0.0109	0.0979±0.0743
100	0.0590±0.0139	0.1548±0.0598
150	0.0525±0.0080	0.1371±0.0346

Archaeal communities were represented by four genera: *Methanobacterium*, *Methanoculleus*, *Methanothermobacter*, and an uncharacterized genus within family *WCHD3-02* (class *Thermoplasmata*). *Methanoculleus* dominated archaeal communities across all treatments (relative abundance >83%, Figure 4 and Table 4). However, at greater QAC concentrations, its relative abundance remained still dominated, but significantly decreased (P = 0.009) in favor of *Methanothermobacter* and an OTU genus within family *WCHD3-02*.

Table 4: Phylogenetic composition of sludge bacterial communities incubated with varying QAC levels as indicated by OTU relative abundance

OTU[a]	QAC (mg l^{-1})					P-value[b]
	0	15	50	100	150	
f__Thermotogaceae	59.47±1.87	58.37±2.29	57.92±1.27	58.91±2.87	57.72±2.39	0.450
f__Clostridiales Family XI. Incertae Sedis	6.74±0.92	7.02±0.68	7.48±0.10	7.74±0.55	7.81±0.64	**0.010**
o__MBA08	4.87±0.54	4.63±0.33	4.54±0.55	4.42±0.37	4.31±0.47	0.081
g__*Thermonema*	3.51±0.87	3.63±0.57	3.79±0.33	3.43±0.48	3.35±0.50	0.438
f__Porphyromonadaceae	2.24±0.32	2.40±0.26	2.21±0.11	2.24±0.12	2.32±0.19	0.992
o__Clostridiales	2.04±0.28	2.15±0.32	2.22±0.41	2.26±0.21	2.39±0.20	0.086
g__*Tepidanaerobacter*	1.84±0.41	1.87±0.43	2.01±0.20	2.14±0.31	2.38±0.35	**0.014**
o__TIBE07	1.98±0.14	2.12±0.19	2.10±0.07	1.71±0.32	1.95±0.45	0.257
f__Ruminococcaceae	1.51±0.05	1.51±0.09	1.45±0.27	1.38±0.36	1.60±0.18	0.774
o__SHA-98	1.28±0.19	1.32±0.17	1.32±0.22	1.27±0.19	1.43±0.17	0.379
o__MBA08	1.43±0.32	1.52±0.41	1.12±0.33	0.85±0.34	1.01±0.28	**0.014**
f__Halanaerobiaceae	0.83±0.16	0.90±0.14	0.95±0.19	0.94±0.14	0.91±0.04	0.526
Total	87.76	87.44	87.11	87.31	87.19	

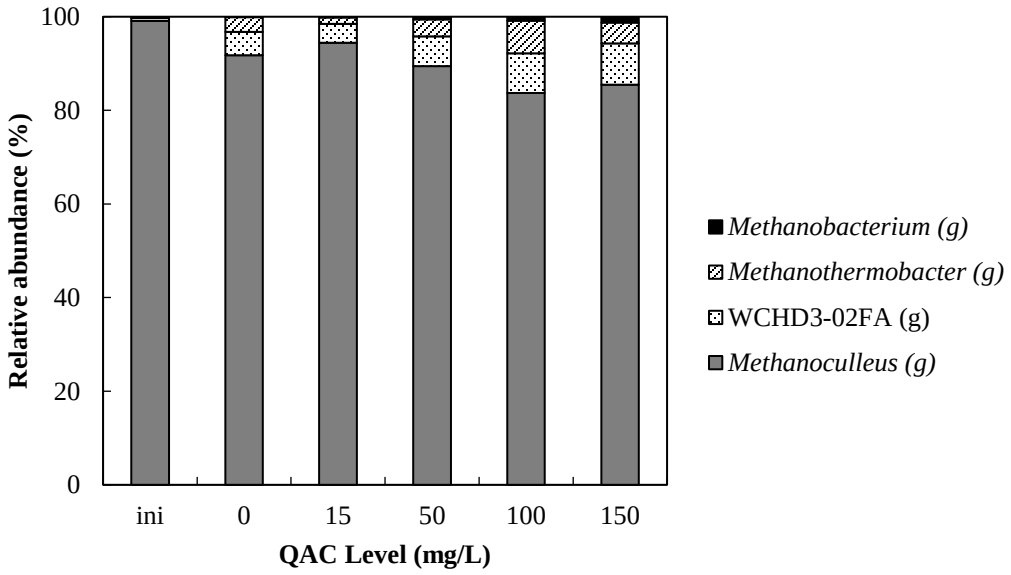
[a]Relative abundance (% of community) data are presented at the lowest classification of the OTU (o, order; f, family; g, genus). [b]P-values for effect test of QAC level on relative abundance of given OTU. P-values less than the critical threshold of 0.05 for pairwise comparisons are bolded. The critical P-value for a familywise error rate of 0.05 is 0.0042.

The abundance of *Methanoculleus* in all cultures suggested that this genus was likely responsible for most of the sludge methanogenic activity. *Methanoculleus* archaea are hydrogenotrophic methane producers (Barret et al., 2013; Wasserfallen et al., 2000). The prominence of hydrogenotrophic methanogens in all communities indicated that the community likely employed syntrophic acetate conversion, where non-methanogenic microorganisms within the sludge community oxidize acetate to produce CO_2 and H_2 for hydrogenotrophic methanogenesis. Syntrophic acetate oxidation is most thermodynamically favorable at elevated temperatures (Karakashev et al., 2006). As a result, the thermophilic communities studied here likely differ considerably from the mesophilic communities examined in prior QAC

contamination studies (Tezel et al., 2006, 2007), which may have relied more on other archaea and methanogenic pathways that are more thermodynamically favorable at lower temperatures, such as acetotrophic methanogenesis. There may be innate differences in the sensitivity of thermophilic and mesophilic archaea to QACs. For instance, it has been observed that thermophilic archaea can be tolerant to a variety of other environmental stresses (Mesbah and Wiegel, 2012). The overall robustness of certain thermophilic archaea may contribute to the greater QAC tolerance observed in this study compared to previous research with mesophilic sludge communities. However, additional research is needed to separate other effects, such as differential adsorption of QACs to suspended solids in thermophilic and mesophilic sludges, that could also affect QAC availability and tolerance in sludges.

Although the culture duration used in this study was sufficient to elucidate differences in biogas production in response to QAC contamination, it was less than the 25 to 30 days hydraulic retention time typically used in anaerobic digesters. It is possible that the pH depression and archaeal restructuring observed at high QAC concentrations could become more drastic over time. Given their abundance within all archaeal sub-communities, *Methanoculleus* sensitivity to QACs is likely a major factor in the overall anaerobic digestion sensitivity to QAC contamination. Similar changes between *Methanoculleus* and other methanogenic archaea has been observed previously in response to digester perturbations (Lee et al., 2014).

Bacterial sub-communities showed no significant relationship between dissimilarity from the initial community state and QAC concentration ($P = 0.79$, Figure 4). Sludge communities contained 20 bacterial phyla spanning 203 genera. The most abundant phyla, *Thermotogae*, *Firmicutes*, and *Bacteroidetes*, showed no significant changes in relative abundance for the QAC levels tested ($P = 0.442$, 0.212 and 0.592, respectively; Figure 5).



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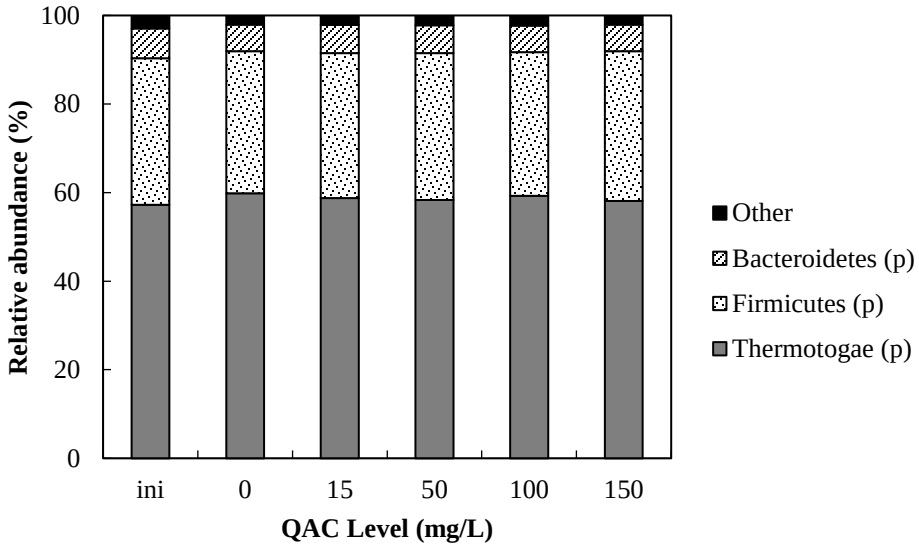

Figure 5: Phylogenetic composition of anaerobic digester microbial communities incubated with various levels of QAC sanitizer and the initial inoculum (ini). Data correspond to (a) archaea and (b) bacteria sub-communities. For clarity, archaea are presented at the lowest resolved phylogenetic classification while bacteria are presented at the phylum level (p, phylum; f, family; g, genus). n = 4

Within these phyla, twelve OTUs accounted for more than 87% of bacterial community abundance for all treatments (Table 4). Three OTUs showed changes in relative abundance in response to varying QAC concentration: Clostridiales Family XI. Incertae Sedis family and Tepidanaerobacter genus increased with increasing QAC concentration whereas an OTU within the MBA08 order showed decreased as QAC levels increased. However, when the Bonferroni correction was used to account for multiple comparisons and establish a new critical P-value ($P \leq 0.0042$), corresponding to a familywise error rate of 0.05 across all OTUs, no OTUs showed significant changes in relative abundance in response to QAC concentration.

The differential response in archaeal and bacterial communities to increasing QAC concentration, as indicated by both phylogenetic restructuring and changes in biogas quality, is consistent with prior research that found that methanogenic archaea were more sensitive to ammonium concentration than sludge bacteria (Sawayama et al., 2004). The differing sensitivity to QAC may relate to physiological differences between certain archaea and bacteria, such as preference for different compatible solutes to manage osmotic stress (da Costa et al., 1998). Additionally, the unique lipids that archaea utilize to withstand thermophilic environments (van de Vossenberg et al., 1998) may ultimately make them less tolerant of QACs. Additional research is needed to explore these possibilities.

3. CONCLUSION

This study suggests a negative impact of QAC on thermophilic digester performance. Further studies to confirm these effects on a larger scale and with a longer retention time are necessary. Data regarding the tolerance of anaerobic digestion microbial communities exposed to QAC sanitizer can inform digester operational procedures and waste treatment practices. QAC sanitizers are often recommended for use at levels up to 400 mg/L (F-29 sanitizer label), considerably greater than the inhibitory threshold for the thermophilic sludge community, sanitizer presence in wastewater has the potential to impact digester operation. Therefore, treatment or dilution of sanitizer wastewater streams with significant QAC concentration will be required ahead of digester loading. Moreover, phylogenetic composition data from anaerobic digestion communities will be useful for predicting QAC susceptibility in other methanogenic communities.

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APPENDIX 1: Supplementary data

PRECAUTIONARY STATEMENTS
HAZARDS TO HUMANS AND DOMESTIC ANIMALS

DANGER. Keep Out of Reach of Children. Corrosive. Causes irreversible eye damage and skin burns. Harmful if swallowed, inhaled or absorbed through the skin. Avoid breathing spray mist. Do not get into eyes, on skin or on clothing. Wear goggles or face shield and rubber gloves and protective clothing when handling. Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum, using tobacco or using the toilet. Remove contaminated clothing and wash clothing before reuse. **ENVIRONMENTAL HAZARD:** This product is toxic to fish. **PHYSICAL OR CHEMICAL HAZARDS:** Do not use or store near heat or open flame. Do not mix with oxidizers, anionic soaps and detergents.

FIRST AID

Have the product container or label with you when calling a poison control center or doctor, or going for treatment. **IF ON SKIN OR CLOTHING:** Take off contaminated clothing. Rinse skin immediately with plenty of water for 15-20 minutes. Call a poison control center or doctor for treatment advice. **IF IN EYES:** Hold eye open and rinse slowly and gently with water for 15-20 minutes. Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. Call a poison control center or doctor for treatment advice. **IF SWALLOWED:** Call a poison control center or doctor immediately for treatment advice. Have person sip a glass of water if able to swallow. Do not induce vomiting unless told to do so by a poison control center or doctor. Do not give anything by mouth to an unconscious person. **IF INHALED:** Move person to fresh air. If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably mouth-to-mouth, if possible. Call a poison control center or doctor for further treatment advice. **NOTE TO PHYSICIAN:** Probable mucosal damage may contraindicate the use of gastric lavage.

DIRECTIONS FOR USE

It is a violation of Federal Law to use this product in a manner inconsistent with its labeling.

DIRECTIONS FOR FOGGING IN DAIRIES, BEVERAGE AND FOOD PROCESSING PLANTS: Prior to fogging, food products and packaging material must be removed from the room or carefully protected. After cleaning, fog desired areas using 1 quart per 1000 cubic feet of room area with a solution containing 1.5 ounces of product to 1 gallon of water (1,200 ppm active). Wear a dust mist respirator when mixing the use solution and pouring it into the fogging apparatus. Vacate the area of all personnel during fogging and for a minimum of 2 hours after fogging and a minimum of 4 air exchanges (ACH) per hour in the facility. All food contact surfaces must be sanitized with an EPA approved food contact sanitizer solution of 150-400 ppm active prior to use. All food contact surfaces must be thoroughly rinsed prior to reuse with potable water. **Note:** The fog generated is irritating to the eyes, skin and mucous membranes. Under no circumstances must a room or building be entered by anyone within two hours of the actual fogging and a minimum of 4 air exchanges (ACH) per hour in the facility. If the building must be entered, then the individuals entering the building must wear a self-contained respirator approved by NIOSH/MSHA, goggles, long sleeves and long pants. **FOGGING IS TO BE USED AS AN ADJUNCT TO ACCEPTABLE MANUAL CLEANING AND DISINFECTING OF ROOM AND MACHINE SURFACES.**

PREPARATION OF NON-FOOD CONTACT SANITIZER USE SOLUTION: For heavily soiled areas, a preliminary cleaning is required. Add 1 ounce of this product to 5 gallons of water (150 ppm active). Apply sanitizer use solution to hard, non-porous non-food contact surfaces with a brush, cloth, mop, sponge, auto scrubber, mechanical spray device or by immersion so as to thoroughly wet surface to be sanitized. For sprayer applications, spray 6-8 inches from surface. Do not breathe spray. Rub with brush, cloth, or sponge. Treated surfaces must remain wet for 3 minutes. Wipe up excess liquid. Change cloth, sponge or towels frequently to avoid redeposition of soil. Prepare a fresh solution at least daily or when use solution becomes visibly dirty.

NON-FOOD CONTACT SANITIZING PERFORMANCE: At 1 ounce of this product per 5 gallons of water use-level (150 ppm active), this product is an effective one-step sanitizer with a 3-minute contact time against the following on hard non-porous surfaces: Staphylococcus aureus, Klebsiella pneumoniae.

SHOE/BOOT BATH SANITIZER DIRECTIONS: To prevent cross contamination from area to area in animal areas, and the packaging and storage areas of food plants, shoe baths containing one inch of freshly made sanitizing solution must be placed at all entrances to buildings, hatcheries and at all the entrances to the production and packaging rooms. Scrape waterproof shoes and place in a 1 ounce of this product per 5 gallons of water (150 ppm active) use solution for 3 minutes prior to entering area. Prepare a fresh solution at least daily or when use solution becomes visibly dirty.

ENTRYWAY SANITIZING SYSTEMS: To prevent cross contamination from area to area, set the system to deliver 1.25 – 2.5 oz. per 2.5 gallons of water (400 – 800 ppm active) of sanitizing solution. The spray/foam must cover the entire path of the doorway. Set the system so that a continuous wet blanket of sanitizer solution is delivered to the floor. Do not mix other foam additives to the sanitizing solution.

FOOD CONTACT SANITIZING DIRECTIONS

Before use as a sanitizer in public eating places and dairies, food products and packaging materials must be removed from the room or carefully protected. Prior to application, remove gross food particles and soil by a pre-flush or pre-scrape and when necessary, pre-soak. Then thoroughly wash or flush objects with a good detergent or compatible cleaner, followed by a potable water rinse before application of the sanitizing solution. **SANITIZING OF FOOD PROCESSING EQUIPMENT AND OTHER HARD NONPOROUS FOOD CONTACT SURFACES IN FOOD PROCESSING LOCATIONS, MEAT PLANTS, DAIRIES, BAKERIES, CANNERIES, BEVERAGE PLANTS, RESTAURANTS AND BARS DIRECTIONS:** For sanitizing precleaned food processing equipment, dairy equipment, food utensils, dishes, silverware, eating utensils, glasses, sink tops, countertops,

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ACTIVE INGREDIENTS:

Octyl Decyl Dimethyl Ammonium Chloride	3.0%
Didecyl Dimethyl Ammonium Chloride	1.5%
Dioctyl Dimethyl Ammonium Chloride	1.5%
Alkyl (C14 50%, C12 40%, C16 10%) dimethyl benzyl ammonium chloride	4.0%
INERT INGREDIENTS:	90.0%
TOTAL:	100.0%

Food processing plants, USDA inspected food-processing facilities, dairy farms, meat/poultry processing plants, meat/poultry producing establishments, canneries, bakeries, and meat packing plants.

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NET CONTENTS: 1 Gallon • 3.785 Liters

Figure S. 1: Label of the sanitizer used in the experiment. This figure was adapted to include the most relevant information.

CHAPTER 3 - LIMONENE TOLERANT MICROBIOME ENRICHMENT DURING BATCH ANEROBIC DIGESTION OF ORANGE PEEL – DISCOVERIES AND CHALLENGES

ABSTRACT

Limonene is one of the most important bioactives in citrus plants because of its citrus-like odor, abundance and antimicrobial activity which can be inhibitory to bioconversion of food residues methods such as Anaerobic Digestion (AD). The objective of this paper was to isolate, through enrichment, a consortium of microorganisms capable to anaerobically digest orange peel while being resistant to limonene. Healthy CO_2 production could still be seen in bioreactors containing concentrations of limonene higher than 15.000 ul/L. However, due to the recalcitrant composition of these digesters, DNA extraction became a challenging step towards isolating and sequencing the microbiota, for this reason, this chapter relies on several unsuccessful methods of extraction to review the literature and create a solid stepping stone for future researches aiming to isolate DNA from high-solids AD systems. Kits that remove inhibitors from plant combined with pectinase are promising tools to isolate and sequence DNA from biodigesters.

1. INTRODUCTION

More than 88 million tons of citrus fruit, the most predominant type being orange, are produced globally each year while in the US alone, 10 million tons of citrus solid waste were generated from the processing of 21.2 million tons of citrus fruit between 2008 and 2011.(1) This solid waste is mainly composed of orange peel (OP) which is rich in citrus essential oils (EO), and its main component is D- limonene (or limonene), with reported concentrations as high as 74.43% (V/W).(2) Limonene is a high-value-added molecule that can be utilized by a variety of industries, ranging from cosmetics to food; however, due to its potential toxicity, the use of

waste rich in OP can be challenging.(1,3,4) Limonene has lipophilic characteristics, and it is believed to pass through cell walls and cytoplasmic membranes and accumulate in microbial plasma. The accumulation disturbs the structures made from layers of polysaccharides, fatty acids, phospholipids, and damages the stability of DNA, which results in a loss of membrane integrity and dissipation of the proton motive force. Ultimately decreases in ATPase activity (Na+K+-ATPase, Ca2+-ATPase) hinders ATP synthesis and the activity of the respiratory complex.(5–7)

Due to this inhibition of ATP synthesis and the respiratory complex, waste rich in limonene is incinerated (8) or dumped into landfills, which is problematic as it can have negative environmental impacts and is not energy efficient.(4) Composting citrus is another method by which to upcycle OP and add value to the waste stream.(4,9) Composting transforms organic matter into stable compounds that can be applied to the land without adverse effects on the environment. However, the excessive moisture in wet OP makes it difficult to compost using the standard methods and consequently it requires pre-treatment, for example, by mixing with lignocellulosic materials, but this creates additional costs.(4)

Anaerobic digestion (AD) is a promising technique for waste valorization, and has reportedly been used with OP waste.(3, 9–11) During AD, organic matter is biologically converted to renewable gaseous biofuels or biogas.(1,12) A consortium of bacteria promotes the hydrolysis, acidogenesis, and the acetogenesis of complex organic compounds to acetic acid, carbon dioxide, and hydrogen gas. These products are the substrates for methanogenic archaea that convert them to carbon dioxide and methane, the main components of biogas.

More research is required to better understand and overcome the toxic effects of essential oils on the microbial communities responsible for AD. Current technologies rely on the pre-

treatments of OP to remove the inhibitory effects of limonene.(1,3) Hexane is a commonly used pretreatment; however, its residues can inhibit the anaerobic digestion process.(13) The identification of tolerant bacteria is a promising method for the enhancement of biobased conversions; however, the main strategy utilized is based solely on the engineering and optimization of these "cell factories" using genome editing tools, such as CRISPR/Cas9.(14) Eng et al. (15) reported that enrichment may be a cheaper strategy by which to isolate microbes tolerant to inhibitors. The authors used laboratory evolution to select Escherichia coli strains that were capable of robust growth and able to restore the production of biofuel in the presence of ionic liquid (IL) stress. Some limonene catabolic pathways are already known, and enzymes such as epoxy hydrolases play an important role in the catabolism of specific carbon sources, such as tartaric acid and limonene. (16) Using enrichment in combination with sequence-based metagenomic approaches could exponentially increase the discovery and use of novel enzymes for AD.(17)

This study aimed to evaluate the laboratory evolution of a thermophilic microbiome in relation to increasing concentrations of limonene during AD. The results will help to improve the anaerobic digestion of orange peels while avoiding the pre-treatment steps currently required to remove limonene. Ultimately, this approach will provide the basic tools with which to identify bacteria that can tolerate and/or consume inhibitors such as limonene.

2. METHODS

2.1 Anaerobic digestion setup

Bioreactors for anaerobic digestion were created according to the scheme shown in Figure 6 and based on the protocols described previously (12-24). Glass bottles (250 mL) with a port were connected to tubing that had an in-line flow check valve (Qosina, Ronkonkoma, NY).

Gas emissions could leave the bioreactor to enter the collection chamber, and the flow check valves blocked the gas from returning to maintain anaerobiosis. From the collection chamber, the gas flowed into the MicroOxymax respirometry system (Columbus Instruments, Columbus OH), where the methane, carbon dioxide, and hydrogen were measured. To accelerate anaerobiosis, oxygen from the headspace was purged with inert nitrogen gas (Airgas Specialty Products, Lawrenceville, GA). Reactors were incubated at 55 °C for 7 d to elucidate the differences in biogas quality between the treatments. Monitoring of the methane and carbon dioxide production during the cultures provided a real-time indication of community vitality in relation to the limonene additions; the larger increments completely inhibited the bacteria causing gas production to cease, and the small increments imposed too small of a challenge causing gas production to rapidly plateau, which confounded the ecological effects and substrate exhaustion.

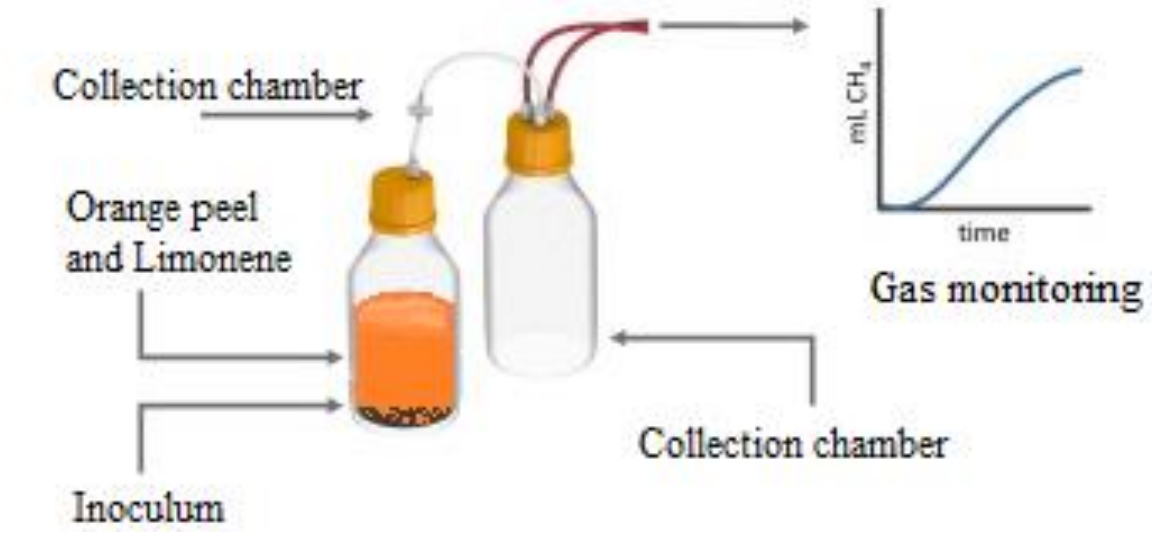


Figure 6. Pictogram of a bioreactor unit. The bioreactor is created by adding orange peel and the inoculum with the anaerobic bacteria, the biosystem is connected to a check valve that allows only gas to leave the reactor to maintain anaerobioses. The gas produced is stored in the collection chamber and analyzed in the respirometer. This set up and figure was adapted from previous works. (12)

2.2 Biomass composition

Bioreactors were composed of orange peel (OP) added of the respective inoculum to achieve a concentration of 3% w/w dry mass and M9 medium (M9 minimal medium with added

trace elements) was added until moisture matched the water holding capacity of the biomass. Organic orange peel was donated after being manually pressed for juice by a food retailer. The OP was first dried for 48 h at 50 °C and then finely milled using a lab blender. Green waste compost (GWC) was used as the first bacterial inoculum on week 0, GWC was a mature compost that originated from yard clippings at a commercial composting site in Zamora, CA. Previous studies have shown that this particular compost is mature and does not induce significant microbial activity without the addition of a labile carbon source.(18) M9 media was chosen to moisten the dry mix, as it is commonly used with microorganisms that are used for downstream microbial biofuel production. (19) After adding the liquid and before adding the inoculum, the wet mix was left to equilibrate overnight in a refrigerator at 4 °C. As so to mimic limonene build up within the bioreactor, liquid (+)-limonene (Across organics, Tracy CA) was added to the bioreactors with weekly increments for 12 consecutive weeks, the increments and limonene incorporation followed the protocol discussed in the subsection 2.2.1. All vessels and M9 media were autoclaved ahead of use.

WEEK-0 (INITIAL SETUP)

Six bioreactors were built according to the procedure described above in subsection 2.1. OP was wetted with M9 media, no limonene, and allowed to equilibrate. After this period, GWC was inoculated into the bioreactors, and oxygen was purged from the headspace. This treatment in week zero is required to acclimate the microflora to the thermophilic conditions and enrich the consortia of microbes involved in methane production. After 7 days, each bioreactor was unhooked from the respirometer, homogenized, and its content was used as inoculum for the next week's set of bioreactors. The moisture content was measured gravimetrically, and the remainder was frozen to extract the genetic material.

ENRICHMENT (WEEK-1 - WEEK-11)

Starting on week 1 of the treatment, the six reactors were split into two sets (limonene and control) with three biological replicates each. The control set was created by inoculating OP with the biomass from the digestion of the previous week, M9 media, and no limonene for the entire treatment period. Controls would inform the optimum evolution of gas using an inoculum specialized in OP digestion but no stress due to limonene build-up.

The limonene group was created in the same way, but limonene was added in weekly increments to the bioreactors, as shown in

Table 5. From week 1–week 8, limonene was added to the M9 media before wetting the

Week of experimentation	Weekly Limonene increments (µL/mL)
Week0	0
Week1	100
Week2	200
Week3	300
Week4	500
Week5	700
Week6	1,000
Week7	1500
Week8	2,000
Week9	5,000
Week10	10,000
Week11	15,000

dry mix and overnight liquid equilibration. From week 9 onwards, limonene concentrations were above 5,000 uL/L of the liquid phase, and solubility became challenging. To overcome this limitation, limonene was added in two steps. Half of the volume was added to the whole M9 volume for overnight equilibration, and the other half was added to the equilibrated wet mix and

prior to inoculation. This method was preferred in lieu of non-ionic surfactants, as they have been reported to affect methane production during anaerobic digestion (20).

Table 5. Weekly increments of the limonene treatment. The increments were based on the readings taken the previous week and calculated according to the liquid content and level of the microbiota stress.

Week of experimentation	Weekly Limonene increments (μL/mL)
Week0	0
Week1	100
Week2	200
Week3	300
Week4	500
Week5	700
Week6	1,000
Week7	1500
Week8	2,000
Week9	5,000
Week10	10,000
Week11	15,000

2.3 Gas evolution kinetics

The cumulative carbon dioxide evolution rate (cCER) was used as a metric for orange peel degradability in the presence of the increasing concentrations of the limonene. For each treatment, the effluent gas from the bioreactors was measured in real time for 7 consecutive days (168 h), which was enough time for the gas emissions of all bioreactors to plateau. Logistic growth curves were fitted to the cCER versus time data to estimate the model parameters for each treatment according to Eq. 2 using R studio (Version 1.3.1093) and JMP software (version

16). The model was applied to individual replicates with an overall fit of 0.9978708, and the mean parameter of each treatment was calculated (n = 3 replicates).

$$\text{cCER} = \frac{\max CO_2}{1 + e^{((k\mu)-(kt^2))}} \quad \textbf{Eq. 2}$$

Where, cCER is the cumulative carbon dioxide evolution rate (cCER) in uL CO_2/g; maxCO_2 is the asymptote or maximum CO_2; "*k*" is the specific production constant; and "μ" is the specific inflection point or (maxCO_2/2). (21–23)

2.4 Isolation and amplification of deoxyribonucleic acid

Extraction of genomic DNA was conducted using several methods and kits (Table 2). DNA was quantified using Qubit® fluorometers following the manufacturer's guidelines for high and broad sensitivity. The quality of the extracts was measured using absorbance values from a Nanodrop (Thermo Fisher Scientific, Wilmington, MA, USA). PCR (24) and electrophoresis was conducted to assess the presence of amplifiable DNA, and they followed the protocols described by Simmons et al. (2014) with several modifications (discussed in subsection 3.2). Extraction and amplification methods were tested separately and in combination, as discussed in the subsection 3.1 and 3.2. The choice of which sample to use as the standard for a given method of extraction was guided by the challenges (e.g. higher limonene concentration in week 11). Sequencing protocols followed the quality guidelines of the Joint Genome Institute (GJI).(25). Green waste compost, sludge from anaerobic digesters and F-29 Quaternary Sanitizers (QACs) were used as positive controls and their description can be found elsewhere (12, 24).

Table 6. Methods used to extract DNA, used as per the manufacturer's instructions, with modifications and combinations. This modifications are discussed in subsection 3.2.

Method	Theoretical hypothesis
Power soil (MO BIO's PowerSoil DNA Isolation Kit)	Basic protocol used in previous works with inhibitors (12, 24) it removes PCR inhibitors from soil types
QIAamp DNA Stool Mini Kit	Contains InhibitEX buffer that separates inhibitory substances from DNA.
PowerPlant MO BIO's Pro Kit	The proprietary (IRT) removes inhibitors from plant extracts during the isolation process
Freeze-thawing	Freeze-thawing rapidly increases the DNA yield even without any purification procedures. (26)
Pectinase	Increased concentrations of pectin work as inhibitors for DNA extraction (27)
PCR mix	Basic conditions (e.g. $MgCl_2$) to improve annealing or additions such as BSA (28) PCR amplification from environmental samples that contain potential inhibitors such as humic and tannic acids.

3. RESULTS AND DISUCUSSION

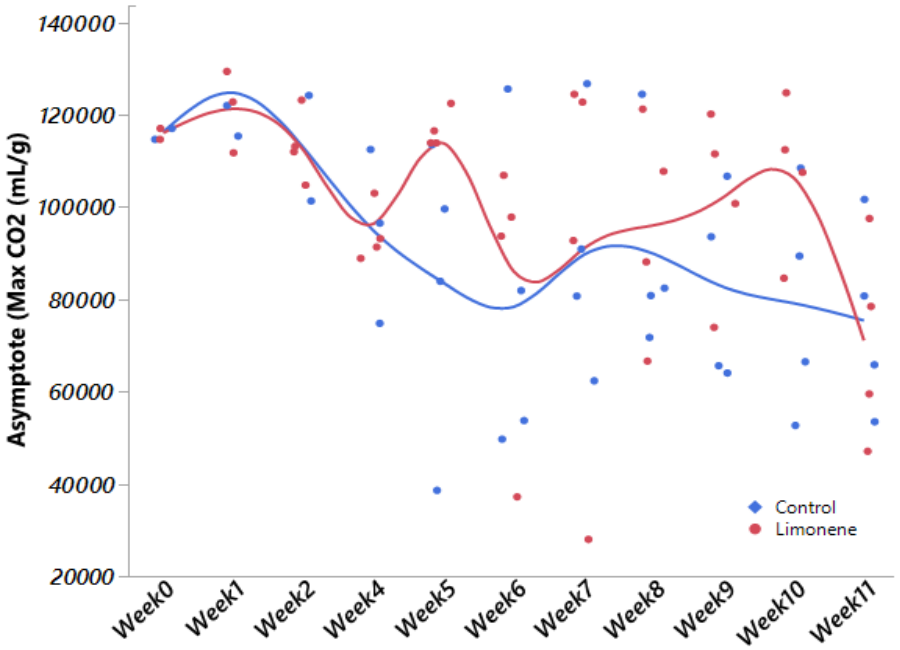
3.1 Gas evolution

The results of this study have shown that orange peel can be used as a substrate for the anaerobic digestion of residues rich in limonene, up to concentrations of at least 15,000 µl/L (Table 7).

Table 7: Estimated maxCO_2 (µL CO_2/g) after 7 days of digestion

		maxCO_2 (µL CO_2/g)	
Week	**Weekly Level (µL/mL)**	**CONTROL**	**LIMONENE**
Week0	0	92,582.62 ± 31,042.80	92,582.62 ± 31,042.80
Week1	100	125,370.35 ± 1,978.87	100,515.45 ± 42,286.44
Week2	200	80,212.99 ± 57,569.59	113,303.90 ± 7,588.17
Week3	300	123,516.40 ± 45,045.30	119,061.50 ± 7,663.35
Week4	500	117,633.41 ± 48,542.77	94,123.85 ± 6,188.27
Week5	700	83,928.27 ± 32,536.67	116,734.57 ± 4,036.76
Week6	1,000	77,753.63 ± 34,983.34	83,919.41 ± 31,627.01
Week7	1500	90,194.12 ± 27,094.48	92,003.99 ± 45,098.28
Week8	2,000	89,887.57 ± 23,543.44	95,952.08 ± 23,807.87
Week9	5,000	82,497.84 ± 21,086.21	101,618.41 ± 20,07.66
Week10	10,000	79,264.92 ± 24,673.62	107,347.46 ± 16,807.52
Week11	15,000	75,441.76 ± 20,743.79	70,657.53 ± 22,088.49

Figure 7 shows the trends for the carbon dioxide evolution rates (cCER, uL CO_2/g) of both treatments followed the same pattern in 9 out of the 11 weeks (Figure 2a). Zema *et al*. (29) were able to produce biogas during the semi-continuous digestion of OP with concentrations of essential oils up to 88.1–111.2 mg/L d^{-1} over 40 d of semi-continuous digestion. In the present study, when inoculated with 3,0000 uL/L of limonene, after 98 days (week 13, data not shown) the bioreactors still produced 20% of the CO_2 produced by the control for the same week.



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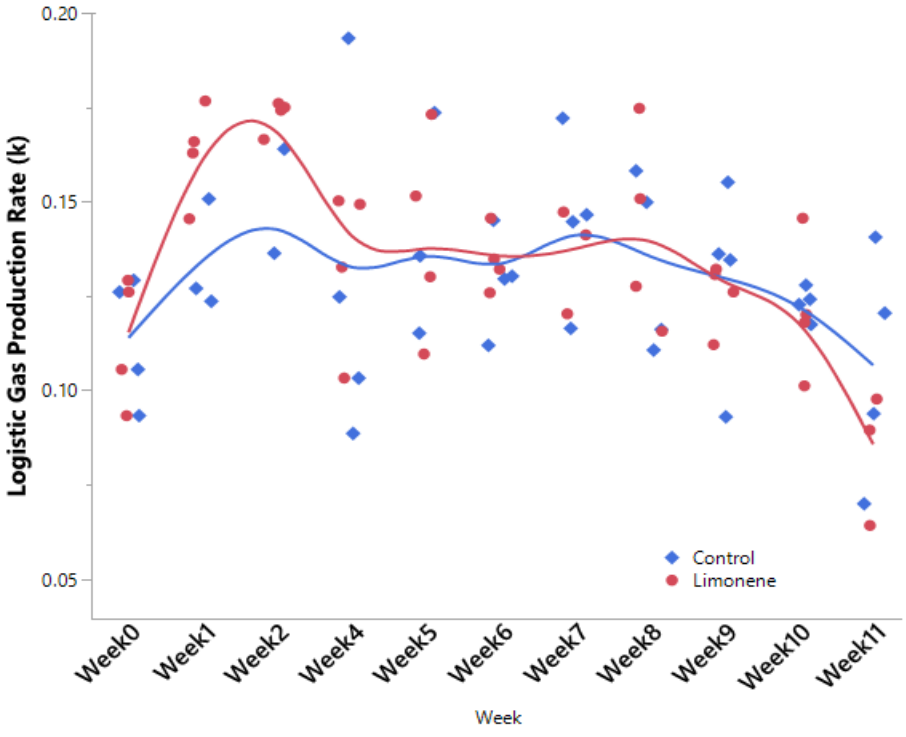

b

Figure 7. Cumulative carbon dioxide evolution rate (cCER, uL CO_2/g). The parameters were calculated from effluent concentration data. (a) Maximum cCER (or the asymptote for that week) and (b) "k" are the specific production constant parameters calculated using EEq. 2. Lines are connecting the average cCER of each week.

Zema et al. (29) also balanced the C/N ratio of the organic load with manure, and this meant that the only selective pressure during enrichment was the limonene tolerance. The results in Figure 7 refer to batch reactors without external nutrient additions. This setup shifts the microbiota towards microorganisms that are tolerant to limonene, but also selects according to the competition for the carbon source in the OP biomass. The limonene-inoculated reactors from week 5 (700 uL/L) and week 10 (10,000 uL/L) showed a higher trend in production when compared to the controls (Figure 2a). It is possible that due to the experimental setup epoxide hydrolase producing taxa were enriched, such as *Rhodococcus erythropolis*, an environmental gram-positive Actinobacterium with a versatile metabolism that can utilize limonene as the sole source of carbon. (30,31)

The specific CO_2 production rate (k parameter) was higher in the first two weeks of digestion for the limonene-added bioreactors (Figure 7b). For these weeks, limonene was added at concentrations of 100 uL/L and 200 uL/L (week 1 and week 2, respectively). These concentrations might have started the enrichment for limonene-degrading taxa, offering extra carbon sources, but could not suppress the other fermenting organisms. Comparing the values from weeks 5 and 10 (Figure 7a and 2b), it is possible to predict that differences might be related to the lag phase, as seen in the higher trends in CO_2 emissions but with similar "k-values." The differences in the gas production rates (k; Figure 7b) between week 1 and week 0 did seem to not evolve following the same trend, and the cCER and the repeats from week 0 and week1 were plotted.

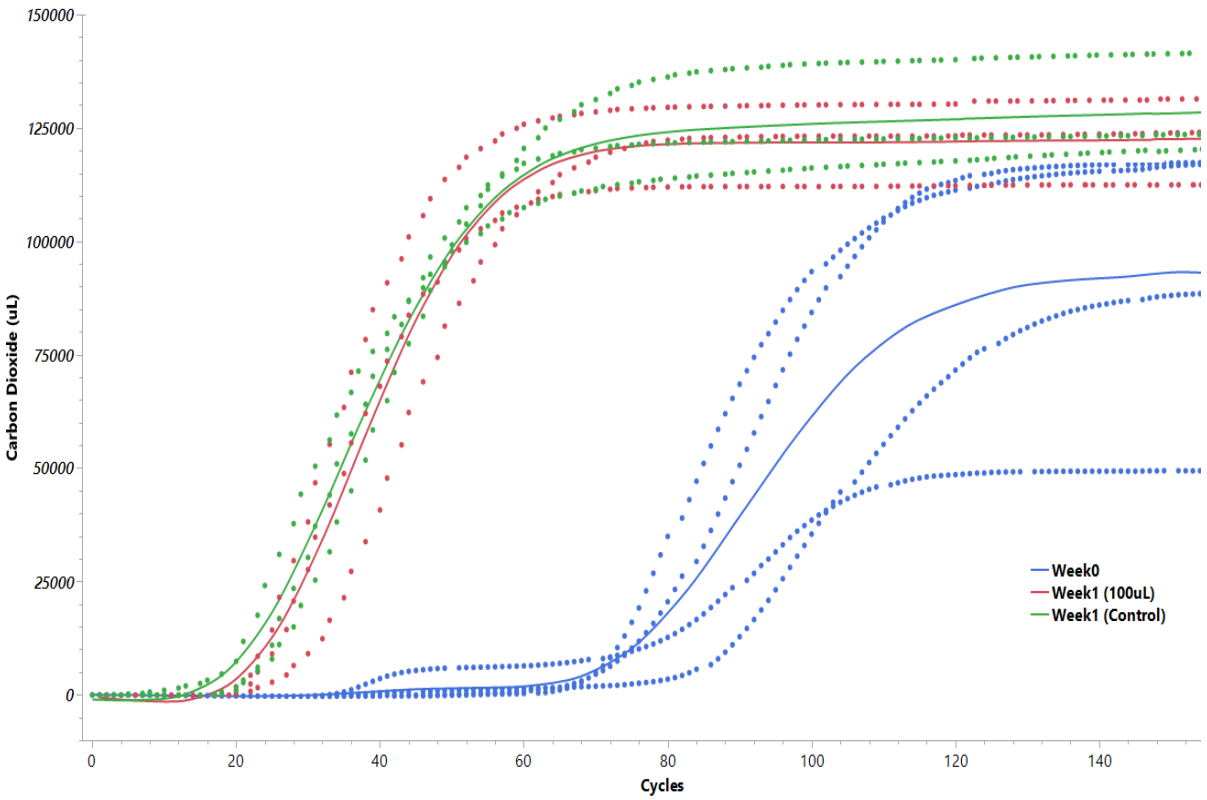

Figure 8. Differences in the cCER between week 0 and week 1. Week 0 (blue) used mature compost as the inoculum following the biomass from these reactors were randomly used as inoculum for week 1 (100 uL of limonene in red and no limonene in green). A solid line represents the curve calculated using a 3-parameter logistic growth curve (Eq. 2) and dotted lines are the measurements from the 3 repeats obtained using the MicroOxymax System. For week 0 and week 1 n = 4 and n = 3 respectively.

Next, the concentration of limonene was plotted as a continuous variable on the x-axis versus the average cumulative CO_2 after 168 h of digestion (Figure 9). It included another two points (25,000 and 30,000 uL/L); however, due to problems with files not running through the R-Studio script, these two points were not included in the analysis of Figure 7; however, the average was manually calculated in Excel and added to Figure 9b for comparison purposes.

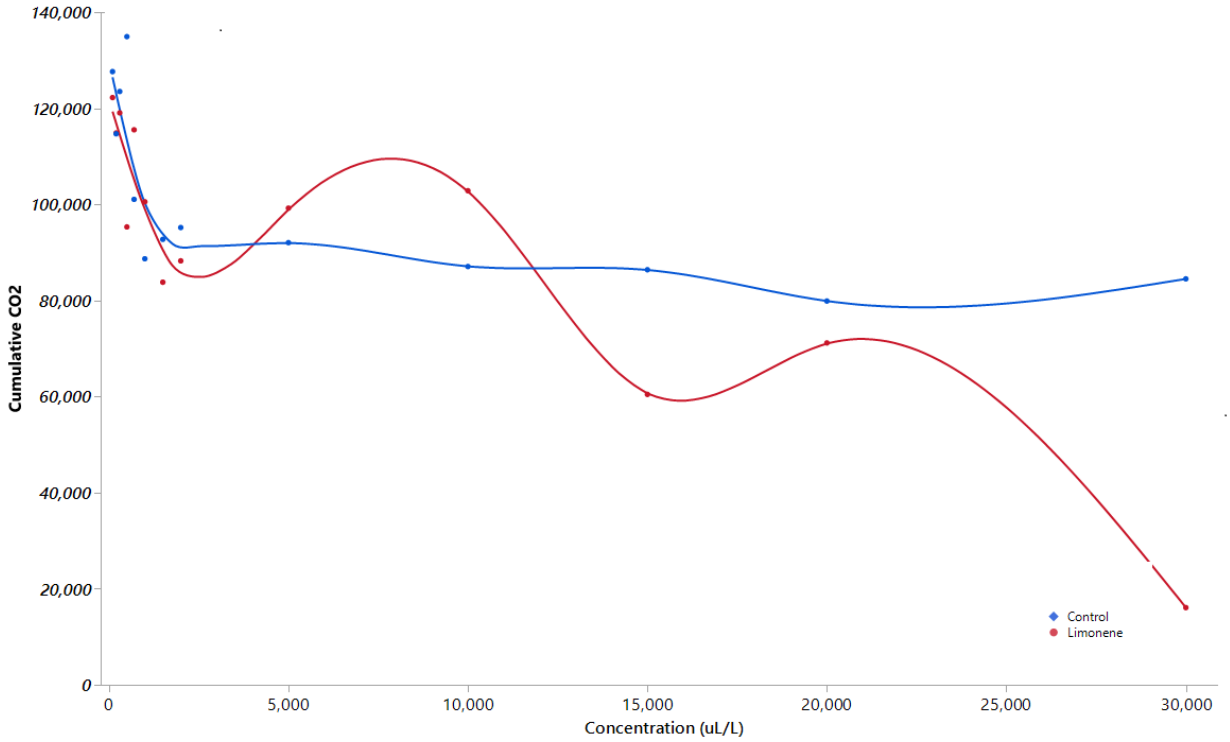

Figure 9. Average of the cumulative CO_2 plotted according to the concentration of limonene. Points for 25,000 and 30,000 uL of limonene were manually obtained from raw files and added to the plot.

According to the Figure 9 and Table 7, the control had a short period of adjustment, after which the calculated cumulative CO_2 remained fairly constant, indicating that the microbiota was better established and adapted to OP digestion. Reactors with limonene also had a similar period of adjustment where the CO_2 production decreased to lower levels than the control (94,123ul CO_2/g) indicating that limonene increments were selecting for organisms that could survive limonene but not necessarily maintain homeostasis and optimum metabolisms; between 5,000 and 10,000 uL/L, the enriched microbiota could survive and metabolize the extra carbon source within the limonene and were more productive than the controls with a maximum of 10,7347ul CO_2/g (control 79264 ul CO_2/g), the following increment of 5,000 uL (15,000 ul/L) had once again imposed a strong selective pressure to the microorganism causing the maxCO2 to drop to 70,657 ul CO_2/g (control 75,441 ul CO_2/g).

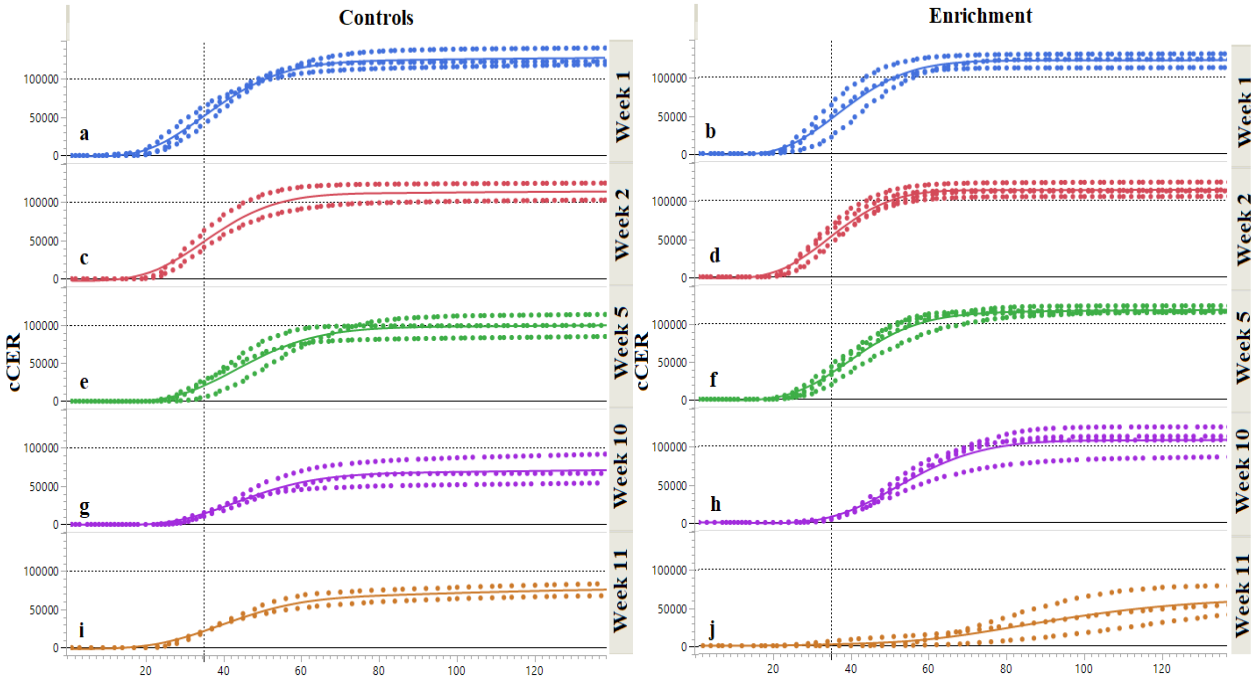

Figure 10. Plot of the cCER versus time for the control and enrichment bioreactors. A solid line represents the curve calculated using the 3 parameter logistic growth curve and dotted lines are the measurements from the 3 repeats obtained using the MicroOxymax system during an 7-days period of incubation. Blue (-------) curves are week 1, red (------) week 2, green (------) week 5, purple (------) week 10, and orange (------) week 11. For limonene, week was 1 – 100 uL/L, week 2 – 200 uL/L, week 5 – 700 uL/L, week 10 – 10,000 uL/L, and week 11 – 150,000 uL/L. Representative weeks were chosen based on results from Figure 7,Figure 9, and 4, (all other data for the weeks can found in Figure S. 2). For the week 2 control, one repeat needed to be restarted in a later cycle and thus to avoid cycle discrepancies the data is not presented here but was used in the calculations.

cCER for week 5 (green) showed the biggest difference between the control and limonene-added bioreactors (83,928 and 116,734 ul CO_2/g respectively. All limonene-enriched bioreactors produced notably more than 100,000 L of CO_2, but only one control bioreactor had similar production (week 3) . Both sets had greater variability between repeats in week 10 (purple); however, the differences followed the trends seen in Figure 7, Figure *8* Figure 9 where CO_2 production was increased possibly because limonene was being metabolized.

These results indicate that we should investigate the gas quality from the bioreactors supplemented with mild quantities of limonene (10,000–15,000 uL/L) but inoculated with the

microbiota from reactors with higher concentrations in future investigations. It is possible that limonene-degrading bacteria could easily catabolize limonene, relieving the selective pressure caused by the inhibitor, thereby reducing the lag phase.

3.2 Evaluation of DNA extraction methods for microbial communities on citrus peel biomass DNA

DNA extraction from plants is usually difficult because these tissues are rich in polyphenolic and polysaccharide compounds (32). For this reason, several methods (Table 6) with several combinations and/or modifications have been tested to check for the presence of amplifiable DNA in their extractions. A compilation of the main results is presented in Nanodrop concentrations and purity were calculated using the ratio between different wavelengths (230, 260, and 280 µm), which can indicate the presence of organic matter and solvent residues. (33) According to the literature (34), the 260/A230 ratio for uncontaminated DNA ranges from 2–2.2, while the 260/A280 ratio for pure DNA is 1.8. The *basic* protocol for three kits (Table 6) had homogenization steps (e.g. chemical lysis of cells and bead beating) and proprietary reagents designed to enhance extraction and inactivate environmental inhibitors; however, according to **Table 6 the power soil, power** plant, and Qiagen Stool Kits alone were not sufficient to yield amplifiable DNA. Nanodrop analysis for these kits yielded very low DNA (A260) with poor quality (skewed 260/233 and 260/280 ratios). This was confirmed using Qubit, with a yield of 0 µg/mL DNA. Even if those kits are optimized to lyse cell wall, and to remove inhibitors, the absence of quality DNA indicates that the proprietary reagents or lysing methods were capable or not in enough to overcome inhibition.

Since sequencing was carried out in collaboration with the Joint Genome Institute (JGI), the quality protocols followed the institute's instructions, DNA quality, quantity, and integrity guidelines (25). According to this document, "It is imperative that users properly assess DNA or RNA mass prior to shipment". The quality check included the DNA concentration from the Nanodrop, quality of DNA extracted (260/230 and 260/280 ratios), and the presence of amplicons after the PCR reaction. However, according to the guidelines, NanoDrop is generally not reliable and for the JGI the concentrations from a four-point standard curve were measured using a Qubit Fluorometer.

Nanodrop concentrations and purity were calculated using the ratio between different wavelengths (230, 260, and 280 μm), which can indicate the presence of organic matter and solvent residues. (33) According to the literature (34), the 260/A230 ratio for uncontaminated DNA ranges from 2–2.2, while the 260/A280 ratio for pure DNA is 1.8. The *basic* protocol for three kits (Table 6) had homogenization steps (e.g. chemical lysis of cells and bead beating) and proprietary reagents designed to enhance extraction and inactivate environmental inhibitors; however, according to Table 6 the power soil, power plant, and Qiagen Stool Kits alone were not sufficient to yield amplifiable DNA. Nanodrop analysis for these kits yielded very low DNA (A260) with poor quality (skewed 260/233 and 260/280 ratios). This was confirmed using Qubit, with a yield of 0 μg/mL DNA. Even if those kits are optimized to lyse cell wall, and to remove inhibitors, the absence of quality DNA indicates that the proprietary reagents or lysing methods were capable or not in enough to overcome inhibition.

Table 8. Quantitative and qualitative analyses of the DNA extracts. The analyses marked with a dash (-) were not part of that trial and those with an asterisk (*) were not performed or problems occurred with the reading.

	Reagents		Purity				Additions to pectinase		PCR
Sample	Basic Kit	Modifications	DNA [µg/mL]	260/280	230/280	Qbit [µg/mL]	Beads	Centrifugation	Amplicons
OP	Power Soil	-	4.90	2.20	0.45	0.00	-	-	No
OP	Power Soil	-	6.00	2.24	0.44	0.00	-	-	No
OP	Power Soil	-	2.50	2.17	0.18	0.00	-	-	No
Week 1 - 0L	Power Soil	-	9.00	1.98	0.37	0.00	-	-	No
Week 1 - 0L	Power Soil	-	4.20	2.00	0.35	0.00	-	-	No
Compost	Power Soil	-	33.70	1.80	1.51	*	-	-	No
Compost	Power Soil	-	18.10	1.83	1.05	*	-	-	No
Week 1	Power Soil	Adjusted volumes	3..50	2.15	*	0.00	-	-	No
Week 1	Power Soil	Adjusted volumes	3.00	1.34	*	0.00	-	-	No
Week 11 - L15000	Power Soil	Adjusted volumes	3.50	2.08	*	0.00	-	-	No
Week 11 - L15000	Power Soil	Adjusted volumes	2.2	3.67	*	0.00	-	-	No
Week 10 - 0L	Stool	+ Stool kit	2.20	3.67	*	0.00	-	-	-
Week 11 - L15000	Stool	+ Stool kit	2.40	1.79	*	0.00	-	-	-
Week 10 - 0L	Stool	+ Stool kit (2h)	30	1.68	2.09	0.00	-	-	-
Week 11 - L15000	Stool	+ Stool kit (2h)	4.30	1.81	1.11	0.00	-	-	-
Week 10 - 0L	Power Soil	+ Stool kit (24h)	2.10	1.78	1.55	0.00	-	-	-
Week 11 - L15000	Power Soil	+ Stool kit (24h)	0.80	0.40	11.2	0.00	-	-	-
Week 10 - 0L	Power Soil	+ Stool kit (24h)	2.20	0.18	1.46	0.00	-	-	-
Week 10 - 0L	Power Soil	+ Stool kit (24h)	2.10	0.19	1.98	0.00	-	-	-
Week 11 - L15000	Power Soil	+ Stool kit (24h)	4.24	0.26	4.04	0.00	-	-	-
Week 11 - L15000	Power Soil	+ Stool kit (24h)	1.60	0.22	1.46	0.00	-	-	-
Week 10 - 0L	Power Plant	-	4.50	1.26	0.25	0.00	-	-	-
Week 10 - 0L	Power Plant	-	3.00	0.55	0.19	0.00	-	-	-
Week 10 - 0L	Power Plant	DNA purification kit	1.70	1.79	0.22	0.00	-	-	-
Week 10 - 0L	Power Plant	DNA purification kit	1.40	2.36	0.21	0.00	-	-	-
Week 10 - 0L	Power Plant	DNA purification kit	4.40	2.51	0.33	0.00	-	-	-
Sludge control	Power Plant	DNA purification kit	33.40	1.82	0.88	27.40	-	-	-
Sludge + limonene (L500)	Power Plant	DNA purification kit	16.50	2.12	1.88	8.20	-	-	-
Sludge + limonene (L500)	Power Plant	DNA purification kit	25.60	1.97	0.86	8.76	-	-	Yes
Sludge + limonene (L2000)	Power Plant	DNA purification kit	11.20	1.80	0.85	13.60	-	-	Yes
Sludge + limonene (L2000)	Power Plant	DNA purification kit	22.50	1.99	2.44	23.60	-	-	Yes
Sludge + QACs	Power Plant	DNA purification kit	1.17	0.86	24.90	4.70	-	-	Yes
Sludge + QACs	Power Plant	DNA purification kit	1.66	0.64	10.90	5.32	-	-	Yes
OP	Power Soil	Pectinase	13.60	0.55	0.10	3.64	Beads	Supernatant	No
OP	Power Soil	Pectinase	64.10	0.56	0.10	4.30	Beads	-	No
Week 11 - L15000	Power Soil	+ Stool kit (24h)	1.60	0.22	1.46	0.00	- -	- -	- -
Week 10 - 0L	Power Plant	-	4.50	1.26	0.25	0.00	-	-	-
Week 10 - 0L	Power Plant	-	3.00	0.55	0.19	0.00	-	-	-
Week 10 - 0L	Power Plant	DNA purification kit	1.70	1.79	0.22	0.00	-	-	-
Week 10 - 0L	Power Plant	DNA purification kit	1.40	2.36	0.21	0.00	-	-	-
Week 10 - 0L	Power Plant	DNA purification kit	4.40	2.51	0.33	0.00	-	-	-

Column "Reagents" refers to the basic kit and the basic modifications added to these kit, Purity refers to Qubit and nanodrop readings according to JGI guidelines. Pectinase yielded the best initial results and modification to this methods were incorporated in the column "additions to pectinase", Beads and "Centrifugation" are discussed in the next paragraph. The column under the name "PCR" refers to visible bands in agarose gel (2%) after polymerase chain reaction. Sluged, compost and Quaternary Sanitizer F-29 (QACs) were used as positive controls.

In attempt to overcome challenges regarding cell wall and inhibitors, several modifications and in different combinations were added to the basic protocol. Extra steps included dilution of initial volume used in the kits; treatments with pectinase or with proteinase K to digest inhibitors; centrifugation to separate inhibitory compounds based on weight and solubility; enhancing homogenization and cell lysis by beating with glass beads or freezing-thaw cycles; extra purification (Qiagen`s Stool kit and DNA purification kit) and extended reaction times. A combination of digestion with pectinase, extra cell wall lysis with glass beads and centrifugation yielded DNA with the best quality, but no amplicons were observed in 2% agarose gel after PCR. References to these protocols and all combinations tested can be found in Table 6 and Table 8 respectively. Compost, Sludge and QACs were used as positive controls.

This difficulty in extracting amplifiable DNA from citrus has been investigated previously (35) by comparing four DNA extraction methods. According to the report, only the method described by Sarkosyl(36) yielded amplifiable DNA. However, this procedure would not be suitable for the present study because the Sarkosyl method has a purification step with a restriction endonuclease (HindIII). This enzyme has been used in previous studies to discriminate closely related taxa using PCR-RFLP in rDNA polymorphisms (37). GJI requires PCR amplification using the 16S polymorphic rDNA as proof that no contaminants are present that would inhibit amplification (25). However, HindIII digestion of DNA introduces confounding factors in closely related taxa, making it a complex tool with which to unravel microbial diversity.

Other alternative DNA methods for citrus typically include detergents (proteases, e.g., proteinase K) or heat denaturation (38), indicating that multiple inhibitors might be present. Bai et al. (27) reported that DNA extraction from orange juice is difficult, specifically because of the

high sugar and pectin concentrations (among others). For this reason, a combination of kits (e.g., Power Plant followed by the Stool Kit or extra digestion with pectinase-K) were tested, but no satisfactory yield was achieved. Another report showed (26) that rapid freeze-thawing increases the DNA yield even without any purification procedures, and that OP freeze-thawing cycles had no effect on DNA quality and yield (data not shown).

As discussed by Bai et al.(27), it is difficult to separate DNA from pectin but is achievable by homogenization and pectinase digestion, increasing the DNA yield. Their method (27) has been tested herein with Power Soil and Power Plant kits, with and without modifications for both orange peel stock and week-13 digestate. Modifications included an extra homogenization step (glass beads) and/or removal of the contaminants through centrifugation. Pectinase yielded higher DNA concentrations in all cases but did not improve the quality and purity. Power Soil combined with pectinase extraction did not yield DNA from OP stock, but week 15 extracts showed measurable DNA (Qubit) and nanodrop concentrations above 10 µm/mL. PCR reactions using several kits and modifications (such varying quantities of bovine serum albumin, magnesium chloride, and annealing temperature) were tested, but no amplicons were detected on 2% agarose gel.

Several other methods have been able to extract genomic DNA from citrus; however, the material was limited to juices and not concentrated OP (39–41) or from leaf and root samples. (41) One method (32) was able to extract DNA from orange peels using the Plant DNA Extraction Kit (TIANGEN Biotech Co., Chengdu, China), this study, however aimed to detect fruit pathogens and the initial material was obtained from cotton swabs on the surface of the orange peel.

This experiment was conducted from September 2013 to May 2014. Surprisingly a search for articles after 2014 and for combination of key words such as “orange peel,” “citrus,” “inhibitors,” and “DNA extraction” revealed no significant updates, and this search included Latin-American database (scielo) where terms were also tested in Portuguese. An interesting update included a successful extraction in semi-continuous AD of pre-treated orange peel conducted by Calabrò et al.(10), and the authors were able to characterize the bacterial community from the liquid taken from each reactor. Calabrò et al. used semi-continuous AD and pre-treated OP, while this study used batch reactors, high-solid setup, and no pre-treatment of orange peel; these differences in experimental design are sufficient to predict that the method by Calabrò et al. would not be applicable to extract DNA from high-solids anaerobic digestion of OP.

4. CONCLUSIONS

The present study was the first to successfully create a microbiota capable of digesting anaerobically organic matter with very high concentrations of the inhibitor limonene (15,000 µL/mL) and to show that these adapted organisms improved CO_2 production, possibly using limonene as a carbon source; however, genomic sequencing was not possible due to obstacles in DNA extraction thus, the optimum microbial consortia could not be identified. The lack of amplifiable DNA showed that OP digestate is recalcitrant as a result of multiple properties such as high pectin and chemical inhibitors (e.g. limonene). Adapting and combining DNA extraction methods could improve DNA yield and quality, however, these genetic material were not amplifiable by PCR. Science is the process of building progress from failure(43), from this perspective, the DNA experiment did not fail; they hughlighted methodological gaps while offering directions for future studies.Gas production and challenges in DNA extraction should be

used as stepping stones future studies aiming a consortium of microorganisms specialized in digesting OP during AD and towards creating a new method for extracting DNA highly concentrated in PCR inhibitors.

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SUPPLEMENTARY FILES

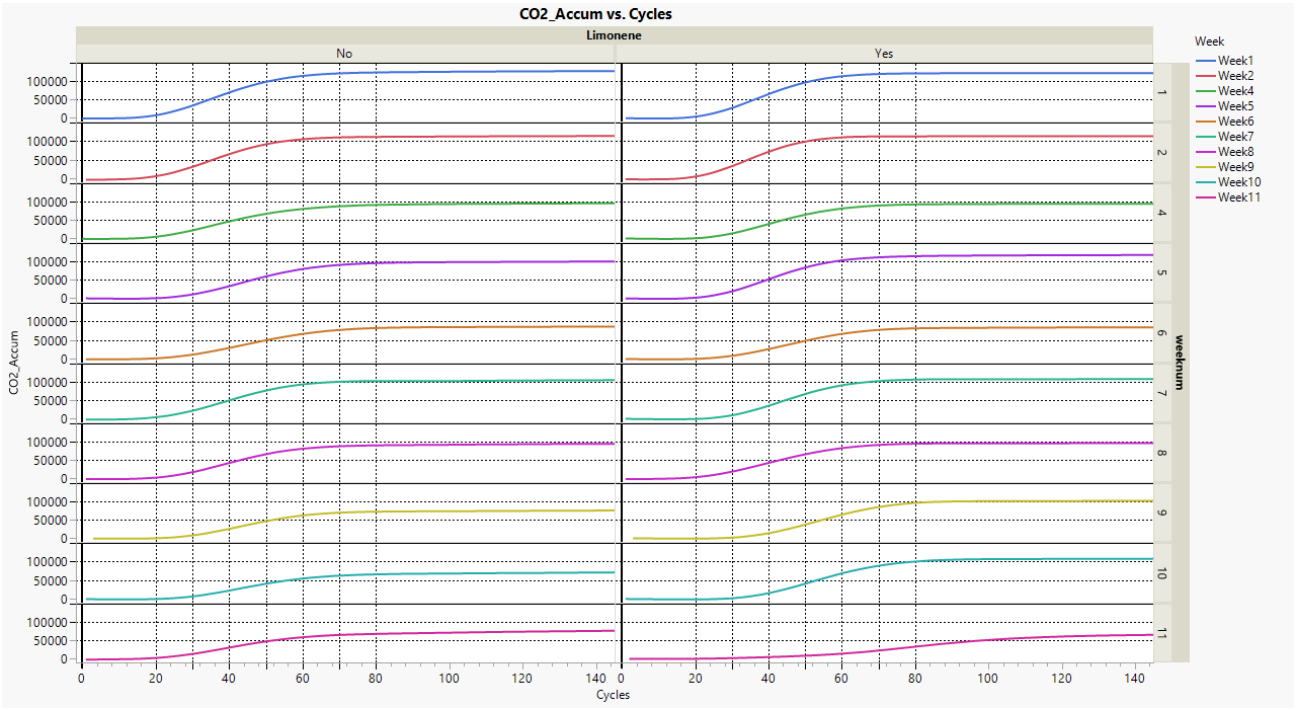


Figure S. 2: Plot of the cCER versus time for the control and enrichment bioreactors. The solid line represents the curve calculated using the 3 parameter logistic growth curve from the 3 repeats obtained using the MicroOxymax system.

CHAPTER 4 - FOOD WASTE STREAMS AS SOIL AMENDMENTS FOR INACTIVATING PATHOGENS DURING BIOSOLARIZATION

ABSTRACT

Aims:

To screen food waste streams as amendments for inactivating *Escherichia coli* during biosolarization (BSB) and identify the synergistic effects of food waste matrices with BSB.

Methods and Results*:*

Bioreactors were used to access log reduction values (LRVs) of food-processing residues as soil amendment streams during BSB. Grape pomace (GP), tomato pomace (TP), sugarcane bagasse, coffee, almond hulls (HS), and mixed almond hulls and shells (HMS) were added to soil (2.5% dry w/w), with or without mature compost, inoculated with a rifampicin-resistant surrogate of *E. coli* O157:H7 (TVS354), and biosolarized for 8 d (12 h cycles of 30 °C/50 °C). Cells were retrieved with PBS, serially diluted, and plated on media to calculate LRVs. GP inactivated *E. coli*; TP mitigated the inactivation; and HS, HMS, sugarcane, and coffee had no effect. Analysis of phenolic and volatile fatty acid (VFA) profiles using HPLC revealed that the synergy between these compounds and temperature is important for inactivation, as they might modulate heat-shock responses. Complementary studies with GP (different varieties and solvent extraction of bioactives) showed that the Maillard reaction may be important for the synergistic inactivation of *E. coli* during BSB.

Conclusions*:*

BSB can be used with food waste streams for inactivating *E. coli* and other soil-borne pathogens. The synergy between the bioactive matrix within the amendment and BSB conditions determines efficiency.

Significance and Impact of the Study*:*

Evaluating the use of BSB as a sustainable, state-of-the-art method against pathogens is needed to replace chemical treatment of soil. Our findings reveal the efficiency of this green method and can be used for future research.

Keywords*:*

Biosolarization, *Escherichia coli*, food waste, grape pomace, tomato pomace, soil amendments

INTRODUCTION

A total of $248 billion USD (Fried *et al*. 2017) is lost in crops globally because of weeds, pests, and pathogens. Every year, additional losses are associated with recalls stemming from disease outbreaks linked to foodborne human pathogens. A report from 2018 (Dewey-Mattia *et al*. 2018) showed that 334 cases and 9,746 illnesses were linked to raw crops. Among these cases, *Listeria monocytogenes, Salmonella spp.*, and Shiga toxin-producing *Escherichia coli* (STEC) were responsible for 82% of all hospitalizations, and 82% of deaths were reported. These pathogens can be harbored and disseminated in agricultural soils (Zhang *et al*. 2020). In soil, *E. coli* and enterococci experience initial growth after soil deposition and survive in the soil for several weeks, whereas fecal coliforms and enterococci can survive for at least 42 weeks (Stocker *et al*. 2015). These soil borne pathogens can then contact humans through runoff and

infiltration waters. Many reports have linked microbial outbreaks with agricultural reuse of wastewater globally. These exposure pathways include agricultural workers, their families, and residents close to areas irrigated with wastewater (Adegoke *et al*. 2018).

Traditionally, soil fumigant pesticides have been used to inactivate soil pathogens. However, such fumigants target plant pathogens and not human pathogens (Gurtler 2017); do not meet the sustainability trends; and have been implicated in human cancer, endocrine syndromes, depletion of the ozone layer, and negative impacts on the soil microbiome (VoPham *et al*. 2015; Feld *et al*. 2015; Attina *et al*. 2016). By contrast, biosolarization inactivates plant pathogens, nematodes, seeds, and weed propagules (Achmon *et al*. 2016; Achmon *et al*. 2017), while avoiding negative environmental impacts (Achmon *et al*. 2017; Fernández-Bayo *et al*. 2017); is compatible with organic practices (e-CFIR); and has low potential for non-target risks to humans (Shea *et al*. 2021). However, the efficacy of biosolarization in controlling soil-borne human pathogens is unknown.

Biosolarization couples the stresses from solarization, such as passive solar heating, and soil fermentation to conditions that are inhibitory to the growth of microorganisms. To this end, soil is amended with organic matter, covered with transparent plastic tarp, and drip-irrigated to induce anaerobioses and promote passive solar heating. These conditions increase exothermic microbial activity, deplete soil oxygen, produce chemical byproducts (volatile fatty acids [VFAs], in particular) (Achmon *et al*. 2017; Fernández-Bayo *et al*. 2018), and cause the temperature to peak above 50 °C. Regarding sustainability, the process adds value to compatible food waste streams by using them as soil amendments to drive fermentation. For example, use of tomato pomace (TP) in biosolarization is predicted to reduce greenhouse gas emissions and primary energy demand by up to 7.7 Mkg CO_2e and 203,000 GJ annually, respectively, by

displacing soil fumigants in California (Oldfield *et al*. 2017). By adding value to waste streams, biosolarization can mitigate the impacts of the increasing biomass residues produced, which in California alone is projected to reach 71 million tons of dry matter per year by 2050 (Breunig *et al*. 2018).

The composition of organic matter used to amend soil for biosolarization is important because the process relies on microbial deconstruction and consumption of the amendments. Many waste stream carbon sources have been shown to be compatible with biosolarization. For example, TP and white grape pomace (GP) induced a drop in pH, and an increase in temperature was positively associated with soil pest inactivation (Achmon *et al*. 2016). Combining solar heating with organic matter amendment resulted in accelerated weed seed inactivation compared with either approach alone (Westerman and Gerowitt 2013). Follow up studies, from the crop yield perspective, reported that plants growing in biosolarized soil, amended with TP and no residual VFAs by the time of planting yielded a significantly greater amount of vegetation, fruit quantity, and fruit ripening, whereas soil residual VFAs did not show significant differences when comparing both treatments to the controls (Achmon *et al*. 2018). The use of almond residues as an amendment for soil biosolarization led to 84%–100% inactivation of the phytoparasitic nematode *Pratylenchus vulnus* (Fernández-Bayo *et al*. 2020). A study using dog food as an amendment for biosolazition reported that after soil biosolarization, levels of *Fusarium oxysporum* f. sp. *lactucae* (a phytopathogenic fungus) were below the limit of quantification. These amendments are important in that they have compositional similarity to food waste from a municipal waste facility (Fernández-Bayo *et al*. 2018). Biosolarizing soil with a readily available organic amendment and compost co-amendment enhanced VFA accumulation, creating a biopesticidal environment even at lower depths (Hestmark *et al*. 2019).

The presence of bioactive compounds within such biomass may inhibit microbiota differentially. Achmon *et al*. (2017) reported that acidogenesis was hindered during soil fermentation of red GP. Volatile fatty acids (VFAs) did not accumulate, indicating that acidogenic bacteria were inactive when the activity of methanogenic archaea was not inhibited. However, during biosolarization with white GP or TP, increased concentrations of VFAs and temperatures known to inactivate unwanted organisms were reported (González-Centeno *et al*. 2013; Katalinić *et al*. 2010). At the molecular level, analysis of microbial co-occurrence after soil biosolarization with TP revealed a unique circular network; the correlation between the clusters and production of soil VFA suggested that *Clostridium* and other genera tolerate, and perhaps drive, VFA production. The analysis also revealed that the relative abundance of fungal genera related to plant pathogenic members (i.e., *Fusarium, Aspergillus*, and *Alternaria)* was reduced (Achmon *et al*. 2020).

The objective of this study was to evaluate the effect of biosolarization using various waste food streams (white GP and TP, spent coffee, sugarcane bagasse, and almond residue) amendments to inactivate non-pathogenic bacteria.

METHODS

Master stock and determination of colony forming units

For all experiments with *E. coli,* the strain TVS 354 was used as the inoculum. The strain was isolated from romaine lettuce (***Lactuca sativa*** L. var. ***longifolia***) and is inherently resistant to rifampicin (Dr. Trevor Suslow, University of California, Davis) (Moyne *et al*. 2013; Tomás-Callejas *et al*. 2011). The colony was grown overnight in Luria–Bertani (LB) medium containing 50 μg ml^{-1} rifampicin at 37 °C. After 24 h, the suspension was washed and

resuspended in phosphate-buffered saline (PBS) three times. The suspension (10^9 colony forming units [CFU] ml^{-1}) was used as the inoculum for all experiments.

The experiments using pathogenic *Salmonella enterica* serovar Typhimurium (*S*Tm) (AJB150), which is derived from *S.* Typhimurium ATCC14028 and is resistant to nalidixic acid (NAL) (Litvak *et al*. 2018), were performed in partnership with the Department of Medical Microbiology and Immunology, School of Medicine, University of California, Davis. The inoculum was grown in LB media with NAL overnight at 37 °C to a load of 10^5 CFU ml^{-1}.

Amendments, co-amendments, and soil characteristics.

Table 9 Properties of soil and biomasses used for simulated biosolarization

Sample	Moisture content (% fresh weight)	VS (% dry weight)	pH
GP	0.10	95.92	4.25
Coffee	0.054	98.38	
Sugar cane	0.043	99.10	7.00 (30) (Messa and Faez 2020)
TP	0.056	97.47	4.53
HMS	0.12	94.2	4.70
AS	0.14	90.6	4.71
Compost	0.080	42.8 ± 1.55	8.00

GP: grape pomace, HMS: mix of almond hulls and shells, TP: tomato pomace, VS: volatile solid

.Table 9 shows the six food and beverage processing waste streams used for the experiments. Moisture content and pH values were measured according to protocols described by Achmon *et al*. 2015 ; Achmon *et al*. 2016). Briefly, pH was measured in the liquid phase obtained after diluting the amendments in distilled water (1:1 w/v). Moisture content was determined gravimetrically by weighing the materials before and after drying in a vacuum oven at 60 °C until the weight stabilized. Total organic matter content was estimated as volatile solid

(VS) content according to Fernandez-Bayo *et al*. (2020). This value was calculated gravimetrically following combustion for 2 h at 360 °C.

White wine GP biomass was obtained from the UC Davis Teaching Winery during the 2016 harvest. The blend is a mix of several varieties that might mimic the diversity seen in the GP pile in wineries. Almonds shells (AH) and a mix of almond hulls and shells (HMS, approximately 70% hull by mass) were obtained from the North State Hulling Cooperative (Chico, CA) in March 2017. TP was obtained from a commercial tomato paste production facility in California. Spent coffee grounds were donated by the Sensory group at the Department of Food Science and Technology (University of California, Davis). Grounds from Peet's Major Dickason's blend (from Latin American and Indonesian origins) with an Agtron score for roasting of 31 were used; the brewing steps can be found (Batali *et al*. 2020). Sugar cane was donated by the Laboratory of Polymeric Materials and Biosorbents, Federal University of São Carlos, UFSCar – BRAZIL, after physical–chemical treatment to remove residues and water-soluble compounds, such as sucrose, followed by milling and sieving through a 125-mesh sieve. The reported biomass contains 166:1 C/N, cellulose/hemicelluloses 1.5:1, cellulose/lignin 19:1, and hemicelluloses/lignin 1.3:1 (Messa and Faez 2020; Sluiter *et al*. 2016; de Moraes Rocha *et al*. 2015).

Compost co-amendment was used for streams that showed a significant difference to the control according to (Equation 3). The use of compost co-amendment has been reported to enhance VFA accumulation (Hestamark *et al*. 2019). Comparing the results from the same amendment with or without compost can rule out that significant differences are a result of the impact and need of exogenous facultative anaerobic and thermophilic bacteria at the beginning of the process (Simmons *et al*. 2013).

Sandy loam soil was used in all experiments and was collected at the Kearney Agriculture Research and Extension Center located in Parlier, CA; details have been presented elsewhere (Achmon *et al*. 2016).

Mesocosms

Soil mesocosms were used as experimental units to capture the in-field combination of biotic and abiotic conditions during biosolarization, while allowing for the recovery of viable soil from a defined volume of inoculated soil. Each mesocosm consisted of an airtight 50-ml centrifuge tube containing 10 g of dry soil and an amendment mixture. For the amended soil, the biomass amendment was mixed into the dry soil to achieve a concentration of 2.5% w/w dry weight. For certain treatments, compost co-amendment was added at 2% w/w dry weight. *E. coli* suspended in PBS was used to wet soil to 80% of water holding capacity and inoculate soil to a final concentration of $3.16 \times 10^8 \pm 0.36 \times 10^8$ CFU/g of dry soil mix. The soil and inoculum were mixed, the tube headspace was flushed with nitrogen, and sealed tubes were placed in an incubator for 8 d to promote anaerobiosis. The incubator was alternated between 30 °C and 50 °C every 12 h.

After 8 d, viable *E. coli* were enumerated by diluting the soil mixture in PBS (1:5 w/v), plating serial dilutions of the liquid phase on LB media, and counting colonies. Extracts from soils not inoculated with *E. coli* were similarly processed to confirm the absence of rifampicin-resistant microbes. All soil samples were analysed in triplicate.

Calculation of log reduction value

CFU values measured in each sample were subtracted from those observed in control soils that were inoculated with *E. coli* and solar heated but were not amended with biomass (i.e., they were solarized but not biosolarized) (Equation 3). The calculated log reduction values

(LRVs) represent the bacterial load stemming from the process being analysed. A positive LRV indicates that the number of culturable/viable or live cells was smaller after the process; a negative LRV suggests that the process mitigates inactivation or promotes bacterial growth; and LRVs close to zero have no effect on the number of surviving cells.

$$LRV = Log\ (CFU\ in\ control) - Log\ (CFU\ in\ treated\ soil) \qquad \text{(Equation 3)}$$

Statistical analysis was performed using JMP (Version *<14.2.0)*, SAS Institute Inc., Cary, NC, 1989–2021). Data were analysed and compared using the ANOVA Tukey-post hoc test.

Kinetic modeling and parameters

For the kinetic studies, GP (BSB), a set of samples (n = 3) was sacrificed every 24 h. Inactivation models and parameters for parametric survival of *E. coli* were calculated according to Geeraerd *et al*. (2005) using GInaFiT, a freeware tool (Geeraerd *et al*. 2005). The decimal reduction value (D-value) and fractional reduction (▿ factor) are not included in GInaFiT and were calculated with equations described elsewhere (Stanbury *et al*.) and Microsoft Excel (2007). Soil control parameters were calculated using only the final load of the solarized soil.

Organic acid measurement of aqueous extracts

The soil was diluted in DI water at a ratio of 2:1 mass (H_2O:sample) for 30 min and shaken. The mixtures were centrifuged at 12,000 × *g* for 10 min, and then the supernatants were filtered through 0.2-µm PTFE syringe filters. Profiles of organic acids (OAs); specifically, formic, acetic, propionic, isobutyric, and butyric acids, were obtained from the filtrates using high-performance liquid chromatography (model UFLC-10Ai; Shimadzu, Columbia, MD) equipped with an ion exchange column (Bio-Rad Aminex HPX-87H, 300 × 7.8 mm) and a UV

detector (SPD-20A Prominence, Shimadzu, Columbia, MD, USA). The filtrates were pretreated with 1:1 10 mM sulfuric acid in Milli-Q water. The mobile phase used was 5 mM sulfuric acid in Milli-Q water at a flow rate of 0.6 ml min^{-1}, the column oven was set at 60 °C, and the UV detector was set at 210 nm.

E. coli inhibition assay with aqueous extracts

Aqueous extracts were obtained from bioreactor soil samples containing 0%, 2.5%, or 5.0% TP or GP (dry w/w amendment) following incubation at 37 °C for 8 d. Soil samples were diluted in distilled water (1:1 w/v) and the liquid phase was filtered using a 0.22-µm filter. Extracts were frozen at −20 °C until use. Aqueous soil extracts were added to LB + RIF media (1:1 v/v) and the mixture was inoculated with *E. coli* using the master stock (1% v/v) (prepared according to described methods). Media spiked with bleach (30%) were used as a negative control. Then, 200 µl of the inoculated media mixture was loaded into the wells of a 96-well plate and incubated at 37 °C for 24 h, under agitation (200 rpm). Optical density (OD) was measured at 590 nm using the ELx800 spectrophotometer (BioTek Instruments, Highland Park, USA).

STm inhibition assay

The bacterial strain IR715 (AJB150) was grown in LB media with NAL overnight at 37 °C. The soil mix was homogenized (vortexed for 3 min) with 1 ml of LB per gram of soil. One milliliter of this soil extract–LB mix was transferred to a 14-ml snapcap tube and mixed with 100 µl of Salmonella at 10^5 CFU ml^{-1} (total 10^4 CFU per tube). These mixtures were incubated at 37 °C or 42 °C for 24 h without agitation, and CFUs were serially diluted in PBS and plated on MacConkey's agar + NAL.

RESULTS AND DISCUSSION

Figure 11 shows the LRV calculated using non-amended soil maintained at 30 °C/50 °C cycles (SC-SBS), and the results revealed three patterns. TP promoted negative LRV (mitigated inactivation); HS, HMS, coffee, and sugarcane bagasse had no effect on LRV; and GP promoted total inactivation (positive LRV).

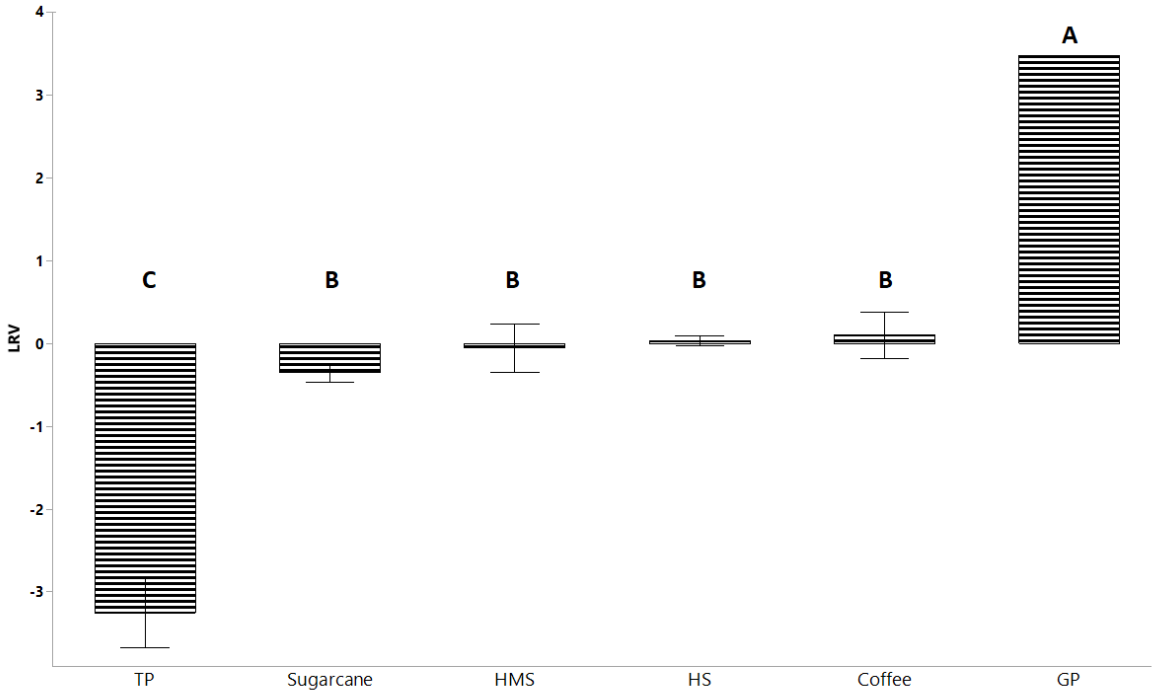


Figure 11: LRV results calculated using biosolarized soil as control. All amendments were added at 2.5% (dry w/w). Samples not connected by the same lowercase letter showed significantly different ($p \leq 0.05$) for n=3

GP: grape pomace, HS: almond hulls, HMS: mix of almond hulls and shells, LRV: log reduction value, TP: tomato pomaceTP biomass composition was reported to be optimal for biosolarization because it inactivates >95% of *Brassica nigra* and *Solanum nigrum* seeds (Achmon *et al*. 2017). Contrarily, Table 10 shows negative LRVs (−3.25 ± 0.4) for TP, indicating that more cells were cultivable after 8 d and amendment mitigated the inactivation

from temperature and anaerobioses (Figure 11). The matrix of bioactive compounds is composed of a multiplex of lycopene and β-carotene flavonoids (Nour et al. 2018), reported to be effective against gram-positive bacteria but ineffective against gram-negative bacteria, such as *E. coli* (Barros *et al*. 2006). Ash content is another important factor; Kearney soil contains 98.13% ± 0.26% ash, whereas TP contains 4.46% ± 1.02% ash (Gurtler 2017). The increased amount of labile carbon and nutrients in TP (i.e., C:N ratio, total sugar (25.73%), and protein (19.27%) (Oldfield *et al*. 2017)) may modulate heat shock proteins, such as DnaK and HtpG (Spence *et al*. 1990; Arsène *et al*. 2000). TP is also high in arginine, an important compound in *acid resistance* systems (Nour *et al*. 2018). TP composition is optimal for anaerobic digestion and the production of short-chain OAs (Achmon *et al*. 2016). The combination of increased temperature, increased labile carbon and arginine content, low pH, and production of weak acids from TP fermentation synergistically triggers the arginine- and glutamate-dependent systems, reported to protect *E. coli* against the effects of weak acids as an adaptive response to host colonization (Lin *et al*. 1996).

Table 10 LRV for all amendments after 8 d of biosolarization

Sample	LRV
Tomato pomace	-3.25 ± 0.4^{c}
Sugarcane	0.35 ± 0.1^{b}
Coffee	0.1 ± 0.3^{b}
HS	-0.03 ± 0.06^{b}
HMS	-0.05 ± 0.3^{b}
Grape pomace	3.48 ± 0^{a}

LRVs are calculated based on the biosolarized soil control (SC-BSB); 3.48 represents the maximum LRV (n=3) reduction at a detection limit of 50 CFU g^{-1} of soil. CFU: colony forming unit, HMS: mix of almond hulls and shells, HS: almond hulls, LRV: log reduction value. Samples not connected by the same lowercase letter showed significantly different ($p \leq 0.05$) for n=3.

Almond is the third most important agricultural product in California; biosolarization with waste can promote conditions known to disinfest soil against nematodes and other agricultural pests (Fernández-Bayo *et al*. 2020). However, for human pathogens, inactivation using almond is not significant, as seen by the close to zero LRV for HS and HMS (−0.03 ± 0.06 and −0.05 ± 0.3, respectively). LRVs close to zero indicate that the decrease in CFUs might be solely related to the effect of temperature at the same rate as seen in the biosolarized soil control (SC-BSB). Previously, we found that HS can slightly lower the pH to 6.0, produce OAs, and increase soil temperature (through fermentation) (Shea *et al*., in press); however, these conditions cannot upregulate the expression of protective genes, as seen with TP. These results are consistent with those reported for the matrix of bioactive compounds (Mandalari *et al*. 2010; Prgomet *et al*. 2017) that are not active against gram-negative bacteria. Thus, the bioactive matrix and biosolarization do not have a synergistic effect on *E. coli* inactivation.

Spent coffee grounds (treated as coffee in this study) are the most abundant byproducts of coffee (45%), with an annual generation of approximately six million tons worldwide (Martinez-Saez *et al*. 2017). Coffee showed similar results to HS and HMS, with an LRV close to zero (0.1 ± 0.3). Coffee is high in carbon and low in nitrogen (<2%) (Pujol *et al*. 2013), which might compromise the synthesis of heat shock proteins, thus exposing the bacteria to the same levels of stress as seen in SC-BSB. The matrix is a potential antimicrobial agent against enterobacteria and a natural preservative (Almeida *et al*. 2006), reported to be effective against yeasts and gram-positive bacteria (Monente *et al*. 2015). These results are consistent with the latter, where coffee waste is not synergistic with biosolarization.

Sugarcane tested with zero activity. As described, sugarcane bagasse was prewashed to remove saccharides, which ferment to produce bioactive byproducts (short-chain OAs). The

resulting high lignin-to-cellulose ratio can shift the microbiome toward lignin-degrading organisms instead of cellulase-degrading (Simmons *et al*., 2014; Yu *et al*. 2017). This condition is unfavorable for the accumulation of antibacterial OAs and does not trigger protective responses against *E. coli* (as seen with TP). The polyphenol composition from sugarcane bagasse was reported to be bacteriostatic against *E. coli* (Zhao *et al*. 2015); our results indicate that coupling to biosolarization is not enough to restore the bactericidal status and inactivate *E. coli*.

Regarding GP, there were no detectable CFUs after 8 d of biosolarization (LRV 3.48 ± 0); a kinetics assessment of the inactivation revealed that this value can be reached in as short as 3 d (data not included). This result is promising as grapes are the top three agricultural commodities in California (Johnson and Cody 2015). Total inactivation may be reached based on the composition of GP. GP does not promote accumulation of OAs or drop in pH (Achmon *et al*. 2016). However, it is more likely that the matrix of bioactives in the amendment inhibit defense mechanisms, which is contrary to that was seen for coffee, sugarcane, HS, and HMS. GP contains flavonoids and non-flavonoids, which are known to have antimicrobial activity (Katalinić *et al*. 2010; Hindi *et al*. 2016). These compounds inhibit heat shock responses by regulating genes involved in bacterial metabolism, formation of persister cells, efflux pumps (Borges *et al*. 2012), and biofilm formation and swarming motility (Lee *et al*. 2009).

The synergy of inactivation (temperature and bioactives for grape) or mitigation (temperature and VFAs) is shown Figure 12.

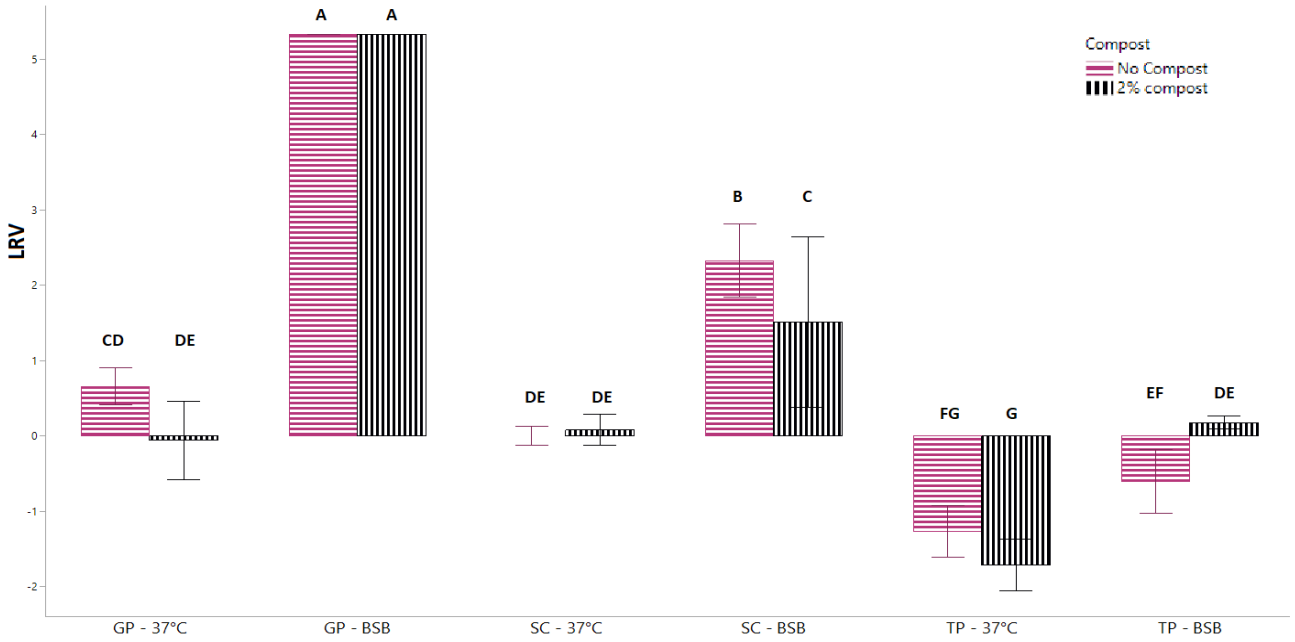


Figure 12: LRV results calculated using SC-37 °C as the control. Samples were incubated at 37 °C for 8 d. TP and GP were at 2.5% (dry w/w) and compost at 2% (dry w/w). Samples not connected by the same letters are significantly different ($p \leq 0.05$) for n=3. BSB: biosolarized soil, GP: grape pomace, LRV: log reduction value, SC: soil control, TP: tomato pomace

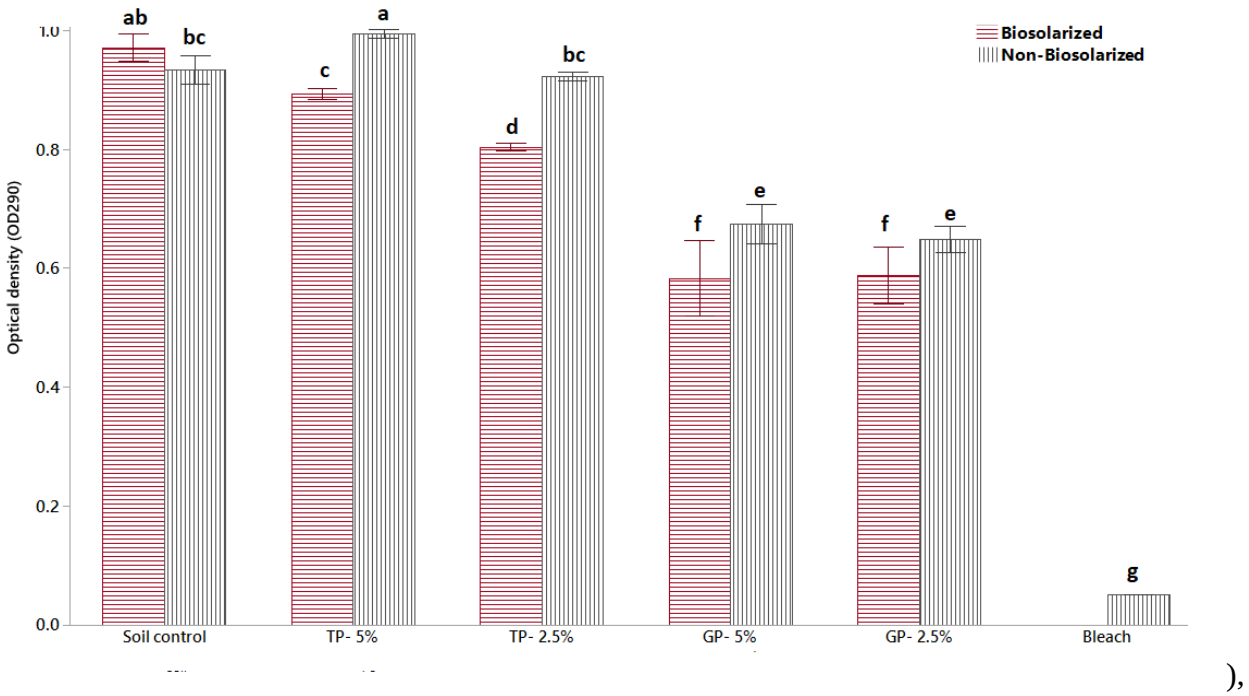
Compost was also added to rule out the effect of the initial load of microbes on the process. HMS, HS, and sugarcane were not included in this figure as they presented an LRV close to zero (not significantly different from SC-BSB).

As shown in Figure 12, LRV was calculated from soil kept at 37 °C; this value represents additional inactivation offered by the amendments, compost, temperature, or a combination of all. The results showed that excluding TP-biosolarized soil (TP-BSB), there was no significant difference between the same amendment with or without compost. For TP-BSB and compost, there was a nearly 1 log increase in the inactivation rate when compared with its non-compost counterpart. These findings are consistent with those in the literature, where this compost does not show significant microbial activity when added to the soil alone. The extra log observed in TP-BSB could be because OA accumulation is skewed in soil destabilized with organic matter

(Achmon *et al*. 2016; Achmon *et al*. 2017; Achmon 2018 *et al*.; Simmons *et al*. 2013; Fernández-Bayo *et al*. 2017) before the bacteria had time to acclimate. However, the cost, such as transportation cost, associated with this 1 log reduction and lack of difference from other treatments makes compost an unnecessary co-amendment for farming systems. These results for compost are consistent with those from other studies, where the inoculum either helped inactivate pathogens or had no effect but did not decrease the inactivation rate (Achmon *et al*. 2016; Achmon *et al*. 2017; Achmon 2018 *et al*.; Simmons *et al*. 2013; Fernández-Bayo *et al*. 2017).

Temperature alone (as seen on SC-BSB) showed positive LRVs but did not promote total inactivation of cells, as seen for GP-biosolarized soil (GP-BSB), confirming that total inactivation is a product of the synergy between GP and biosolarization (Figure 12). TP under optimal temperature (TP-37 °C) had the lowest inactivation rate (negative LRV), which can be explained by the presence of nutrients and fermentation byproducts and the absence of a stressor (temperature) in TP.

GP under optimal temperature (GP-37 °C) had values not different from those of the control. These results are not translatable to the inhibition effect on growth shown by GP extracts during the inhibition assay.



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indicating that the composition, concentration, and availability of bioactive compounds within reactors are not optimal to alter cell inactivation rate alone but impose stresses that inhibit growth. These results corroborate the hypothesis that inactivation using GP and *thermophilic temperature* is a result of inhibited heat shock responses (e.g., ROS formation) in addition to direct damage (e.g., increase in ROS species), as suggested by other polyphenol-synergistic studies (Wang *et al*. 2017).

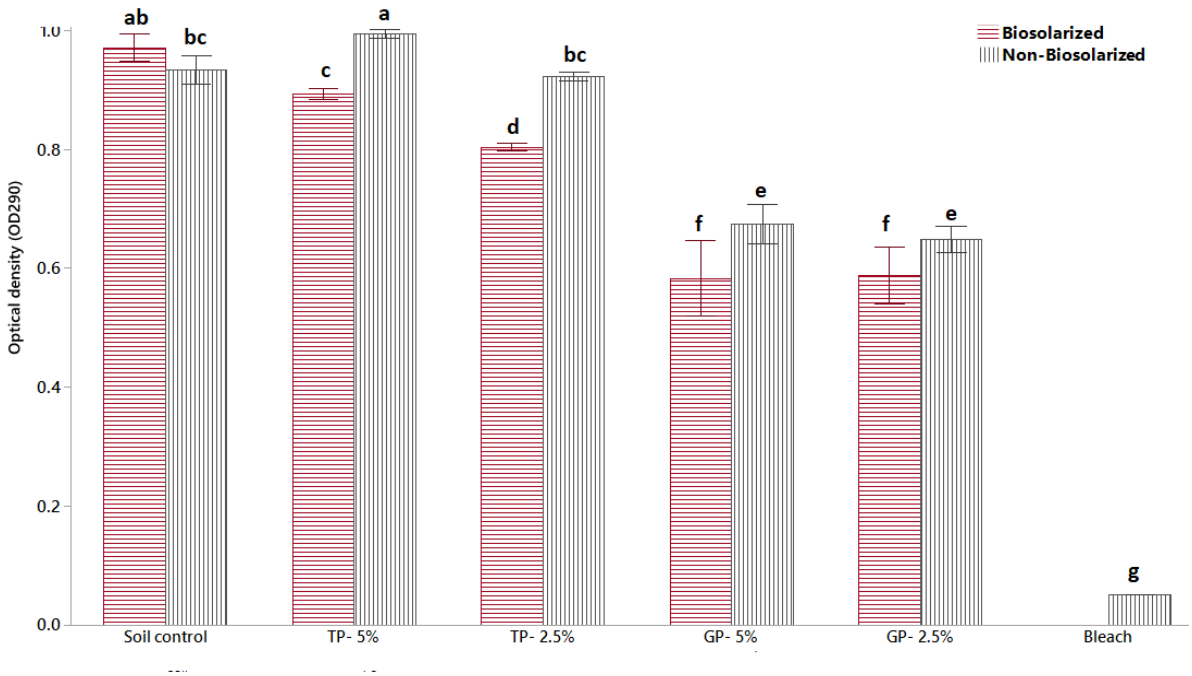
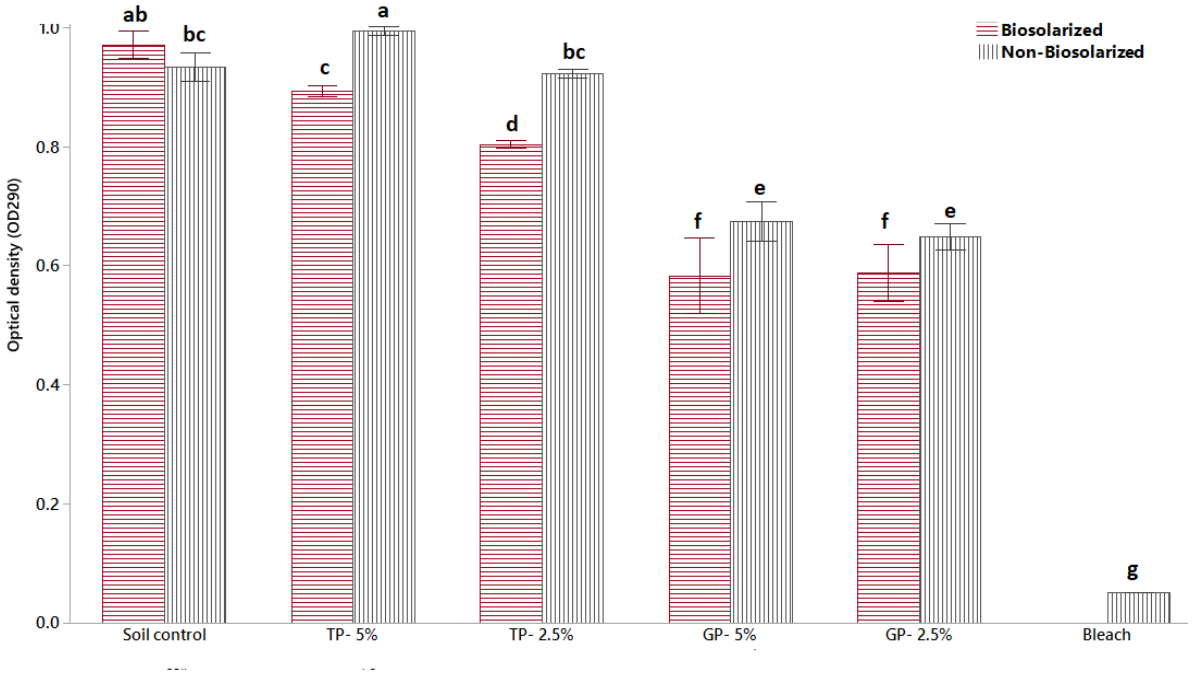


Figure 13: Optical density of LB media added to pomace extracts. After inoculation, LB media was incubated for 24 h at 37 °C, with agitation under aerobic conditions. The number represents the concentration used in the bioreactors. Bleach represents the positive control for total inhibition. Samples not connected by the same letters are significantly different (P > 0.05) for n=3. GP: grape pomace, LB: Luria–Bertani, TP: tomato pomace

Figure 12



shows the inhibitory activity of several extracts on the growth of *E. coli* in LB media. Except for the soil control, all samples had different growth rates when comparing biosolarized samples with their non-solarized counterparts. Our findings reveal that fermentation compounds affect cellular activity. GP had the biggest inhibitory effect, which was not related to concentration as inhibition for both treatments was not different. By contrast, both biosolarized GP extracts (GP2.5% and GP5%) samples showed increased inhibition compared with their non-solarized counterparts, which can be explained by difference in available bioactives. Grape polyphenols are mildly soluble in water (Panprivech *et al*. 2015), but biomass decomposition during fermentation might hasten their release into the liquid phase.

Inhibition with TP-5% was lower than that with TP-2.5% and was not statistically different from the non-biosolarized soil control. This is consistent with findings (Achmon *et al*. 2018), where soil biosolarized with two concentrations of TP (2.5% and 5%) had the same pH

after biosolarization but different OA profiles produced during fermentation. The presence of OAs produced during fermentation might mitigate the inactivation rate (Figure 11 Figure 12) by upregulating metabolic responses; however, different OAs might impose different metabolic challenges on growth. To explore these hypotheses, the inhibitory activity of the aqueous extracts from several biosolarized bioreactors using 5% amendments was measured against *S*Tm (Litvak *et al*. 2018), an important gram-negative pathogen. The aqueous extracts showed no statistical difference (Figure 14) in *E. coli.* However, there is a trend with the highest variability in LRV for GP-amended soils. The analysis included three biological repeats; more repeats may increase the power of the analysis. In addition, a preliminary test analysed the effect of adding compost against *S*Tm (data not shown); the aqueous extracts from Grape pomace and compost GPC (5%) at 30 °C increased the LRV to 5.3 ± 0.15, which was significantly different from that of GP at 30 °C (LRV = 1.20 ± 0.89).

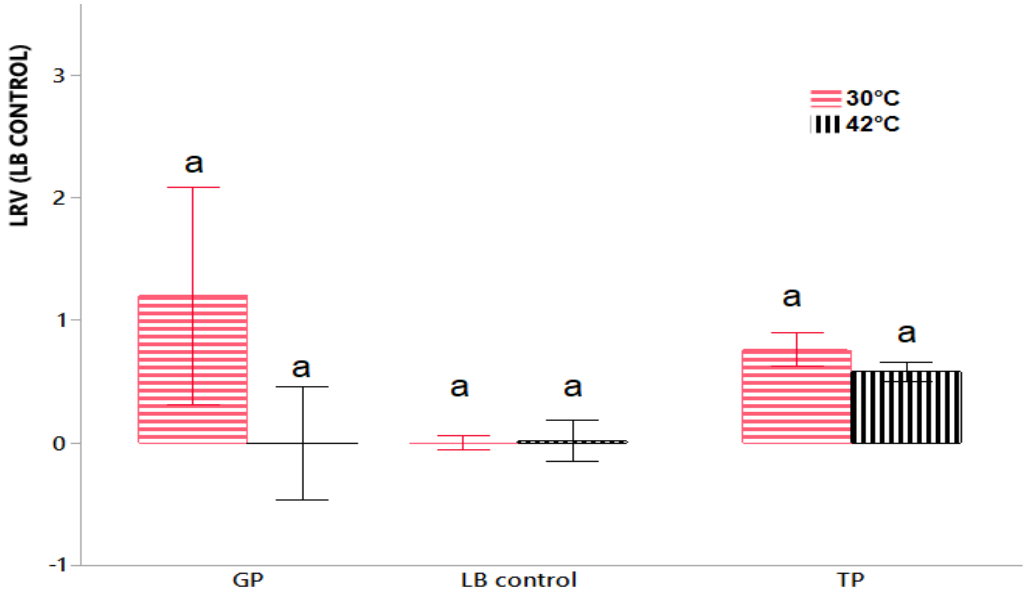


Figure 14: LRV results were calculated using LB media as control. Soil was amended with 5% TP. Samples not connected by the same letter show significant differences (P > 0.05)

for n=3. After mixing LB with the aqueous extracts, the media was incubated at two temperatures (30 °C and 42 °C) (n = 3). LB: Luria–Bertani, LRV: log reduction value, TP: tomato pomace

As discussed before, this finding is consistent with those reported in the literature (Achmon *et al*. 2017; Achmon *et al*. 2018; Hestmark *et al*. 2019; Simmons *et al*. 2013; Simmons *et al*. 2014). Compost does not decrease the inhibitory capacity of the amendment. *Salmonella* is not the model organism adopted in this thesis; therefore, it warrants further investigation; however, this preliminary result suggests that compost affects *S*Tm inhibition and should be further explored as *Salmonella* can survive for more than 98 d in animal compost and under greenhouse conditions (Chen *et al*. 2018).

To determine the importance of VFAs in these data, OA profiles were obtained for aqueous extracts (Figure 15).

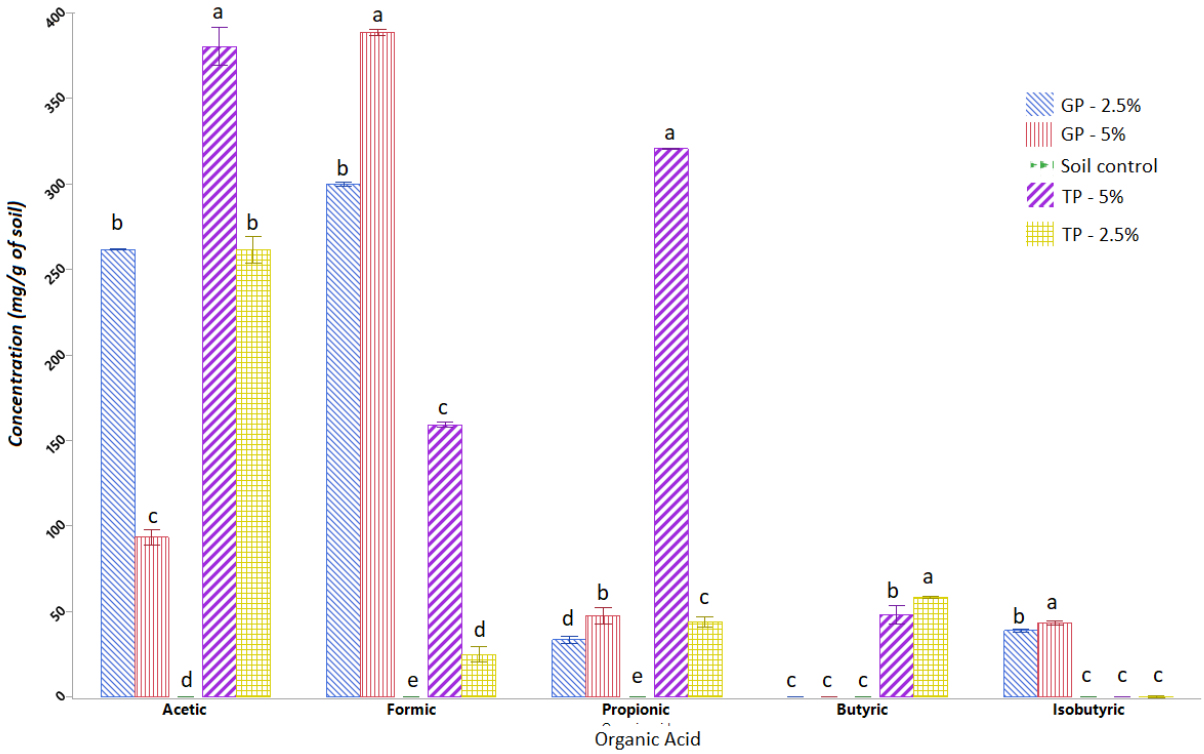


Figure 15: HPLC results for the concentration of organic acids in aqueous extracts from biosolarized bioreactors. For a given acid, each letter indicates significant difference ($P > 0.05$) for n=3. GP: grape pomace, HPLC: high performance liquid chromatography:, TP: tomato pomace.

Formic and propionic acid concentrations were more than 5 times lower in TP-2.5% than in TP-5%; acetic acid was higher in TP-5% but TP-2.5% also showed high levels. High levels of these three acids are sufficient to quickly regulate the acid-stress response and enable the cells to grow at the same rate as those in the non-biosolarized soil control. By contrast, the much lower concentration of formic and propionic acids and the presence of acetic acid in TP-2.5% might not be able to upregulate these genes, but is enough to impose metabolic challenges that, for example, might increase the lag phase of growth or modulate the generation time. In addition, the synergy between temperature and OAs during biosolarization (Figure 11Figure 12) can induce the expression of defensive genes and mitigate cell inactivation; because growth is not expected to occur during biosolarization, any change in growth parameters is not relevant.

Acetic acid and formic acid were also present in both GP extracts; however, their concentrations were much higher in TP-2.5%, whereas isobutyric acid was present in GP but not in TP. These data suggest that isobutyric acid is sufficient to either inhibit *E. coli* growth (Figure 14), inactivate *E. coli* (figure 12) or bioactives in GP suppress acid stress responses, such as attachment and biofilm formation (25,42,61) (Katalinić *et al*. 2010; Mandalari *et al*. 2010; Carraro *et al*. 2014).

For *Salmonella*, the lack of a statistically significant difference is consistent with the literature. In general, high concentrations (≥4%) of acids are required to inactivate 2 log of cells (Tamblyn and Conner 1997). In a study (Jiang *et al*. 2018), the minimum inhibitory concentration (MIC) of VFAs were evaluated; pH was reported to have a greater impact than VFA concentration. For example, VFAs at pH 6 decreased the MICs of the VFAs and caused greater harm to *Salmonella with* sharp reductions close to 100%. *This may explain the data shown in* Table 11 Change in pH during the heating process (biosolarization)*and* Figure 14.

Table 11 Change in pH during the heating process (biosolarization)

Treatment		Initial pH	Final pH
GP	2.50%	6.20	4.92 ± 0.17
	5.00%	5.73	4.65 ± 0.44
TP	2.50%	6.68	7.06 ± 0.04
	5.00%	6.11	6.27 ± 0.11
GPC	2.50%	6.50	4.69 ± 0.08
	5.00%	6.20	4.79 ± 0.04

GP: grape pomace, GPC Grape Pomace and Compost, TP: tomato pomace (n=3)

The final pH is also viable for GP-amended soils, which might be the reason for the high variability. The drop in pH and the presence of polyphenolics might explain these differences and will be covered in the next sections of this chapter.

Typically GP is stored for a period ranging from several days to a few weeks that would could ferment, consuming any residuals fermentable sugars and result in a production of 4–10% (w/w) of ethanol (Muhlack et al. 2018). GP used in this experiments were dried after storage causing the ethanol to evaporate. Considering that neglectable quantities of residual sugar for alcoholic fermentation and no leftover alcohol were present during biosolarization, ethanol was not measured and won`t be further discussed.

Validation of GP as an amendment against E. coli

AEROBIOSIS

According to previous results (Figure 11 Figure 12), the mixture of white grape varieties could inactivate the surrogate strain *E. coli* TVS 354 isolated from lettuce leaves (Tomás-Callejas *et al*. 2011). Figure 16 expands the LRV and reveals the effect of each physical–chemical process (temperature, amendment, and aerobiosis). We also included a comparison to the initial number of retrievable cells per gram of soil (initial load/g). Our findings reveal that amendment, the presence of oxygen, or temperature alone has no effect on reducing the bacterial load. Results from comparing GP and GPC in anaerobioses reiterates the importance of the cell inactivation process; the presence of compost in GPC (aerobic) decreased the LRV 1.3 times compared with GP (aerobic). The present data is comparable to previous studies (Achmon et al. 2018; Simmons et al. 2013; Simmons et al. 2014 and Fernández-Bayo et al. 2019) where compost only showed either beneficial or no effect on biosolarization patterns against soil and plant pathogens, in this studies, compost did not revert the inhibition conditions for E.coli during biosolarization with GP but it reverted the protective effect of compost in soil that allows the

bacteria to survive for periods longer than 168 d (Stocker et al. 2015; Hestmark et al. 2019 Chen et al. 2018). An enrichment test was performed for reactors with no detectable cells, GP (BSB) and GPC (BSB). After 24 h of enrichment (data not shown), no cultivable cells were found, indicating that the synergy between GP and temperature reduced the LRV by killing the cells and not rendering them uncultivable.

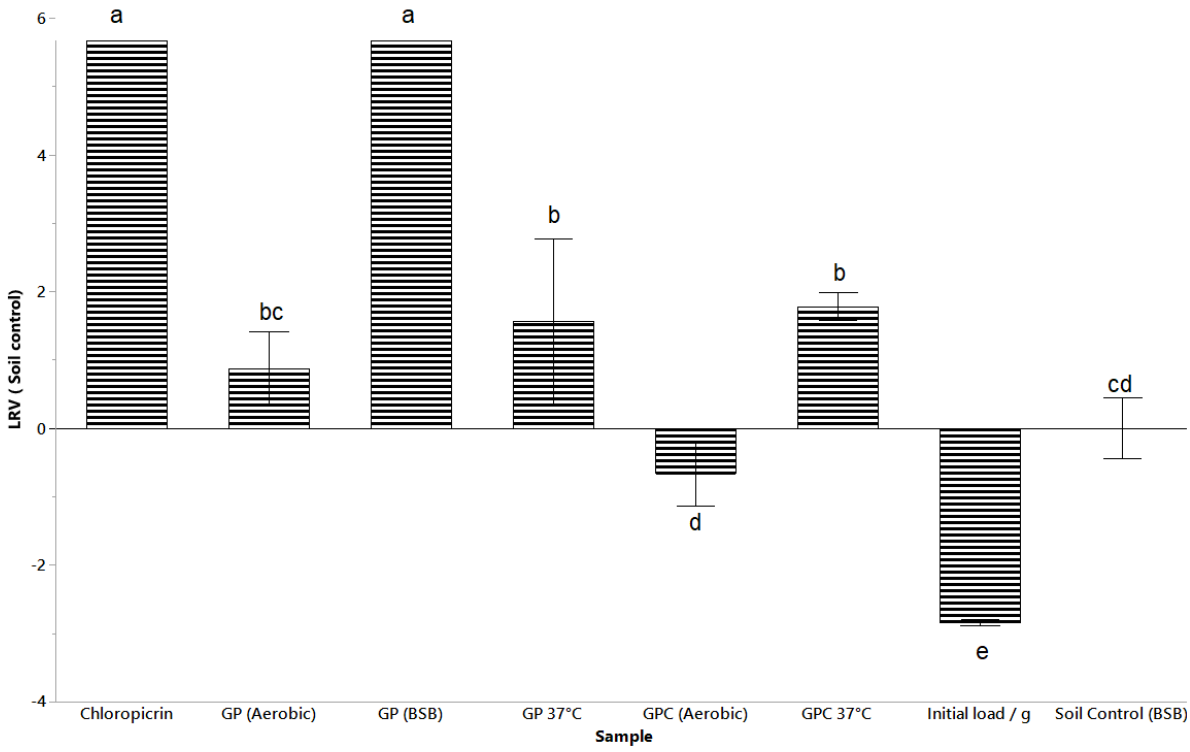


Figure 16: LRV values from different biosolarization conditions compared with the soil control, aerobic means amended soil kept under room temperature and aerobiosis, 37 °C indicates samples that were kept under anaerobiosis and constant temperature of 37 °C for 8 d. Samples not connected by the same letter show significant differences ($P < 0.05$) for n=3. BSB: biosolarized soil, GP: grape pomace, LRV: log reduction value, TP: tomato pomace

ANTAGONISM TO NON-E. COLI TVS 354 MICROBIAL COMMUNITIES

Some undetermined species, possibly filamentous fungus, grew in some plates after 8 d in a seemingly inverse relationship with E. coli (Figure *16*).

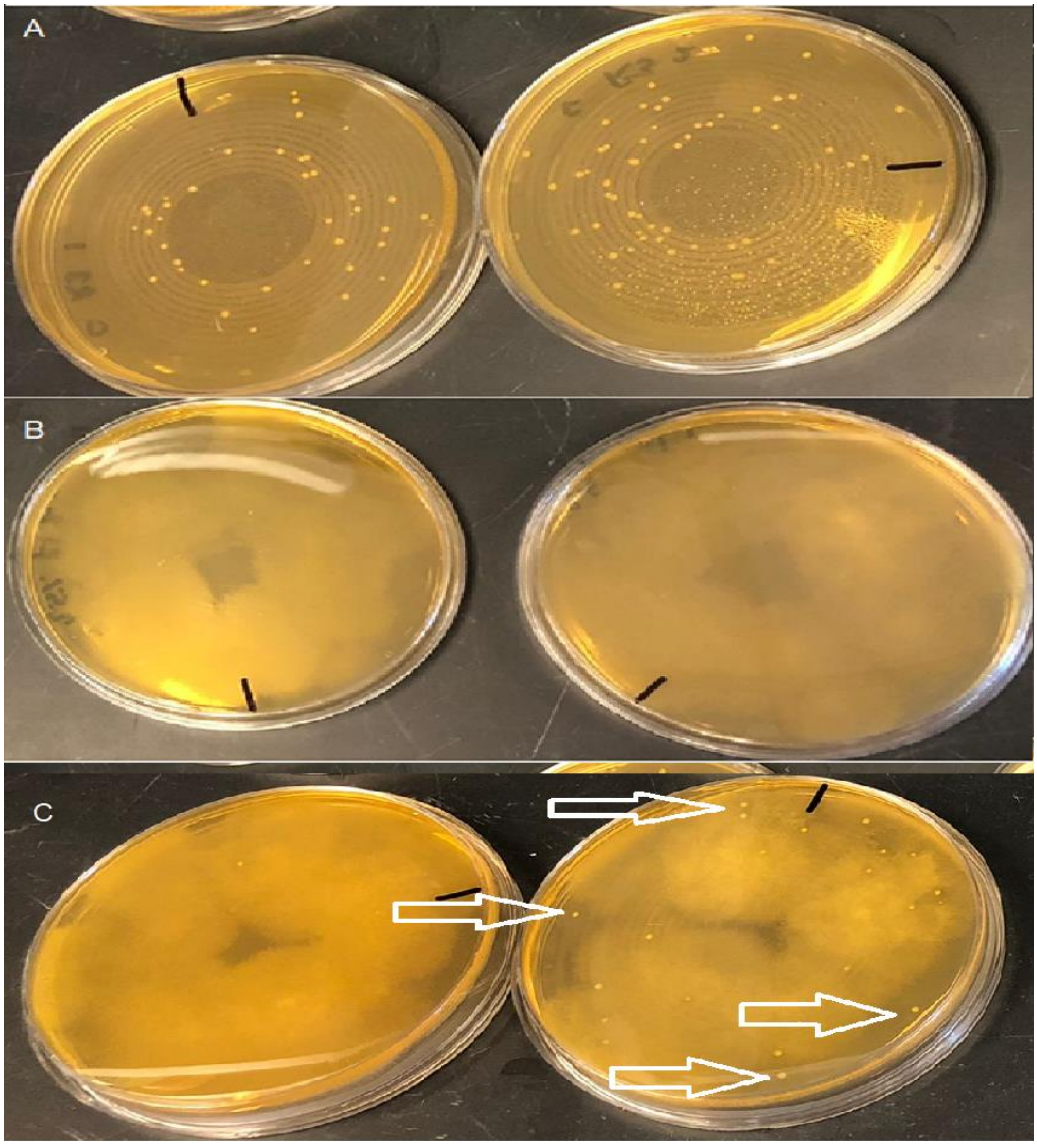


Figure 17: Pictures of plates from the serial dilution assay after 8 d of biosolarization with grape pomace. A shows the presence of only Escherichia coli, with fungus was absent. B has no E. coli but may contain filamentous fungus. Arrows in C indicate a few E. coli colonies growing concomitantly with the contaminating microbe.

To test this hypothesis, reactors were created with either autoclaved GP or soil to investigate whether the presence of these microbes is an antagonist to E. coli. In all cases (data not shown), there was no difference in the autoclaved counterpart, with no detectable cells after 8 d of biosolarization with GP. This indicates that E. coli is not antagonized by such contaminants.

Although further studies are necessary, it is possible to infer that the antagonism, if present, is from E. coli toward the fungi. This is consistent with the literature. An in vitro study from 2013 (Sivanantham et al. 2013) screened the antagonistic effect of soil-borne bacteria on phytopathogenic fungi. Eight bacterial species, including E. coli, showed biocontrol potential, induced vegetative germination, and inhibited spore germination.

KINETICS STUDY OF THE INACTIVATION WITH GP

Figure 18 shows bacterial inactivation during the 8-d process. GInaFiT was used to generate a log-linear curve with the shoulder and tail for GP (BSB). The curve for soil control was not included, as the parameters were calculated using initial and final microbial loads. However, it was possible to use the same approach to estimate the specific death rate (*k*)

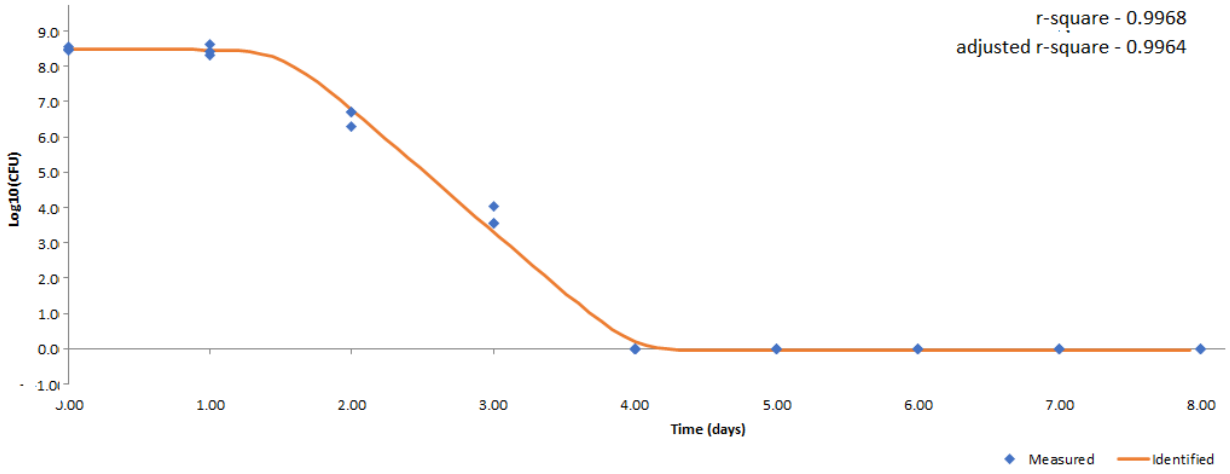


Figure 18*:* Inactivation curve calculated for GP amended soil (2.5% dry w/w). This curve was generated using GInaFiT, a free tool for MS-office, using log-linear modeling with shoulder and tail. CFU: colony forming unit, GP: grape pomace and n=3.

As seen in Figure 18, there were no culturable cells after day 3 of biosolarization, the slope of inactivation (constant-*k*) between days 1 and 4 was estimated at 7.94 (d^{-1}). For soil control, we estimated that it would take 44.4 h (D-value of 1.85) for one log reduction to occur after 1.85 d (Table 12Table **12** Kinetic parameters from the inactivation with GP or soil control

(no amendment)summarizes and compares the calculated kinetic parameters and includes the D-value, fractional reduction (▿ factor), *k* constant, and model parameter r^2, where applicable.

Table 12 Kinetic parameters from the inactivation with GP or soil control (no amendment)

	D-value (d)	▿ factor (8 d)	-*k* (time^{-1})
GP	0.63	−19.571	7.94*
SC	1.85	−9.9376	1.24

**k_{max}: slope of the curve or maximum death rate, D-value: decimal reduction value, k: specific death rate, GP: grape pomace, SC: soil control, ▿factor: fractional reduction*

EFFECT OF TYPE OF SOIL, GP VARIETY, AND E. COLI STRAIN

We investigated whether the inactivation of *E. coli* was linked or limited to specific parts of the system, such as the type of soil, variety of grapes, or *E. coli* strain. Figure 19 shows the LRV related to soil control. For comparison purposes, soil control was renamed *unamended sandy loam soil*.

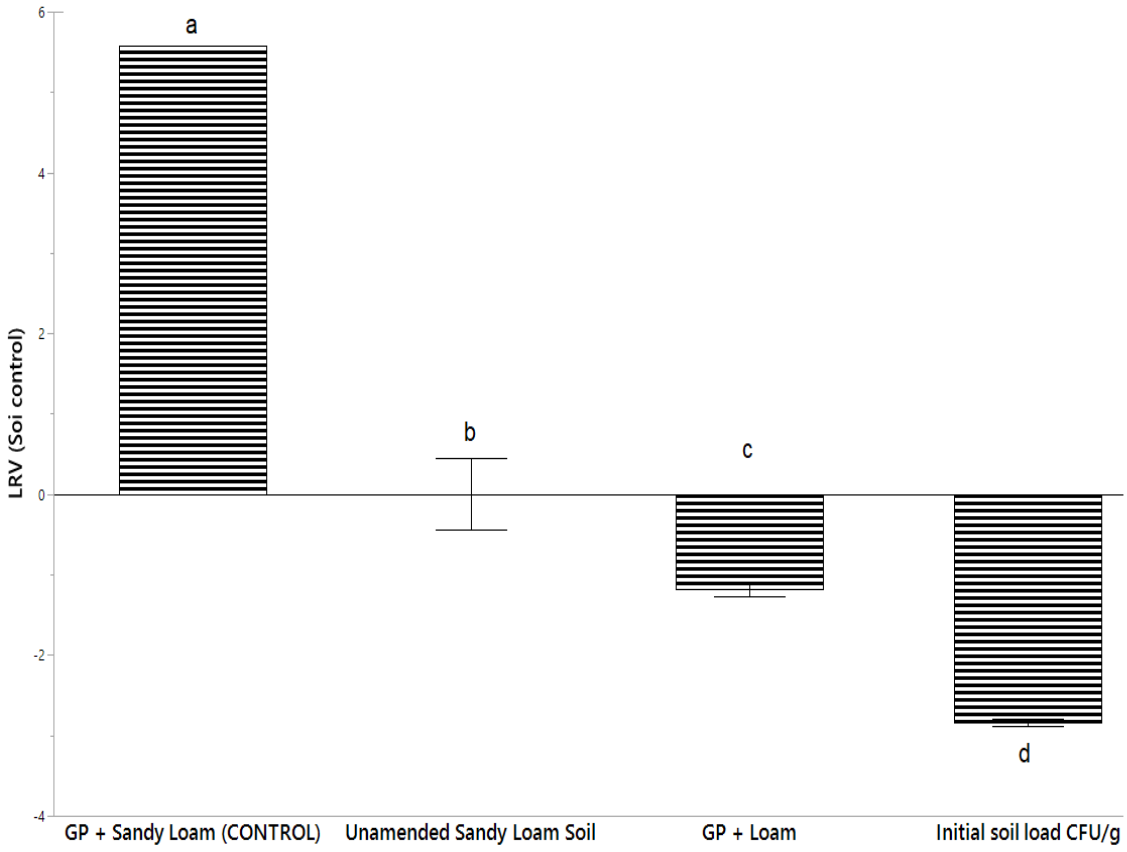
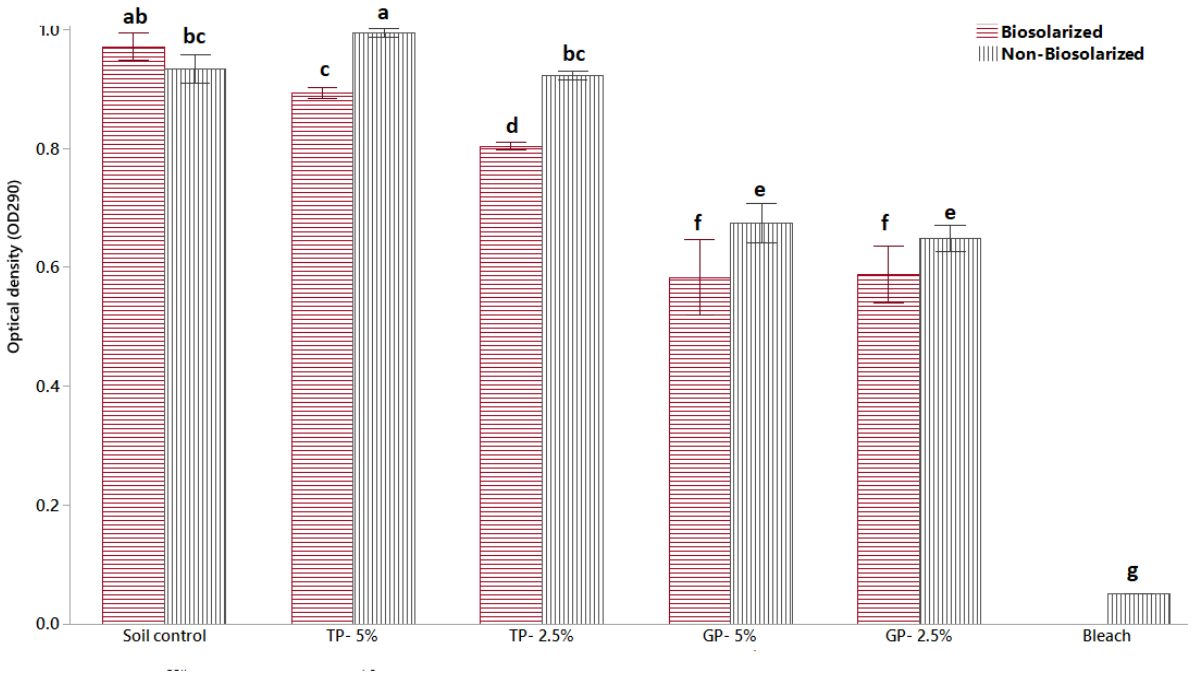


Figure 19: LRV of different soil types compared with the unamended soil control. For clarity, soil control was renamed unamended sandy loam soil. LRV was calculated using this value. CFU: colony forming unit, GP: grape pomace, LRV: log reduction value. Samples not connected by the same lowercase letter showed significantly different ($p \leq 0.05$)

A study from 2020 (Ivanov *et al*. 2020) assessed the short-term effects of stimulated microbial activity on the microstructure of loamy and sandy soils. Microbial activity altered the enmeshing of soil solid particles; as a result, cohesion increased in sandy soils and compressive strength decreased in loams in response to binding agents, such as extracellular polymeric substances. On one hand, cohesion modulates ultimate torque (gradient vs. flagellar movement), which dictates the direction of bacterial swimming; this motility is known as bacterial rheotaxis and can interfere with bacterial chemiotaxis. Bacterial rheotaxis is a purely physical phenomenon, in contrast to fish rheotaxis (Marcos *et al*. 2012). On the other hand, the physical

and chemical structure of soil that has undergone cohesion dictates the spatial distribution of cells and community behavior, such as quorum sensing, intercellular communication, and biofilm formation. Such microenvironment factors and complexities modulating microbial synergism and evolution (Aufrecht *et al*. 2019) could explain the difference seen in these two soils; the resulting LRVs can be a direct response to stress being modulated by the inhibitory potential of GP bioactives and soil properties (cohesion and pore size) to accommodate biofilm formation (a bacterial shield to GP bioactives). As discussed , heat shock responses are regulated by genes related to bacterial metabolic state (Borges *et al*. 2012), as well as genes related to biofilm formation and swarming motility (Lee *et al*. 2009), which are potentially inhibited by GP (Figure 11 and Figure 12



).

Six other batches of grapes were tested. Figure *20* shows all seven batches and the four varieties tested. The varieties tested included Verdello (batches 1 and 2), Viognier (batches 1 and 2), Torrontes, and two mixed varieties batches (2016 and 2017). The 2016 batch was used as the

standard in all assays. Figure *20* also accounts for uncontrollable experimental uncertainty; for example, systematic errors when all results within the same essays (or experimental "batches") are shifted by the same amount (Kramer et al. 2016). As explained in (Equation 3), soil control was added to all experimental batches; by applying LRV to these soil controls, we could account for variations and compare experimental batches (Figure *20*). All results above the red line show total inactivation (no detectable cells after 8 d). From all samples tested, only mixed variety 2018 did not show the same inactivation level; however, it mitigated the inactivation in a pattern similar to that of TP (Figure 11).

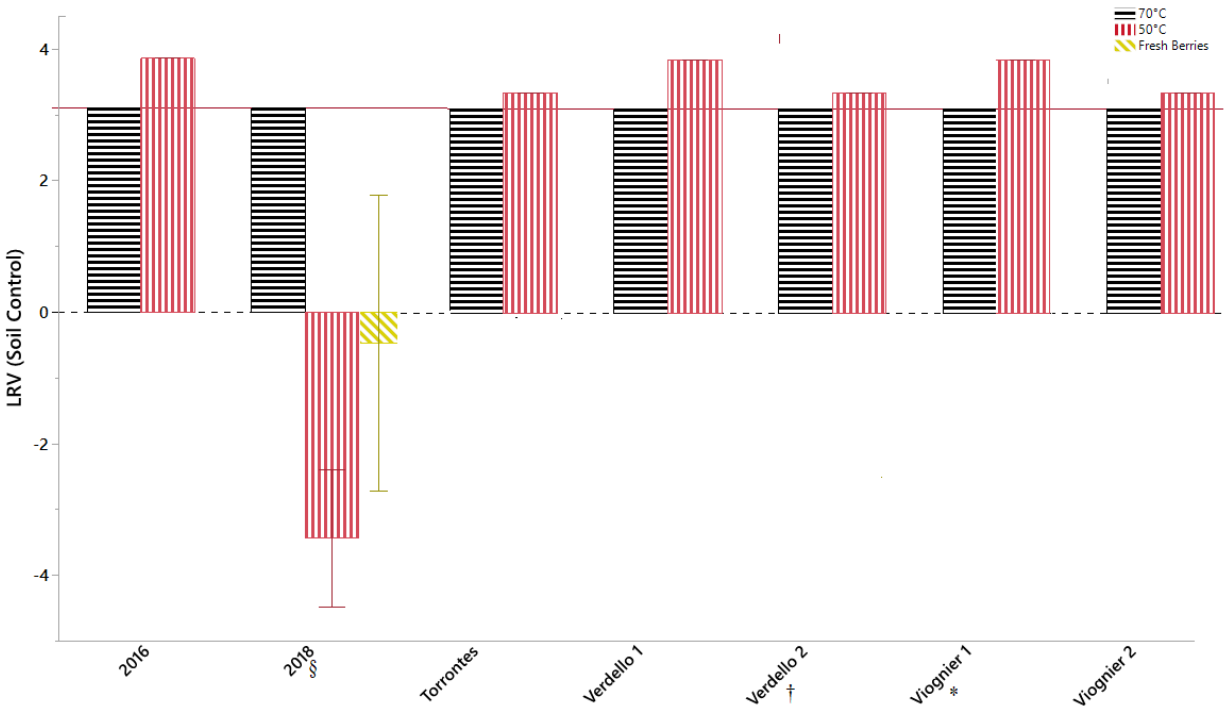


Figure 20: BSB using soil and 6 varieties of GP. All samples were amended at the standard level of 2.5% dry w/w. *Treated as GP-2017 throughout the text; in this figure it was used to highlight any variability due to different batches of the same variety. †This sample required a different regimen of drying, for comparison reason a new regimen of 70 °C for 48 hs was created to guarantee that the results were comparable. Results above the red line show total inactivation with no detectable cells after 8 d; variations observed are intrinsic to biological systems. LRV was chosen to allow comparison and minimize this variability. Sample marked as § did not show significant difference ($P < 0.05$) between fresh berries and dried biomass (n=3). 2016 is a mixed variety and is the batch used, unless mentioned otherwise. BSB: biosolarization, GP: grape pomace, LRV: log reduction value

Sample Verdello 2 (marked †) required adjustments to the drying period. The sample required an initial drying period of 2 h at 70 °C prior to the standard 48 h of drying at 50 °C. Irreversible oxidative reactions with phenolics were reported to be important during drying and significantly affected by air temperature (Mrad et al. 2012). For this reason, all batches were resubmitted to an extra "drying" period of 24 h at 70 °C. After the new drying period, sample 2018 also exhibited no detectable cells after 8 d of biosolarization. The change in inactivation indicates that temperature affects biomass; however, it hints that temperature-related reactions, for example Maillard, can be impactful as phenolic inhibition. In pears, increased drying temperatures influenced phenolic content and significantly induced an increase in *a** and *b** colorimetric parameters due to non-enzymatic browning (Mrad et al. 2012). A 2019 study reported the bactericidal effect of glucosamine, an alpha-aminocarbonyl compound synthesized through the Maillard reaction. Glucosamine can also undergo non-enzymatic browning, and convert to fructosazine, for example. These compounds were shown to damage the outer membrane of heat-resistant E. coli through the fructosazine-induced production of reactive oxygen species (Bhattacherjee et al. 2019) and act synergistically with UV-B to inactivate E. coli. These data indicate that Maillard compounds are an important factor to be considered in the inactivation of E. coli during biosolarization using white GP.

CONCLUSIONS

Our findings reveal that biosolarization is a promising technique for inactivating *E. coli*. These results highlight the importance of the synergy between the bioactive matrix of the biomass and temperature. Although temperature is an important stressor, biomass composition seems to modulate heat shock responses. For example, the presence of fermentation byproducts

seems to upregulate resistance genes, whereas grape bioactives (i.e., polyphenols) suppress these responses; moreover, both the level and type of bioactives are important for the level of regulation achieved. Our findings reveal the potential of GP to inactivate *E. coli* during biosolarization as a surrogate to other gram-negative pathogens. Gram-negative bacteria develop complex mechanisms, such as sturdier cell wall composition, to survive host infection that differ from those of gram-positive bacteria. Considering the microbial activity of food bioactive matrices against gram-positive bacteria, as reported in the literature, the number of amendments capable of inactivating gram-positive bacteria during biosolarization might be much higher than the one shown here against gram-negative bacteria.

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APPENDIX:

Original manuscript from which chapter 2 was modified from. The online document can be found on https://pubmed.ncbi.nlm.nih.gov/28571852/ and

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STRUCTURE AND ACTIVITY OF THERMOPHILIC METHANOGENIC MICROBIAL COMMUNITIES EXPOSED TO QUATERNARY AMMONIUM SANITIZER

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Abstract

Food processing facilities often use antimicrobial quaternary ammonium compound (QAC) sanitizers to maintain cleanliness. These QAC can end into wastewater used as feedstock for anaerobic digestion. The aim of this study was to measure the effect of QAC contamination on biogas production and structure of microbial communities in thermophilic digester sludge. Methane production and biogas quality data were analyzed in batch anaerobic digesters containing QAC at 0, 15, 50, 100 and 150 mg/L. Increasing sanitizer concentration in the bioreactors negatively impacted methane production rate and biogas quality. Microbial community composition data was obtained through 16S rRNA gene sequencing from the QAC-contaminated sludges. Sequencing data showed no significant restructuring of the bacterial communities. However, significant restructuring was observed within the archaeal communities as QAC concentration increased. QACs in thermophilic anaerobic digester sludge impacted the level and quality of biogas production, which corresponded to a significant changes in the sludge archaea. Further studies to confirm these effects on a larger scale and with a longer retention time are necessary.

Keywords: anaerobic digestion; antimicrobials; biofuels; microbial ecology; waste management

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Introduction

Anaerobic digestion is a waste management technology that can biologically convert wastewater organic matter to renewable gaseous biofuel, or biogas (Khalid et al., 2011; McCarty et al., 2011). Bacteria in digester sludge are responsible for the hydrolysis, acidogenesis and acetogenesis of complex organic compounds to acetic acid, carbon dioxide and hydrogen gas. These products serve as the substrates for the methanogenic archaea that ultimately convert them to methane and carbon dioxide, the principal components of biogas.

Quaternary ammonium compound (QAC) sanitizers are frequently used in food facilities due to their efficacy as a disinfectant (Gerba, 2015). Previous studies have shown that QAC contamination can affect methane production in mesophilic digesters operating at 35 ˚C (Tezel et al., 2006, 2007). However, thermophilic digesters employing temperatures of 50-60 ˚C are often used as well, which can have considerable differences in sludge microbial community structure (Chachkhiani et al., 2004; Shi et al., 2013) that can lead to more rapid cellulose degradation (Shi et al., 2013) and elevated methane production rates (Hashimoto, 1983). However, these benefits may come at the cost of decreased community stability (Dinsdale et al., 1996; Kim et al., 2002). To date, the susceptibility of thermophilic sludges to QACs has not been determined nor has the phylogenetic composition of QAC-contaminated sludge microbial communities been linked to biogas production data. In this study, the structure of thermophilic sludge microbial communities exposed to varying levels of QACs was determined via 16S rRNA gene sequencing. Changes in community composition were related to biogas production rate and quality data to identify microorganisms that may be sensitive to QACs.

1. Experimental

1.1. Anaerobic digestion

Batch anaerobic digesters were comprised of 250-mL glass media bottles fitted with modified caps containing a port connected to tubing and an in-line check valve (catalog #80103, Qosina). The methanogenic sludge used for these digesters was obtained from a thermophilic anaerobic digester located near the University of California, Davis campus that processes food scraps, spoiled packaged food, manure, yard waste, and paper waste. The digester did not process any rinse water from facilities using QAC sanitizers and thus the background QAC level in the

sludge was assumed to be negligible. The sludge had a total solids content of 3.5%. The commercial QAC sanitizer F-29 (Rochester Midland Corporation, Rochester, NY, USA) was added to sludge to at varying levels. The QAC content of F-29 sanitizer consisted of 4% (*W/W*) alkyl (C12-16) dimethylbenzylammonium chloride, 3% decyldimethyloctylammonium chloride, 1.5% didecyldimethylammonium chloride, and 1.5% dioctyldimethylammonium chloride.

To establish methanogenic cultures, sludge was initially incubated for 2 days at 55°C to exhaust most residual methane production. Each digester was then loaded with 100 ml of sludge and 0.5 mg of finely-milled tomato pomace to simulate organic matter that may be found in food processing wastewater. Varying volumes of F-29 sanitizer were loaded into digesters to achieve 0, 15, 50, 100 or 150 mg QAC/L. Reactor headspace was flushed with nitrogen gas. Reactors were incubated at 55°C for 4.5 to 7.25 days to elucidate differences in methane production between treatments without the confounding ecological effects of substrate exhaustion. Methane, carbon dioxide, and hydrogen content in biogas was measured via a MicroOxymax respirometry system (Columbus Instruments). The pH of the sludge was measured for two reactors from each treatment at the end of the incubation.

1.2. DNA isolation and 16S rRNA gene sequencing

Genomic DNA was purified from sludge microbial communities using a PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc). The V4 region of the 16S rRNA gene was amplified and sequenced according to previously described methods (Simmons et al., 2014) with one alteration. Under the altered protocol, a qPCR library quantification kit (KAPA Biosystems) was used to determine the concentration of V4 amplicons capable of being sequenced ahead of sequencing.

1.3. Data processing and analysis

DNA sequencing reads were filtered, assembled, clustered, and assigned taxonomy using iTagger, a custom PERL script developed by the Joint Genome Institute, as described elsewhere (Hausmann et al., 2016). Ecological analyses were performed using RStudio (version 0.98.1103) with the vegan and entropart packages. Prior to analysis, singletons were removed from operating taxonomic unit (OTU) read count data to reduce noise. Linear regression analyses of

community diversity, dissimilarity, OTU abundance, and biogas production data were performed using JMP software (version 12.0.1, SAS). For comparison of bacterial OTU changes in response to QAC concentration, critical P-values were adjusted for multiple comparisons to achieve a familywise error rate of 0.05 using the Bonferroni method (Bland and Altman, 1995).

2. Results and discussion

2.1. Biomethane production

A significant negative trend was observed between QAC level and methane production over the culture period (**Fig. 1a**, P = 0.002). Differences in cumulative methane production between treatments related to changes in methane production rates (**Fig. 1b**). Sludges containing 0, 15, or 50 mg QAC/L appeared to maintain more similar methane generation rates compared to sludges with 100 or 150 mg QAC/L. Specifically, sludges with at least 100 mg QAC/L showed a marked decrease in methane production rate 48 hr post-QAC addition compared to those with lower QAC levels. These data suggest a critical QAC level between 50 and 100 mg QAC/L for the thermophilic sludge. Previous studies observed inhibitory effects above 25 mg QAC/L for mesophilic methanogenic communities (Tezel et al., 2006, 2007). QAC concentration also affected the quality of biogas produced by sludge (**Fig. 1c**). The methane content of the biogas produced by sludge significantly decreased as QAC concentration increased (P = 0.008). These data indicate that methanogenesis in the thermophilic sludge was more sensitive to the concentration of QAC compared to upstream metabolic processes that produce carbon dioxide. A similar response was previously observed for mesophilic sludge (Tezel et al., 2006). Although some of these upstream processes, such as the production of acetate from other organic acids, produce gaseous hydrogen in tandem with carbon dioxide, no accumulation of hydrogen gas was detected for any treatment (data not shown). A significant negative correlation was observed between the final sludge pH and the QAC level (P = 0.004, **Fig. 1d**). However, the lowest pH measured (7.85) was still well within the tolerable range for anaerobic digestion (Cioabla et al., 2012).

2.2. Microbial community composition

Calculation of Good's coverage values predicted that over 99.9% of OTUs were accounted for in the sequencing data for each microbial community analyzed. At the whole community level, diversity index (H') did not show any significant difference in response to QAC level (**Table 1**).

Separate analysis of archaea and bacteria within the communities revealed differing trends between these sub-communities in response to QAC contamination. Alternately, increasing QAC levels corresponded to a significant increase in dissimilarity for archaeal sub-communities compared to archaea in the sludge prior to treatment (P = 0.009, **Fig. 2**). Archaeal communities were represented by four genera: *Methanobacterium*, *Methanoculleus*, *Methanothermobacter*, and an uncharacterized genus within family *WCHD3-02* (class *Thermoplasmata*). *Methanoculleus* dominated archaeal communities across all treatments (relative abundance >83%, **Fig. 3**). However, its relative abundance significantly decreased (P = 0.009) in favor of *Methanothermobacter* and an OTU genus within family *WCHD3-02* at greater QAC concentrations. The abundance of *Methanoculleus* in all cultures suggested that this genus was likely responsible for most of the sludge methanogenic activity. *Methanoculleus* archaea are hydrogenotrophic methane producers (Barret et al., 2013; Wasserfallen et al., 2000). The prominence of hydrogenotrophic methanogens in all communities indicated that the community likely employed syntrophic acetate conversion, where non-methanogenic microorganisms within the sludge community oxidize acetate to produce CO_2 and H_2 for hydrogenotrophic methanogenesis. Syntrophic acetate oxidation is most thermodynamically favorable at elevated temperatures (Karakashev et al., 2006). As a result, the thermophilic communities studied here likely differ considerably from the mesophilic communities examined in prior QAC contamination studies (Tezel et al., 2006, 2007), which may have relied more on other archaea and methanogenic pathways that are more thermodynamically favorable at lower temperatures, such as acetotrophic methanogenesis. There may be innate differences in the sensitivity of thermophilic and mesophilic archaea to QACs. For instance, it has been observed that thermophilic archaea can be tolerant to a variety of other environmental stresses (Mesbah and Wiegel, 2012). The overall robustness of certain thermophilic archaea may contribute to the greater QAC tolerance observed in this study compared to previous research with mesophilic sludge communities. However, additional research is needed to separate other effects, such as differential adsorption of QACs to suspended solids in thermophilic and mesophilic sludges, that could also affect QAC availability and tolerance in sludges.

Although the culture duration used in this study was sufficient to elucidate differences in biogas production in response to QAC contamination, it was less than the 25 to 30 days hydraulic retention time typically used in anaerobic digesters. It is possible that the pH depression and

archaeal restructuring observed at high QAC concentrations could become more drastic over time. Given their abundance within all archaeal sub-communities, *Methanoculleus* sensitivity to QACs is likely a major factor in the overall anaerobic digestion sensitivity to QAC contamination. Similar changes between *Methanoculleus* and other methanogenic archaea has been observed previously in response to digester perturbations (Lee et al., 2014).

Bacterial sub-communities showed no significant relationship between dissimilarity from the initial community state and QAC concentration (P = 0.79, **Fig. 2**). Sludge communities contained 20 bacterial phyla spanning 203 genera. The most abundant phyla, *Thermotogae*, *Firmicutes*, and *Bacteroidetes*, showed no significant changes in relative abundance for the QAC levels tested (P = 0.442, 0.212 and 0.592, respectively; **Fig. 3**). Within these phyla, twelve OTUs accounted for more than 87% of bacterial community abundance for all treatments (**Table S1**). Three OTUs showed changes in relative abundance in response to varying QAC concentration: Clostridiales Family XI. Incertae Sedis family and *Tepidanaerobacter* genus increased with increasing QAC concentration whereas an OTU within the MBA08 order showed decreased as QAC levels increased. However, when the Bonferroni correction was used to account for multiple comparisons and establish a new critical P-value ($P \leq 0.0042$), corresponding to a familywise error rate of 0.05 across all OTUs, no OTUs showed significant changes in relative abundance in response to QAC concentration.

The differential response in archaeal and bacterial communities to increasing QAC concentration, as indicated by both phylogenetic restructuring and changes in biogas quality, is consistent with prior research that found that methanogenic archaea were more sensitive to ammonium concentration than sludge bacteria (Sawayama et al., 2004). The differing sensitivity to QAC may relate to physiological differences between certain archaea and bacteria, such as preference for different compatible solutes to manage osmotic stress (da Costa et al., 1998). Additionally, the unique lipids that archaea utilize to withstand thermophilic environments (van de Vossenberg et al., 1998) may ultimately make them less tolerant of QACs. Additional research is needed to explore these possibilities.

3. Conclusion

This study suggests a negative impact of QAC on thermophilic digester performance. Further studies to confirm these effects on a larger scale and with a longer retention time are

necessary. Data regarding the tolerance of anaerobic digestion microbial communities exposed to QAC sanitizer can inform digester operational procedures and waste treatment practices. QAC sanitizers are often recommended for use at levels up to 400 mg/L (F-29 sanitizer label), considerably greater than the inhibitory threshold for the thermophilic sludge community, sanitizer presence in wastewater has the potential to impact digester operation. Therefore, treatment or dilution of sanitizer wastewater streams with significant QAC concentration will be required ahead of digester loading. Moreover, phylogenetic composition data from anaerobic digestion communities will be useful for predicting QAC susceptibility in other methanogenic communities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in online version at xxxxxx.

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Table 1 Sequencing coverage and community diversity indicators for sludge communities exposed to varying levels of QAC. Values are presented as mean ± standard deviation (n = 4).

QAC level (mg/L)	Good's coverage	Shannon (H')
0	$0.9997 \pm 5 \times 10^{-5}$	2.07±0.07
15	$0.9996 \pm 5 \times 10^{-5}$	2.11±0.09
50	$0.99975 \pm 5 \times 10^{-5}$	2.13±0.05
100	$0.9996 \pm 5 \times 10^{-5}$	2.09±0.11
150	$0.9997 \pm 5 \times 10^{-5}$	2.13±0.11

Figure captions

Fig. 1 Biomethane production from sludge containing varying levels of quaternary ammonium compounds (QAC). (a) Final cumulative methane production expressed as a percentage of that observed in control reactors lacking QAC. **(b)** Cumulative methane production over the first 4.5 days of culture. **(c)** Biogas quality estimated from cumulative production of methane and carbon dioxide over the culture period. **(d)** Sludge pH at the conclusion of the incubation. Dotted lines indicate the line of best fit for the data. Error bars represent one standard deviation. n=4 for a-c, n=2 for d

Fig. 2 Bray-Curtis dissimilarity of bacteria and archaea within sludge communities exposed to QACs relative to the initial sludge community. Solid and dotted lines represent lines of best fit for archaeal and bacterial sub-communities, respectively. Error bars indicate one standard deviation. n = 4.

Fig. 3 Phylogenetic composition of anaerobic digester microbial communities exposed to various levels of QAC sanitizer and the initial inoculum (ini). Data correspond to (a) archaea and (b) bacteria sub-communities. For clarity, archaea are presented at the lowest resolved phylogenetic classification while bacteria are presented at the phylum level (p, phylum; f, family; g, genus). n = 4.

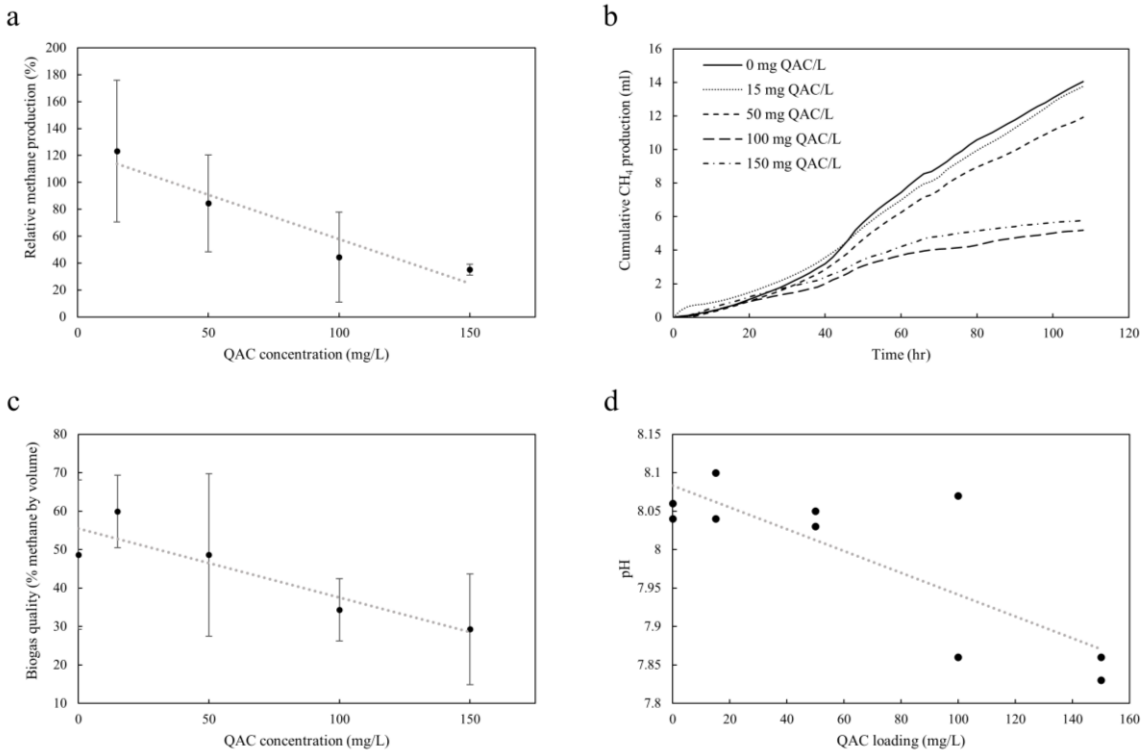
a
Relative methane production (%)
QAC concentration (mg/L)
b
Cumulative CH4 production (ml)
0 mg QAC/L
15 mg QAC/L
50 mg QAC/L
100 mg QAC/L
150 mg QAC/L
Time (hr)
c
Biogas quality (% methane by volume)
QAC concentration (mg/L)
d
pH
QAC loading (mg/L)

Fig. 1

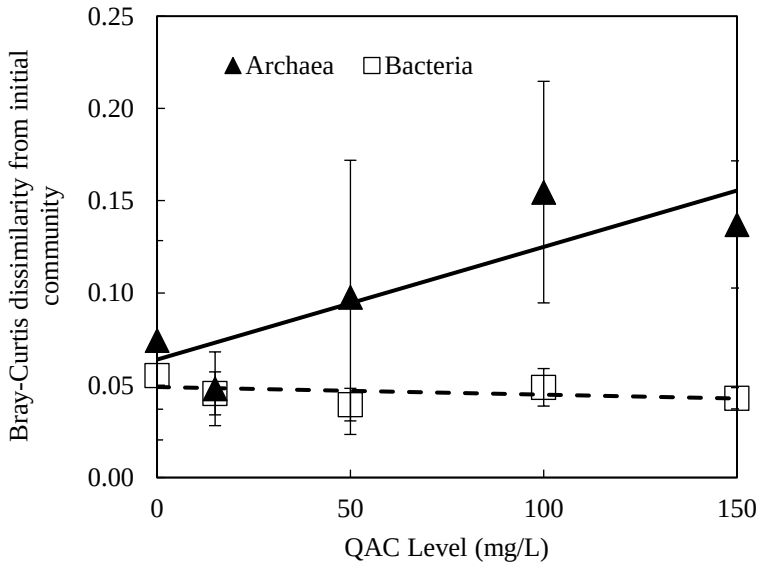
Archaea
Bacteria
Bray-Curtis dissimilarity from initial community
QAC Level (mg/L)

Fig. 2

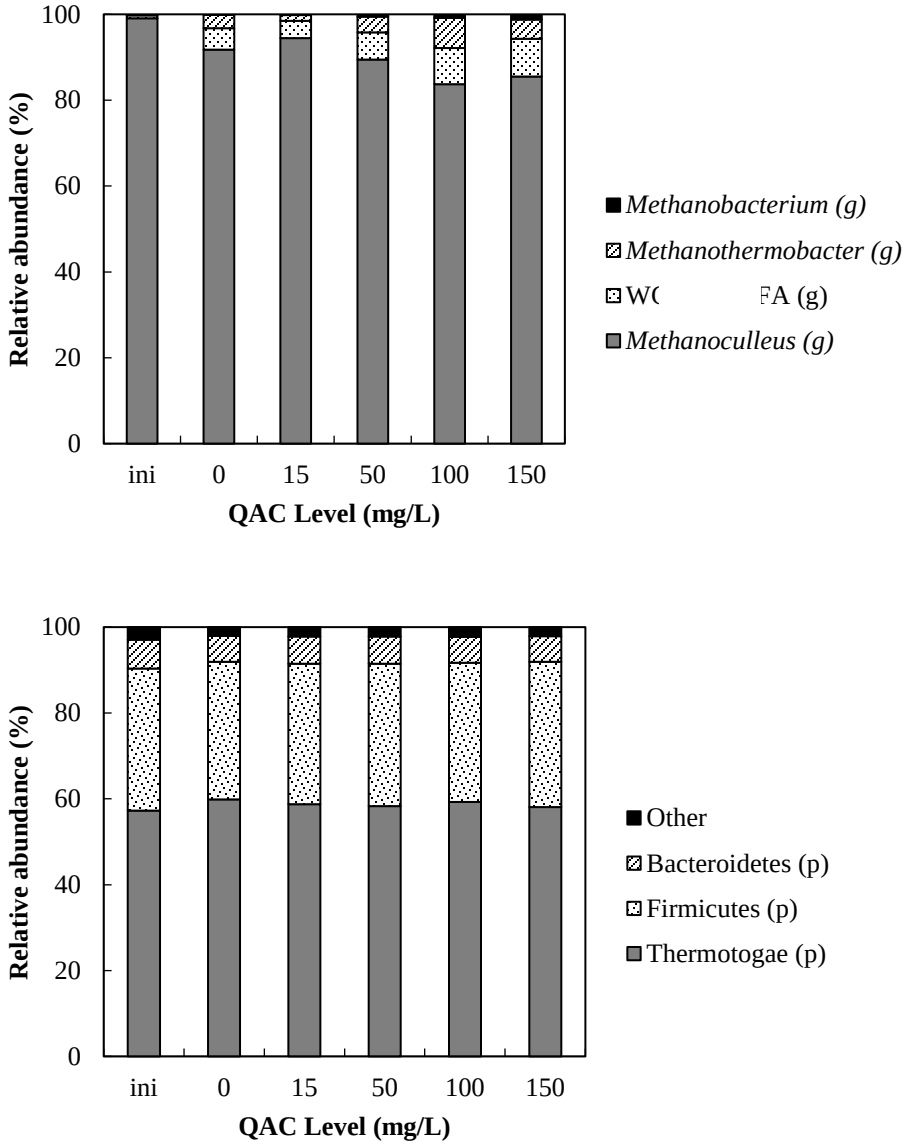
100
80
60
40
20
0
Relative abundance (%)
ini
0
15
50
100
150
QAC Level (mg/L)
Methanobacterium (g)
Methanothermobacter (g)
WC FA (g)
Methanoculleus (g)
100
80
60
40
20
0
Relative abundance (%)
ini
0
15
50
100
150
QAC Level (mg/L)
Other
Bacteroidetes (p)
Firmicutes (p)
Thermotogae (p)

Fig. 3