PRODUCTION OF VOLATILE FATTY ACIDS FROM FOOD WASTE

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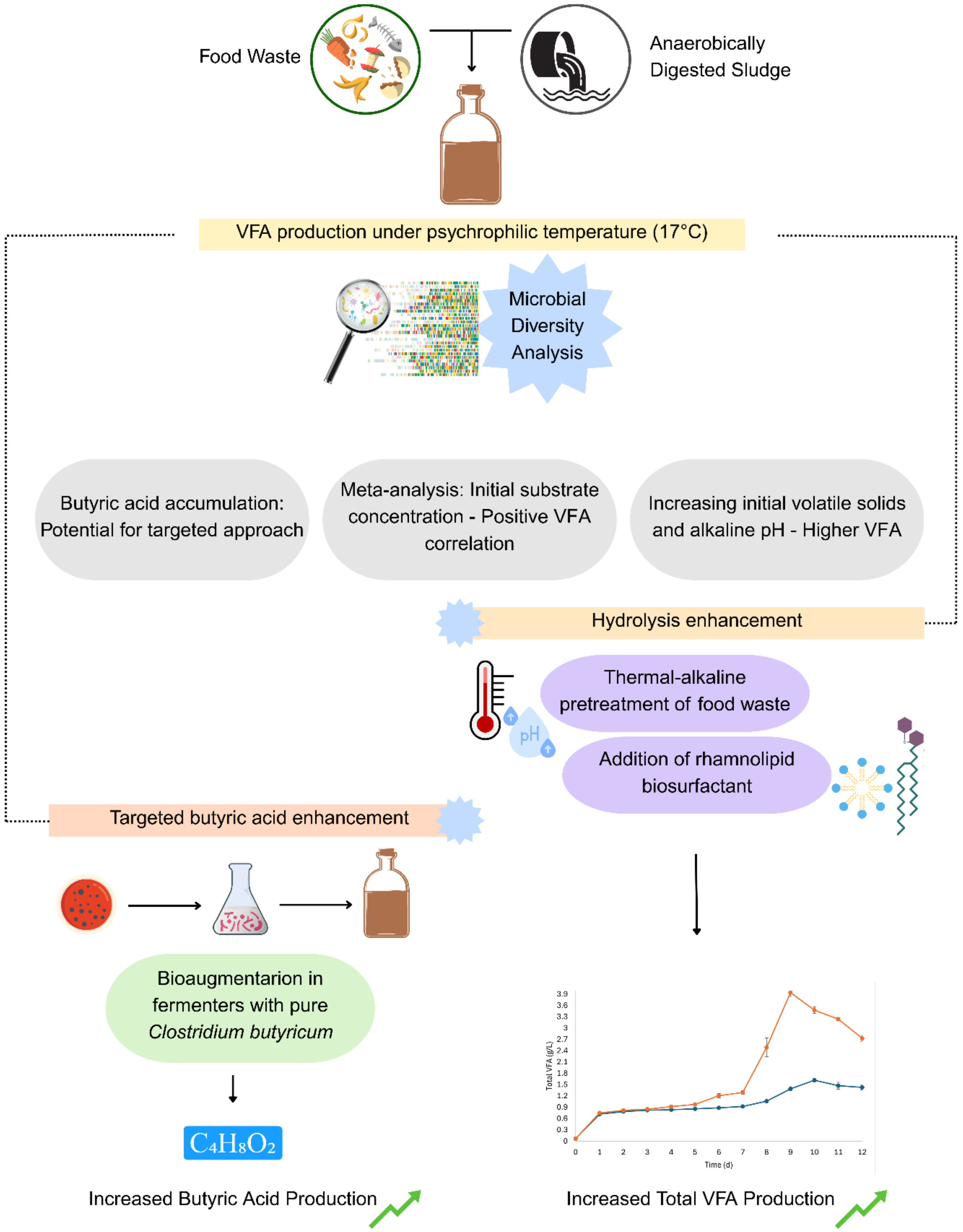
ABSTRACT

Food waste is a major environmental concern, often ending up in landfills or incinerators, contributing to greenhouse gas emissions and the loss of valuable organic matter. Conventional treatment methods like composting or anaerobic digestion offer limited resource recovery, particularly in colder climates where energy demands for heating remain high. As the demand for sustainable and climate-adaptable solutions grows, volatile fatty acids (VFAs) have emerged as valuable intermediates for bio-based products such as bioplastics and biofuels This research explores the microbial production of VFAs from food waste under psychrophilic conditions (≤20 °C), presenting a low-energy alternative aligned with cold-climate needs. Compared to traditional mesophilic systems, fermentation at 17 °C resulted in slower hydrolysis but showed a distinct shift in the VFA profile, with enhanced butyric acid accumulation. Microbial community analysis revealed the dominance of psychrotolerant genera such as *Solibacillus, Sporosarcina*, and *Paenibacillus*, which supported butyrate-producing *Clostridium* species. These findings highlight the potential for pathway-specific adaptation at low temperatures. To improve process efficiency, substrate solubilization was enhanced using thermal-alkaline pretreatment and rhamnolipid biosurfactants, which led to a twofold increase in VFA yield (up to 4.4 g/L). The addition of rhamnolipids not only improved lipid accessibility but also favored acidogenic microbial populations over lactic acid producers, promoting more efficient fermentation. Further targeted butyric acid was enhanced through bioaugmentation with *Clostridium butyricum*, a known butyrate producer. Its introduction significantly increased butyric acid concentration by sevenfold (reaching 1.4 g/L), validating the approach of targeted microbial steering even under low-temperature conditions. Overall, this study demonstrates the feasibility of psychrophilic fermentation as a sustainable platform for producing VFAs from food waste. By integrating pretreatment, microbial community insights, and bioaugmentation, the research offers a practical framework for resource recovery in cold regions, advancing circular bioeconomy goals while addressing food waste challenges.

GRAPHICAL ABSTRACT



Food Waste

Anaerobically Digested Sludge

VFA production under psychrophilic temperature (17°C)

Microbial Diversity Analysis

Butyric acid accumulation: Potential for targeted approach

Meta-analysis: Initial substrate concentration - Positive VFA correlation

Increasing initial volatile solids and alkaline pH - Higher VFA

Hydrolysis enhancement

Thermal-alkaline pretreatment of food waste

Addition of rhamnolipid biosurfactant

Targeted butyric acid enhancement

Bioaugmentarion in fermenters with pure *Clostridium butyricum*

$C_4H_8O_2$

Increased Butyric Acid Production

Increased Total VFA Production

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ABBREVIATIONS

VFA – Volatile Fatty Acids
VS – Volatile Solids
SCOD – Soluble Chemical Oxygen Demand
BES - 2-bromoethanesulfonate
MES – Mercaptoethanesulfonate
ABE - Acetone-butanol-ethanol
AET - Acetate-ethanol type
HRT – Hydraulic Retention Time
OLR – Organic Loading Rate
EMP - Embden–Meyerhof–Parnas
BTF - Butyrate-type fermentation
LTF – Lactate-type fermentation
PTF - Propionate-type fermentation
MAF - Mixed-acid fermentation
AD – Anerobic Digestion
OFMSW - Organic Fraction Municipal Solid Waste
CSTR - Continuous stirred-tank reactors
COD – Chemical Oxygen Demand
TS – Total Solids
TKN – Total Kjehldahl Nitrogen
TCOD – Total Chemical Oxygen Demand
SNF – Solids-not-fat
LC-MS - Liquid Chromatography-Tandem mass Spectrometry
TSS – Total Suspended Solids

VSS – Volatile Suspended Solids

SDS – Sodium Dodecyl Sulfate

DA – Degree of Acidification

RDA – Redundancy Analysis

PCA – Prinicipal Component Analysis

OD – Optical Density

RCM – Reinforced Clostridial Medium

PCoA – Principal Coordinates Analysis

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CHAPTER ONE

Background

1. Introduction

The global rise in food waste generation poses a significant challenge for municipalities, industries, and governments striving to manage organic waste sustainably. Food waste, rich in biodegradable organics, is often landfilled or incinerated, leading to greenhouse gas emissions, leachate issues, and loss of potentially valuable resources [1]. Traditional waste treatment options such as composting or anaerobic digestion for biogas production offer limited value recovery and face operational constraints, especially in colder climates. There is a growing demand for more resource-efficient and climate-adaptable strategies that not only mitigate environmental harm but also enable the recovery of value-added products from such organic waste streams. In this context, volatile fatty acids (VFAs) have emerged as promising intermediates in the bioeconomy. These short-chain carboxylic acids (C2-C6) are platform chemicals for downstream applications such as the synthesis of bioplastics, biofuels, preservatives, industrial solvents, dyes, lubricating oils, animal feed, antimicrobial agents, and artificial flavours, among others [2]. They had a global market demand of USD 1.2 billion in the year 2024, which is projected to reach USD 2 billion by 2033 [3], [4]. Conventionally, VFAs have been derived from petroleum-based resources via petrochemical pathways [5]. Microbial fermentation of organic waste offers a sustainable alternative for VFA production, with the dual benefit of waste reduction and resource recovery. However, most existing studies and technologies operate under mesophilic temperatures, typically around 35–37 °C, requiring controlled heating and incurring significant energy costs. The potential of psychrophilic fermentation, operating at temperatures below 20 °C, remains largely untapped despite its suitability for cold or ambient environments such as those in Canada.

This doctoral research explores, for the first time, in a comprehensive and integrated manner, the production of VFAs from food waste under psychrophilic conditions. The study addresses three interconnected objectives: understanding the influence of temperature on VFA yield and microbial community dynamics, enhancing the hydrolysis step to improve process efficiency under psychrophilic conditions, and selectively steering fermentation towards a target VFA (butyric acid) production through the manipulation of underlying microbial systems.

In the initial phase (discussed in Chapter 5), VFA production was compared under mesophilic (37 °C and 27 °C) and psychrophilic (17 °C) temperatures using food waste as a substrate and anaerobically digested sludge as the inoculum. While psychrophilic conditions led to a reduction in overall microbial activity and hydrolysis rate, a distinctive observation of selective accumulation of butyric acid at psychrophilic temperature was made. This shift in metabolic profile correlated with changes in the microbial community structure, as determined through high-throughput sequencing. Psychrophilic environments favored specific genera such as *Solibacillus, Sporosarcina* and *Paenibacillus*, which further supported acidogenic *Clostridium* species contributing to the sustained production of butyric acid accumulating at 522 mg/L. This indicated the potential for pathway-specific adaptation and providing a foundation for steered fermentation targeting butyric acid under psychrophilic temperature. Furthermore, a comprehensive meta-analysis of studies conducted in the North American context was undertaken to explore the inconsistencies in reported outcomes and operational parameters for food waste fermentation (Chapter 5). The analysis identified a strong positive correlation between the initial volatile solids (VS) concentration and the total VFA yield. This finding led to assessing different initial VS concentrations resulting in maximum VFA production under psychrophilic temperature, providing the basis for experimentally-tested selection of 3 g VS as an optimal substrate concentration for subsequent experiments.

In parallel, Chapter 6 discusses another critical aspect constraining the VFA production - efficient hydrolysis of the substrate. This step is a known bottleneck for fermentation, especially under psychrophilic temperatures with reduced microbial and enzymatic activity. While a targeted VFA accumulation was observed, the overall VFA concentration remained constrained. Various pretreatment strategies were explored to enhance solubilization, among which thermal-alkaline treatment demonstrated the highest efficacy. To further enhance lipid hydrolysis, rhamnolipid biosurfactants were introduced in the fermentation system. This strategy improved the accessibility of lipid components for microbial uptake and subsequent fermentation. The combined approach led to a two-fold increase in the VFA yield over the control, reaching up to 4.4 g/L. Microbial profiling revealed a marked enrichment of acidogenic bacteria alongside a suppression of lactic acid producers, which typically divert substrate catabolism away from VFA production.

The third and final research objective investigated the targeted enhancement of butyric acid through bioaugmentation, as discussed in Chapter 7. Drawing on microbial community insights from earlier phases of this study (Chapter 5 and 6), *Clostridium butyricum*, a known butyrate producer, was introduced into the system. This enrichment strategy was directly informed by observed microbial abundance and shifts in the previous experiments which indicated the potential for targeted butyric acid production [6]. This strategic inoculation of enriching *Clostridium* *butyricum* was derived led to a significant 7-fold increase in butyric acid concentration reaching 1.4 g/L from 240 mg/L, validating the hypothesis that psychrophilic fermentation can be effectively directed towards a selective VFA formation without using pure cultures, typically considered as the norm.

The research offers new scientific insights and practical strategies for transforming food waste into high-value biochemicals through low-temperature fermentation. By advancing our understanding of microbial dynamics, improving hydrolysis efficiency, and enabling selective product formation, this work lays a strong foundation for the development of energy-efficient biotechnology that aligns with the principles of waste valorization and circular economy.

CHAPTER 2

Review of Literature

2.1. Production of volatile fatty acids (VFA) from Organic Waste

Acidogenic fermentation of organic wastes provides a value-added method for organic waste management and resource-recovery through the production of metabolites such as volatile fatty acids (VFA). These intermediate compounds are monocarboxylic acids comprising of two to six carbon atoms (C2 to C6), named acetic, propionic, butyric, valeric, and caproic acid in the order of increasing carbon chain. VFA have a vital role as building blocks in a wide range of applications in food, pharmaceutical, plastic production industries, and wastewater treatment [2]. The acidogenic fermentation process thus provides an environment-friendly alternative over the conventional petroleum-derived production of VFA. Furthermore, the use of waste streams as feedstock for mixed-culture fermentation can be employed by the conversion of existing anaerobic digestion systems, saving both energy and associated costs of producing biogas. In contrast to the complete anaerobic digestion process, which proceeds through acetogenesis and methanogenesis to produce biogas, acidogenic fermentation selectively restricts methanogenic activity to favor VFA accumulation. This partial oxidation of organic matter results in the production of mixed VFAs, which can subsequently serve as precursors for downstream processes such as chain elongation, biopolymer synthesis, or biohydrogen production. While the suppression of methanogenesis reduces overall methane yield, it enhances carbon recovery in the form of soluble metabolites with higher market value and greater application versatility. While the anaerobic digestion is a well-established technology, there are several challenges to the process, such as extended residence times (exceeding 4 weeks), requiring large reactor volumes, inefficient carbon conversion (40-60%) and slow digestion rates [7], [8], [9]. This increases the overall capital costs of the system. The production of VFA alternatively, requires shorter residence time, has higher carbon conversion efficiency (70%) and revenue generation due to their higher market value than biogas [10]. For instance, using food waste (FW) as the feedstock, the revenue generation by producing VFA is estimated to be $23.62 per tonne of FW, while it is $12.07 per tonne of FW for producing biogas [11].

The commercial production of VFAs typically relies on chemical synthesis using petrochemical raw materials [2]. Acetic acid is primarily produced through methanol carbonylation, along with other methods such as the catalytic oxidation of acetaldehyde, ethylene, or butane.[12] Propionic acid is conventionally obtained as a by-product of acetic acid production or via hydroxycarboxylation of ethylene using a rhodium or nickel carbonyl catalyst [13]. Butyric acid is commonly synthesized by oxidizing butyraldehyde, which is produced through the oxosynthesis of propylene derived from crude oil. Another traditional approach involves extracting butyric acid from butter, but this process is expensive [14]. Given the concerns about climate change and the depletion of fossil fuels, microbial fermentation for VFA production is becoming increasingly attractive. Additionally, the cost of petrochemically produced VFAs is closely tied to fossil fuel prices. Moreover, most of the biological production of VFA is derived from pure substrates such as glucose, xylose, glycerol etc. Although these substrates provide higher yield, but it overall increases the costs of the process. Hence, using waste streams such as food waste, wastewater, agricultural or forestry wastes offer both waste management and resource recovery simultaneously.

During acidogenic fermentation of organic wastes (Figure 1), the organic matter, including carbohydrates, proteins, and lipids, undergoes hydrolysis, breaking down into soluble forms and increasing the soluble chemical oxygen demand (sCOD). This step, known as hydrolysis, converts complex organic matter into simpler soluble compounds like sugars, amino acids, and fatty acids. These soluble compounds are then quickly fermented into pyruvate via glycolysis and subsequently into volatile fatty acids (VFAs), with hydrogen, carbon dioxide, and small amounts of alcohol produced as by-products during the acidogenic phase [15]. Within an anerobic digester, the methanogenic activity is suppressed to prevent VFAs from being further utilized to produce biogas. Suppression methods include modifying operational parameters—such as reducing reaction time, adjusting pH, and controlling the food-to-microorganism (F/M) ratio—to enhance acidogenic bacterial activity [16], [17], using inhibitors like carbon monoxide, 2-bromoethanesulfonate (BES), or 2-mercaptoethanesulfonate (MES) to inhibit methanogens [18], [19], or applying substrate pretreatments to limit the activity [20].

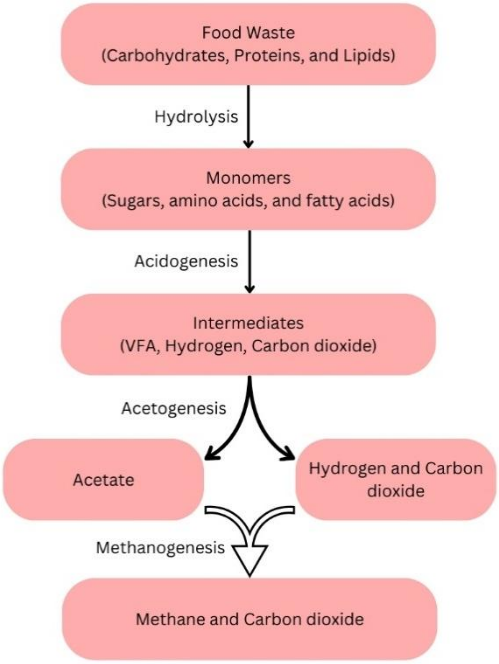

Figure 1. Process flow of VFA and methane production

The primary VFAs generated during acidogenic fermentation include acetic acid, propionic acid, butyric acid, valeric acid, and caproic acid. These acids serve as renewable carbon sources for numerous biological applications, such as biopolymer production, bioenergy generation, and biological nutrient removal. Additionally, they find use in industries such as food additives, pharmaceuticals, cosmetics, and chemical manufacturing [20]. The distribution of VFAs depends on the dominant metabolic pathways, which are influenced by environmental factors like pH and temperature. Variations in these conditions drive the development of distinct microbial communities, leading to different fermentation profiles [21]. Depending on the main fermentation product, acidogenic fermentation can be categorized into types such as acetate-ethanol, propionate, butyrate, mixed-acid, or lactate fermentation.

2.2 Metabolic pathways for VFA production from organic wastes

Acetate can be formed either through the acetyl-CoA pathway or via the syntrophic oxidation of ethanol or long-chain fatty acids. Ethanol is produced by converting pyruvate into acetaldehyde through pyruvate decarboxylation, followed by the reduction of acetaldehyde to ethanol. During this process, acetone and butanol can also be produced by certain Clostridia species, such as *Clostridium acetobutylicum*, through acetone-butanol-ethanol (ABE) fermentation [22], [23]. The prevalence of acetate in acetate-ethanol type (AET) fermentation is strongly linked to the activity of the functional enzymes in the acetyl-CoA pathway and syntrophic oxidation (Figure 2) [22]. In Enterobacteriaceae, the conversion of pyruvate to ethanol involves three steps, with acetyl-CoA and acetaldehyde serving as intermediates [24]. However, in some other bacteria, this process is simplified to two key steps: pyruvate decarboxylation to acetaldehyde, followed by the reduction of acetaldehyde to ethanol [8]. Acetate production is significantly influenced by the digester's microenvironment, which includes factors such as substrate type, inoculum, pH, temperature, configuration, organic loading rate (OLR), hydraulic retention time (HRT), operational modes, and headspace H_2 pressure. The composition of food waste, particularly the proportions of carbohydrates, proteins, and lipids, plays a critical role in determining the distribution of fermentation products—for instance, a high carbohydrate content favors acetate production [25].

Propionate is generated via two different pathways. In the pyruvate catalyzation pathway, lactate is first produced by lactate dehydrogenase, and then lactate is reduced to propionate by propionate dehydrogenase. Alternatively, propionate can also be produced through the trans-carboxylate cycle carried out by acidogenic bacteria such as Corynebacteria, Propionibacterium and Bifidobacterium [26].

In the butyrate-type metabolic pathway, butyrate is produced through the reduction and decarboxylation of pyruvate, accompanied by the consumption of acetate. Butyrate is synthesized through the Embden–Meyerhof–Parnas (EMP) pathway, where glucose is converted to pyruvate, which is then transformed into butyryl-CoA, with intermediates including acetoacetyl-CoA, 3-hydroxybutyryl-CoA, and crotonyl-CoA. These intermediates are then catalyzed sequentially by thiolase, 3-hydroxybutyryl-CoA dehydrogenase, and butyryl-CoA dehydrogenase. Finally, butyryl-CoA is converted into butyrate by the enzymes phosphotransbutyrylase and butyrate kinase. During butyrate production, two molecules of $NADH_2$ are utilized to reduce the intermediates involved in the pathway. [22].

In lactic acid fermentation, glucose or other organic materials are converted to lactic acid by bacteria such as *Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus thermophilus*, etc. Pyruvate from glycolysis is converted into lactate by lactate dehydrogenase. Lactic acid production can be classified into two types: homolactic fermentation (where one mole of glucose is converted into two moles of lactate) and heterolactic fermentation (where acetate or ethanol are co-products alongside lactate), which occurs through the phosphoketolase and bifidus pathways [27], [28].

Mixed-acid fermentation is another pathway that can occur during acidogenic fermentation, where the microorganisms use two or more metabolic pathways to convert pyruvate into a variety of fermentation products. In this case, there is no dominant VFA in the fermentation products, though acetic acid, propionic acid, and butyric acid are generally present in relatively high concentrations, with other products like lactate and ethanol appearing in lower amounts [22], [29].

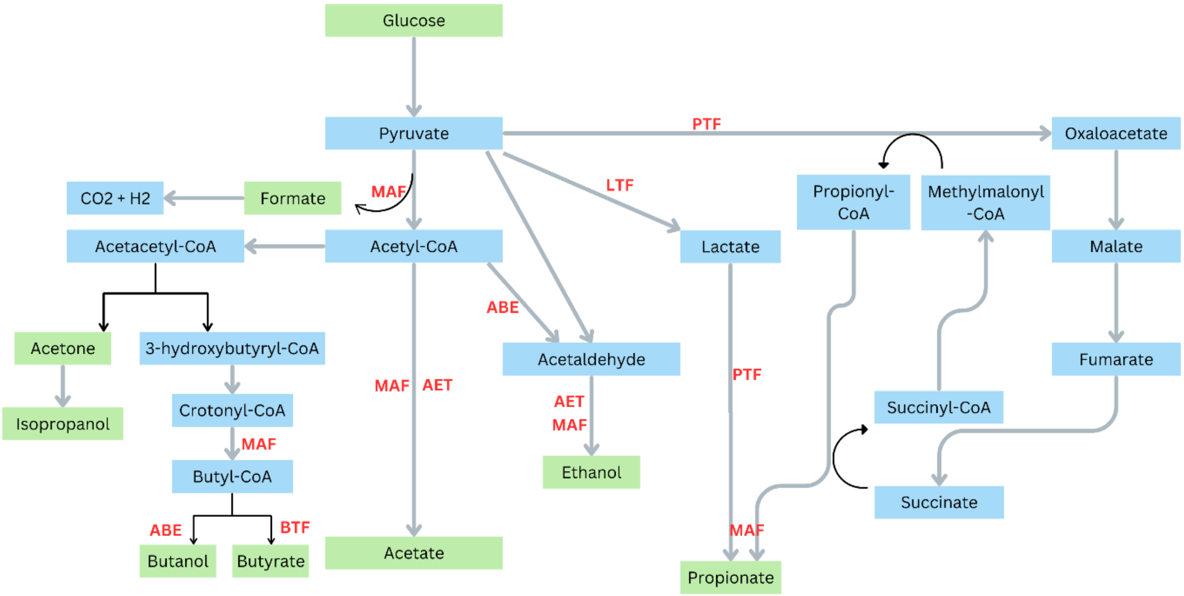

Figure 2. Key metabolic pathways of acidogenic fermentation – Acetate ethanol-type fermentation (AET), acetone-butanol-type fermentation (ABE), butyrate-type fermentation (BTF), lactate-type fermentation (LTF), propionate-type fermentation (PTF), mixed-acid fermentation (MAF).

2.3. Microbial Diversity in Acidogenic Fermentation

Complex organic wastes such as food waste, an inexpensive raw material, can undergo bioconversion into VFA, biohydrogen, and ethanol during hydrolysis and acidogenic stages of AD. This bioconversion requires the microbial cells to synthesize hydrolysing enzymes which can break the complex substrate chains into simpler molecules, e.g. carbohydrates, proteins, and fatty acids [30]. The fermentative bacteria include phyla Bacteroidetes, Proteobacteria, Actinobacteria, Chloroflexi, and Firmicutes [31], [32], [33]. Among fungal organisms, the dominant phyla include Ascomycota, Basidiomycota, and Rozellomycota. They play a crucial role in the degradation of carbohydrates and better adapt to harsh conditions than bacteria while reducing the limitations faced in the hydrolysis step [34]. The hydrolyzed simpler molecules are further assimilated by these microbes into the before-mentioned bioproducts, which can be digested to produce biogas or biomethane by acetoclastic methanogens such as *Methanosarcinales* and *Methanosaeta* and hydrogenotrophic methanogens like *Methanobacteriales*, *Methanomicrobium*, *Methanomassiliicoccales* [35], [36]. Any alteration in the microbiome of the reactor can affect these processes and similarly, any alteration in the operational or environmental conditions can affect the microbiome [32]. For instance, the accumulation of VFA negatively impacts the activity of methanogens [30] which is thus positive feedback for the acidogenic fermentation process by halting methanogenesis.

Acetogenic microorganisms like *Moorella thermoacetica*, *Clostridium formicaceticum*, *Clostridium aceticum*, *Acetobacterium woodii*, and *Thermoanaerobacter kivui* are capable of producing acetic acid exclusively from various hexoses and pentoses. Additionally, other key organisms involved in acetic acid production include *Streptococcus lactis*, *Clostridium thermoaceticum*, *Acetobacter pasteurianus*, *Acetobacter aceti*, *Acetobacterium wieringae*, *Acetomicrobium*, and *Gluconobacter* species [37]. In anaerobic environments, homoacetogens are obligate anaerobes that use H2 to reduce CO2 to acetate through the homoacetogenesis process [38]. They rely on CO2 and/or organic compounds for energy and carbon. In the autotrophic process, they consume H2 and CO2 to produce acetate via the Wood-Ljungdahl pathway, which has two branches: the methyl and carbonyl branches. The methyl branch reduces CO2 to formate, consuming reducing equivalents, while the carbonyl branch reduces CO2 to CO. Methyl groups and CO are combined to form Acetyl-CoA, which is then converted to acetate or used for cell growth. The process is energy-intensive, but in certain conditions, like acidic environments, low temperatures, or high hydrogen levels, homoacetogens can outcompete other acidogens [39].

Butyric acid can be produced by various anaerobic bacteria, including Clostridium species (especially *Clostridium tyrobutyricum*, *Clostridium butyricum*, *Clostridium acetobutylicum*, and *Clostridium beijerinckii*), *Butyrivibrio*, *Butyribacterium,* *Coprococcus*, *Eubacterium*, *Fusobacterium*, *Megasphaera*, *Roseburia*, and *Sarcina* [40]. During butyric acid fermentation, acetyl-CoA from a hexose is converted into either acetate or butyryl-CoA. Butyryl-CoA is then transformed into butyrate by two key enzymes, phosphotransbutyrylase and butyrate kinase, which are commonly found in butyrate-producing *Clostridia* [14], [41].

The microbial community responsible for propionic acid production includes propionibacteria, a group of gram-positive, anaerobic, rod-shaped bacteria in the *Actinobacteria* phylum. Key species of propionibacteria include *Propionibacterium freudenreichii* and *Propionibacterium shermanii*, which can produce acetic acid using enzymes such as phosphotransacetylase, acetate kinase, acetate-CoA ligase, or acetyl-CoA synthetase [42]. In the obligate anaerobe *Clostridium propionicum*, propionic acid is produced via the acrylic acid pathway. Other species of Propionibacterium involved in propionic acid production include *P. acidipropionici*, *P. thoenii*, and *P. jensenii* [43].

2.4 Effect of Different Operational Parameters on VFA production

The efficiency and yield of VFA production are strongly governed by operational parameters, which influence substrate hydrolysis, microbial metabolism, and the overall biochemical pathways involved in fermentation. Key factors such as substrate composition, pH, temperature, hydraulic retention time (HRT), organic loading rate (OLR), and microbial community structure play significant roles in directing metabolic fluxes toward VFA accumulation. For instance, pH is a critical regulator that determines whether acidogenic bacteria thrive, while temperature influences enzyme activity and microbial diversity. Likewise, the choice of substrate dictates the availability of fermentable carbon, which in turn affects VFA composition and distribution. Optimizing these parameters not only enhances VFA yield and selectivity but also provides insights into microbial ecology and metabolic shifts under different fermentation conditions. A systematic understanding of their effects is essential for designing efficient anaerobic fermentation systems that maximize VFA production. Table 1 enlists different studies on VFA production using food waste as a substrate, emphasising the operational conditions and the dominant microorganisms. This section delves into the influence of various operational parameters on VFA production, highlighting their mechanistic roles, interactions, and practical implications for process optimization.

Table 1. Studies utilising food waste as a substrate for producing VFAs

Substrate and Inoculum	**Operating conditions**	**VFA Yield**	**Order of VFA composition**	**Dominant microorganisms**	**Reference**
Household food waste and digestate wastewater	pH - 6, 15% total solids	20.5 g/L	Acetic acid > propionic acid > butyric acid > valeric acid		[44]
Household food waste and digested food waste	Temperature - 35ºC, pH -10, and retention time – 15 days	22±1 gCOD/L	Acetic acid > propionic acid > butyric acid > isovaleric acid > valeric acid > caproic acid	*Ruminococcaceae, Veillonellaceae* under *Firmicutes* phylum; *Anaerolineaceae* under *Chloroflexi* phylum	[45]
Household food waste (protein rich) and agricultural digestate	Temperature - 37ºC, pH 7, time – 5 days	13.7 gCOD/L	Butyric acid > isovaleric acid > acetic acid > isobutyric acid > propionic acid > caproic acid	*Lactobacillaceae, Enterobacteriaceae, Atopobiaceae*	[46]
Supermarket food waste and digested sludge	Temperature - 35ºC, pH – 7, organic loading rate – 10 gCOD/L/d	22 gCOD/L or 3.9 gCOD-VFA/L/d	Acetic acid > propionic acid > butyric acid > valeric acid > caproic acid		[47]
Household food waste (protein rich) and digested sludge	Temperature - 35ºC, I/S ratio – 0.3, uncontrolled pH and incubation time – 5 days	80 g/L	Acetic acid > valeric acid > propionic acid > butyric acid		[48]
Kitchen waste and digested sludge	Temperature - 37ºC, pH – 7, S/I ratio – 1:1,	22.3 g/L	Acetic acid > butyric acid > propionic acid	*Bacilli, Clostridia*	[49]

	retention time – 10 days, organic loading – 5 gVS/L/d				
Food waste	Temperature - 37ºC, S/I ratio – 3, uncontrolled pH, retention time – 10 days	18.8 g/L	Acetic acid > caproic acid > butyric acid > valeric acid > propionic acid > isovaleric acid	*Lactobacillus*	[50]
Canteen food waste and digested sludge	Temperature - 35ºC, pH 7, organic loading – 22 gVS/L	11.8 gCOD/L	Acetic acid > butyric acid > propionic acid	*Clostridia, Bacteroidia, Bacilli*	[51]
Household food waste	Temperature - 55ºC, pH 7, organic loading – 11 gVS/L/d	23.5 g/L or 0.38 gVFA per gVS fed	Butyric acid > caproic acid, propionic acid, acetic acid	*Clostridium, Lactobacillus*	[52]
Municipal food waste (carbohydrate rich)	Temperature - 50ºC, pH 6.5, and hydraulic retention time – 7 days	15 gCOD/L	Butyric acid > propionic acid > acetic acid > valeric aicd > isovaleric acid	*Clostridium sensu stricto 1, Sporanaerobacter, Proteiniphilum*	[53]

2.4.1 Temperature

Fermentations are generally categorized based on operating temperature into psychrophilic (<25 °C), mesophilic (25–45 °C), or thermophilic (>45 °C) conditions. Temperature plays a pivotal role in determining the production and composition of VFAs, as it directly influences microbial activity—particularly hydrolytic and acidogenic bacteria kinetics [24] as well as the efficiency of physical and chemical processes. A recent statistical analysis of 551 experiments identified a negative correlation between VFA yields and temperatures exceeding 55°C [54]. A study observed a significant drop in VFA production from 17 g/L to 11 g/L when the operating temperature was increased from 35 °C to 55 °C without altering the pH [55]. This decrease might be attributed to enhanced solubilization of organics at higher temperatures but a reduced acidogenesis rate [56]. This suggests that temperatures within the mesophilic to thermophilic range can promote microbial growth and organic solubilization, although mesophilic conditions are optimal for maximizing VFA yields. Additionally, specific VFAs can be preferentially produced at certain temperatures, as different microbial species and waste materials respond uniquely to temperature variations. Identifying the optimal temperature for specific microbial species is crucial since many acidogenic bacteria are unable to withstand extreme temperatures [57], [58]. While temperature can impact the type of VFA produced, current findings are inconsistent [59], likely due to limited understanding of the interactions between various parameters. For instance, temperature may influence ammonia release [56], complicating the isolation of its independent effects. Studies such as those by Garcia-Aguirre et al. observed that temperature did not significantly affect product distribution in the treatment of slaughterhouse wastewater and paper mill wastewater [60]. Similarly, Yu and Fang reported no notable impact of temperature on product distribution in protein-rich wastewater [61].

With mesophilic temperature conditions found to be optimal for VFA production, it is still an energy-intensive process. In cold countries such as Canada, the average ambient temperature is below 20°C. Thus, the energy expenditure for maintaining higher operational temperatures can be significant. At an industrial scale, VFAs have been produced through sludge fermentation at temperatures between 20-25°C for downstream applications like nitrogen removal from wastewater. However, research on acidogenic fermentation at temperatures below 30°C, or in psychrophilic ranges, is limited due to slow microbial activity and low VFA production. Despite this, operating at these lower temperatures can reduce capital and operational costs by 20-40% compared to thermophilic processes [62], [63]. Most studies reporting waste valorisation under psychrophilic temperatures have focused on biogas production, reporting VFA yields as intermediate metabolites. However, psychrophilic temperatures were shown to inherently decrease methanogenic conversion of the substrates, favouring persistence of VFAs in the system [64]. Additionally, the complex mixture of metabolites produced at mesophilic and thermophilic temperatures makes single VFA extraction more challenging. The persistence of VFA under low temperature conditions can thus support its downstream extraction. Temperature affects the metabolism of the acid-forming microorganisms at a biochemical level by altering the changeable shift between glycolysis and the pentose-phosphate pathway [65]. *Enterobacter* and Bacillus species have been observed to show increased pentose-phosphate pathway activity at lower temperatures and increased glycolysis activity at higher temperatures [66]. The slower metabolic rate extends the time required for the complete breakdown of organic matter in the food waste. Moreover, the active microbial diversity is limited which may require additional process optimization. Psychrophilic conditions can result in incomplete degradation along with the accumulation of other metabolites such as alcohols and lactic acid which can be inhibitory for eventual VFA generation. This happens because the syntrophy between hydrolytic and acidogenic bacteria gets affected when interspecies electron transfer gets affected [67]. Certain strategies such as pretreatment of substrate could be used to increase the rate of hydrolysis since it is known to promote substrate hydrolysis by increasing the surface area of food waste constituents for effective biodegradation and lowering the degree of polymerization [68], [69]. Thermophilic pretreatment has been validated in sludge treatment systems, where pretreatment increased SCOD and led to improved VFA yields, even when subsequent digestion was performed at lower temperatures [70].

2.4.2 pH

During fermentation, pH plays a critical role in hydrolysis and acidogenesis, making it essential to maintain an optimal range that supports both processes. pH significantly impacts microbial enzymatic activity during the hydrolytic and acidogenic stages of fermentation [24]. Specifically, pH variations alter microbial community composition, leading to changes in metabolite profiles [71]. Acidogenic bacteria thrive at a pH of 5.5–6.5, while methanogens are most active around pH 7.0. Maintaining the pH in the acidic range can effectively inhibit methanogens, promoting VFA accumulation [72]. Additionally, pH regulates the transport of undissociated acids across cell membranes, with acidic conditions requiring more energy for permeation and alkaline conditions facilitating energy gain [73]. The relationship between pH and the dissociation constant (pKa) of volatile fatty acids plays a critical role in governing their production and accumulation during acidogenic fermentation. The pKa of most short-chain VFAs, including acetic, propionic, and butyric acids, is approximately 4.8. When the system pH falls below this value, VFAs primarily exist in their undissociated form, which readily diffuses across cell membranes and can inhibit methanogenic and syntrophic activity, thereby promoting acid accumulation. Conversely, at pH values above the pKa, the acids remain largely dissociated as carboxylate ions, facilitating their solubility in the aqueous phase and enabling further conversion to methane or longer-chain fatty acids under favorable conditions. Therefore, the pH–pKa relationship determines not only the metabolic pathway preference (acidogenesis versus methanogenesis) but also the degree of VFA retention in the system [74]. A comprehensive study examining the effects of pH, ranging from acidic (pH 4–6) to alkaline (pH 8–11), found that the highest VFA concentration of 53.87 gCOD/L was achieved at pH 6 using food waste (FW) as a substrate [75]. Conversely, during the AD of spent mushroom compost, the maximum VFA concentration of 3.8 g/L was recorded at pH 10 when pH conditions ranged from 4 to 12 [76]. Across these studies, acetic acid consistently accounted for more than 50% of the total VFAs.

While substrate type largely determines the VFA composition, pH also significantly influences it during acidogenic fermentation [77]. For instance, Wang et al. found that during the anaerobic digestion of food waste, butyric acid was the dominant product (80%) at pH below 5.0 [78]. At a constant pH of 5.0, acetic acid was the major product, followed by butyric, propionic, and valeric acids [56]. Other studies reported that butyric acid was the primary VFA at pH 8.0, while propionic acid dominated at pH 6.5 [79], [80]. Begum et al. observed that at pH 5.5, acetic acid was the major VFA, followed by formic, butyric, and propionic acids, whereas an alkaline pH of 11 favored butyric acid production, followed by acetic, formic, and propionic acids [16]. Cheah et al., using organic fraction municipal solid waste (OFMSW) as a substrate, reported VFA concentrations exceeding 10 g/L at pH 9, with acetic acid comprising over 48% of the VFAs [81]. Similarly, in co-fermentation tests of sludge (SS) and artichokes, they observed that the highest VFA yield (0.36 gVFA/COD_{in}) occurred at alkaline pH 9, with a metabolic shift from butyrate-dominated pathways under acidic conditions (pH 5.5) to acetate-dominated pathways under alkaline conditions.

Li et al. tested pH conditions of 6, 7, and 8 and found the highest VFA concentration of 19.92 g/L at pH 8, characterized by a mixed-type fermentation where acetic and butyric acids collectively accounted for 86.4% [25]. Protein fermentation studies at pH 7 and 9 also showed a predominance of acetic acid, representing 55–60% for casein and 65–75% for gelatin [82]. Khatami et al. further highlighted that under acidic conditions (pH 5), the fraction of acetate decreased, while propionic and valeric acids increased. In contrast, at alkaline pH 10, acetate levels rose while caproic acid production was completely suppressed [83].

Table 2. Dominant VFA types under different ranges of pH and substrate conditions

pH Range	**Dominant VFA**	**Dominant phyla/genera**	**Substrate conditions**	**References**
<5.0	Acetic acid, butyric acid	*Clostridium, Lactobacillus, Bacteroides*	Food waste, protein-rich waste	[84]
5.0-6.5	Acetic acid, propionic/butyric acid	*Syntrophomonas, Treponema, Geobacter*	Food waste, organic fraction of municipal solid waste	[84]
6.5-7.5	Propionic acid	*Syntrophus, Desulfovibrio, Methanosaeta, Methanoculleus*	Organic fraction of municipal solid waste, sewage sludge	[84]
8-9	Mixed- Acetic and butyric acid	*Syntrophomonas, Desulfovibrio, Anaerostipes*	Sewage sludge, artichokes	[85]
9-10	Acetic and butyric acid	*Anaerobrance, Alkalibacterium, Oscillospira*	Alkaline co-fermentation	[86]
>10	Butyric and acetic acid	A*lkaliphilus, Thermovibrio*	Protein fermentation	[86]

Based on the reported studies, significant beneficial impact of alkaline pH on VFA production could be inferred. At elevated pH levels, specific fermentative bacteria and hydrolytic enzymes thrive, facilitating more efficient hydrolysis of complex organic matter, including recalcitrant materials like cellulose and proteins. This enhanced hydrolysis increases the pool of readily available substrates for acidogenesis, the stage where VFAs are generated. Simultaneously, alkaline conditions inhibit methanogenic bacteria, leading to a net accumulation of VFAs in the system. The enhanced buffering capacity provided by alkaline conditions further contributes to process stability, mitigating drastic pH drops caused by VFA accumulation and maintaining an environment conducive to microbial activity. However, this requires to be balanced against potential inhibitory effects: high pH increases the concentration of free ammonia, which can be toxic to VFA-producing bacteria. Moreover, the solubility and availability of essential nutrients, such as phosphates, may be reduced at alkaline pH, potentially limiting microbial growth.

Metagenomic insights into anaerobic fermentation, largely based on mesophilic systems, indicate that pH significantly shapes microbial functional gene expression (Appendix 9), providing scope for baseline interpretation under psychrophilic conditions (Figure 3). At acidic pH values (4.5–6.5), there is a clear enrichment of genes involved in acidogenesis and hydrolysis, including *ldh* (lactate dehydrogenase), *ackA/pta* (acetate kinase/phosphotransacetylase), and *hydA* (hydrogenase), aligning with the dominance of fermentative bacteria such as *Clostridium* and *Bacteroides* [87], [88]. Neutral pH (6.8–7.5) facilitates a more balanced microbial ecosystem where genes linked to syntrophic metabolism and acetoclastic methanogenesis such as *mcrA* (methanogenesis marker), *fhs* (formate metabolism), and *por* (pyruvate-ferredoxin oxidoreductase) become active, enabling conversion of VFAs to methane [85], [86]. In alkaline ranges (8–10), the system favors expression of redox-balancing and stress-adapted genes like *adhE* (alcohol dehydrogenase), *pfl* (pyruvate-formate lyase), and *narG* (nitrate reductase) [89], while extremely alkaline pH (>10) induces survival pathways through upregulation of stress proteins (*groEL, dnaK*) and alkaline enzymes like *phoA* [90]. Thus, strategic pH control in psychrophilic fermentation can serve as a lever to direct microbial gene activity toward targeted volatile fatty acid production.

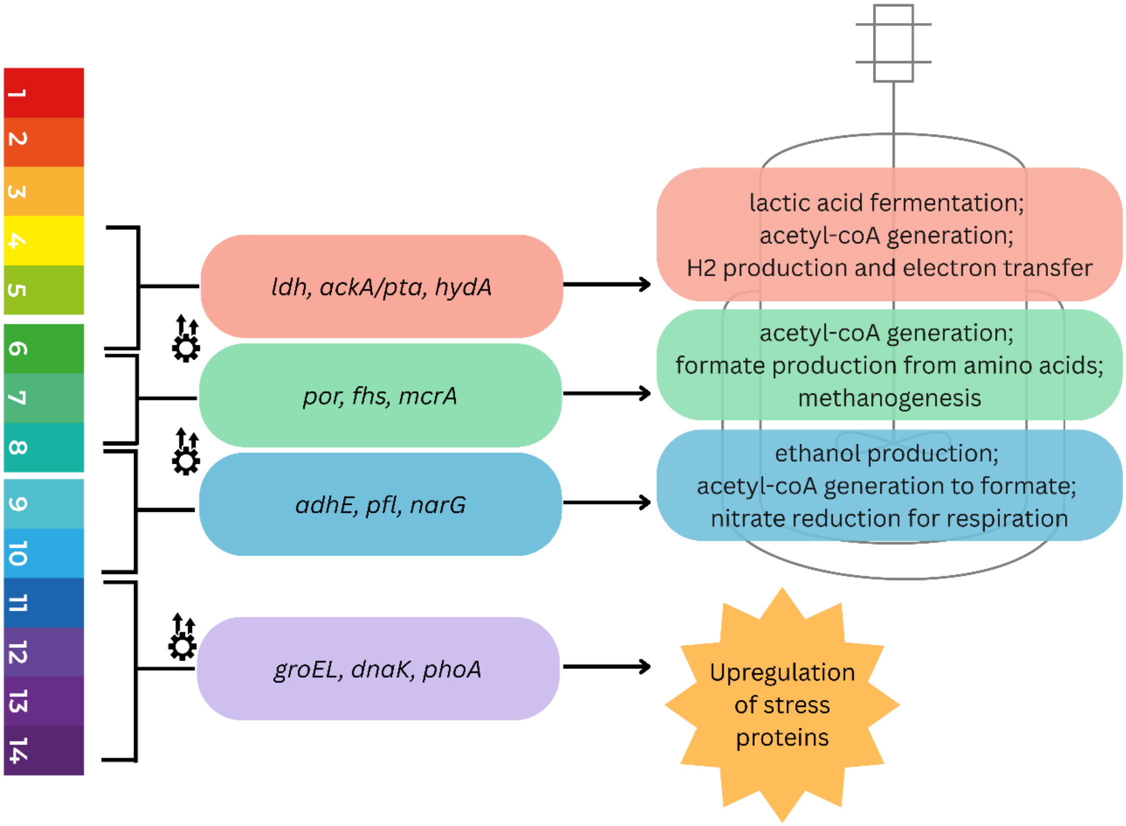

Figure 3. Effect of different pH values on regulating specific genes and their respective roles in fermentation mechanisms. The pH scale on the left shows different ranges of acidic, neutral, alkaline, and highly alkaline pH values. These four ranges correspond to their effect on gene groups. The gene group – *ldh, ackA/pta, and hydA* – are upregulated under acidic pH and their subsequent effect is enhanced in lactic acid fermentation, acetyl-coA generation, and hydrogen production and electron transfer respectively. Under a neutral pH range, the gene group – *por, fhs, mcrA* – are upregulated and promote acetyl-coA generation, amino acid-derived formate production, and methanogenesis respectively. The alkaline pH upregulates the gene group – *adhE, pfl, narG* – resulting in ethanol production, acetyl-CoA to formate conversion, and nitrate reduction respectively. Highly alkaline conditions induce a stress response by upregulating genes, *groEL, dnaK*, and *phoA*.

2.4.3 Substrate concentration

The organic loading rate (OLR) refers to the quantity of organic material, measured in volatile solids (VS), introduced per unit volume of the reactor per day. It determines the availability of substrates for fermentation, with higher OLRs providing more substrates, potentially benefiting VFA production. However, excessively high OLRs can cause the reactor medium to become highly viscous, leading to process instability [91]. For anaerobic digestion (AD), recommended OLR values typically range from 2 to 7 g VS L^{-1} day^{-1} [92], [93]. Exceeding these values can inhibit methane production and favor acidogenesis. When the OLR surpasses the AD threshold of 7 g VS L^{-1} day^{-1}, higher VFA concentrations are observed, though yields tend to decrease [94]. Studies have shown that VFA production increases with OLR up to a certain threshold, beyond which it gradually declines. This indicates the existence of an optimal OLR for maximum VFA production [95]. Therefore, an optimal balance between VFA yield and concentration is desirable. Using dry substrates with a total solids content of approximately 20% can slow methanogen activity and enhance VFA production, although this approach may result in lower VS destruction [96]. In moderate OLRs (2-5 gVS/L/day), microbial diversity tends to be higher with fermentative bacteria expressing genes like *ldh* and *ackA* [97]. Functional metagenomics reveals that at high OLRs, bacteria express stress-related and acid-tolerance genes, including *dnaK*, *groEL*, and *phoA*, alongside upregulation of formate and lactate fermentation pathways (*pfl, adhE*) [98], [99]. Additionally, OLR influences the composition of VFAs, with lower OLRs favouring the production of propionic and butyric acids, while higher OLRs lead to increased production of acetic and valeric acids [56].

OLR plays a critical role in governing the metabolic pathways activated during fermentation, as different microbial consortia respond uniquely to varying substrate availabilities. At moderate OLRs, acidogenic bacteria thrive and efficiently convert complex organic compounds into VFAs, while methanogenic archaea remain suppressed due to suboptimal conditions for methane production in instances where no measures are taken for arresting methanogens. However, excessively high OLRs can lead to reactor overloading, causing an accumulation of intermediate metabolites such as lactic acid and ethanol, which inhibit further acidogenesis and shift the metabolic balance towards solventogenesis rather than VFA production. Additionally, high OLRs can lead to acid accumulation and pH fluctuations, which disrupt microbial homeostasis and potentially trigger process failure [82].

Additionally, OLR influences the composition of VFAs, with lower OLRs favoring the production of propionic and butyric acids, while higher OLRs lead to increased production of acetic and valeric acids [56]. This shift in VFA profile is attributed to differences in microbial metabolic pathways. Lower OLR conditions tend to favor the activity of propionibacteria and butyrate-producing bacteria, which preferentially metabolize carbohydrates and proteins into propionic and butyric acids through the methylmalonyl-CoA and butyrate fermentation pathways, respectively. In contrast, at higher OLRs, the predominance of acetogenic bacteria, such as Clostridium spp., leads to increased acetate and valerate formation through the Wood-Ljungdahl pathway and β-oxidation of longer-chain fatty acids [83]. These compositional shifts are crucial when targeting specific VFAs for industrial applications, as acetate and butyrate are preferred for bioenergy and biochemical production, while propionate is valuable in food preservation and pharmaceutical industries.

The impact of OLR on VFA production efficiency is also influenced by factors such as reactor configuration, hydraulic retention time (HRT), and substrate composition. Continuous stirred-tank reactors (CSTRs) tend to tolerate higher OLRs due to their effective mixing and substrate homogenization, while plug-flow and batch reactors may experience localized substrate overloading and uneven microbial distribution. Similarly, adjusting HRT in response to OLR variations can help maintain process stability by allowing sufficient time for microbial adaptation and substrate conversion. Studies have shown that decreasing HRT at higher OLRs can counteract acid accumulation by promoting faster substrate turnover and preventing prolonged exposure to inhibitory conditions [84].

2.4.4 Retention time

Hydraulic retention time (HRT) is a critical factor in acidogenic fermentation Short HRTs, typically less than 10 days, are favored as they help eliminate slower-growing methanogens [92], [93]. However, for solid waste substrates, shorter retention times can lead to reduced yields since hydrolysis is often the rate-limiting step [100]. According to Pant et al., a minimum HRT of 3 days is necessary to achieve optimal conversion during the fermentation process [101]. In batch operations, peak VFA concentrations are generally reached within 4 to 9 days, suggesting that relatively short retention times are sufficient for effective fermentation.

One study found that the concentration of VFAs increased when the hydraulic retention time (HRT) was extended from 1 day to 2 days during co-digestion of mixed waste. However, further increasing the HRT to 3 or 4 days did not significantly improve VFA concentration [29]. Specific acid production can be influenced by HRT, as longer retention times allow slower-growing organisms to dominate, while faster-growing microbes are washed out at shorter HRTs. For example, in whey fermentation, propionic acid production increased as the HRT was extended from 20 hours to 95 hours, whereas butyric acid production was suppressed [102]. In contrast, co-fermentation of waste active sludge and fruit/vegetable waste showed little change in the proportion of VFAs within an HRT range of 1 to 4 days [103].

The relationship between HRT and microbial activity is particularly important when optimizing VFA production. Shorter HRTs not only suppress methanogens but also encourage acidogenic bacteria to proliferate rapidly, leading to enhanced VFA accumulation. However, extremely short HRTs (<1 day) may limit substrate availability for microbial metabolism, causing incomplete hydrolysis and fermentation inefficiencies. Conversely, prolonged HRTs (>10 days) can facilitate further microbial processing of VFAs into secondary metabolites, thereby reducing net VFA yields. This trade-off highlights the need for precise control over HRT to maintain favorable conditions for acidogenesis while preventing excessive conversion of VFAs into methane or other undesirable byproducts [86].

HRT also plays a crucial role in determining the type of VFAs produced. For instance, shorter HRTs (1–3 days) tend to favor the production of acetic and butyric acids, which are intermediates in metabolic pathways such as glycolysis and the butyrate fermentation route. In contrast, longer HRTs allow for extended microbial processing, shifting the balance towards propionic and valeric acid production. This shift is attributed to slower metabolic pathways such as the methylmalonyl-CoA pathway, which require more time for completion [87]. The impact of HRT on VFA profiles is particularly relevant in applications where specific VFAs are desired, such as acetic acid for bioplastics or butyric acid for biofuel precursors.

The impact of HRT is also substrate-dependent. In high-protein waste streams, extended HRTs can lead to the accumulation of ammonia due to amino acid deamination, which can be inhibitory to acidogenic bacteria. Similarly, in carbohydrate-rich feedstocks, prolonged HRTs can increase lactic acid accumulation, which alters fermentation pathways and may negatively affect VFA yields [88]. Optimizing HRT for different feedstocks requires careful consideration of both microbial dynamics and substrate composition. From an operational perspective, HRT optimization is highly dependent on reactor configuration. In continuous stirred-tank reactors (CSTRs), HRT is directly linked to the reactor volume and influent flow rate, meaning that adjusting HRT requires modifications in system throughput. In contrast, plug-flow and sequencing batch reactors (SBRs) offer more flexibility in HRT control, allowing for periodic adjustments based on process monitoring. Hybrid systems that integrate multiple reactor types may provide additional advantages by enabling tailored HRT strategies to maximize VFA yields while maintaining system stability [89].

2.5. Pretreatment of substrate for enhanced hydrolysis

Achieving effective substrate hydrolysis under psychrophilic temperatures poses challenges due to reduced microbial activity and slower biochemical reactions. Pretreatment of the substrate could be used to increase the rate of hydrolysis since it is known to promote substrate hydrolysis and solubilization by increasing the surface area of substrate constituents for effective biodegradation and lowering the degree of polymerization [69]. This, in turn, can increase VFA production by increasing the availability of simpler or smaller-sized molecules in the aqueous phase in less process time. However, studies focusing on VFAs using pretreatment are scarce. The pretreatment studies for anaerobic digestion could be used to infer the effect on VFA, though these are not optimized for VFAs. Pre-treatment technologies are employed for various purposes, including the production of methane (CH4), hydrogen (H2), glucose (C6H12O6), fermentable soluble sugars, and bioethanol. Pretreatment strategies can be classified as mechanical, physical, chemical, physiochemical, and biological.

2.5.1 Mechanical methods

Reducing particle size through processes like milling, grinding, and chipping enhances surface area, thereby improving substrate solubility and biodegradability. In anaerobic digestion (AD), finer particle sizes can hasten volatile fatty acid (VFA) production, as shown by studies indicating that smaller particles (e.g., 0.4 mm) promote acetic acid formation, whereas larger particles (e.g., 0.9 mm) favor butyric acid production [104], [105], [106]. Mechanical pretreatment offers the benefits of low energy requirements for dry feedstocks, ease of implementation, and improved dewaterability. However, it is less effective for breaking down lignin, does not aid in pathogen removal [104], and involves significant equipment maintenance costs [105].

One of the mechanical methods – crashing method - reduces food waste (FW) particle size, facilitating microbial degradation of organic solids for enhanced gas production. Agyeman and Tao observed that reducing FW to a particle size of 2.5 mm significantly improved digestate dewaterability and increased methane production rates [107]. Similarly, crashing FW to ≤30 mm particle size enhanced methane production by 30% and improved process stability [108]. This method is also widely applied in the anaerobic digestion (AD) of other substrates, including agricultural and animal wastes, due to its effectiveness in reducing particle size [109].

2.5.2 Chemical methods

Acid treatment primarily hydrolyzes hemicellulose, improving substrate digestibility and enhancing yields of hydrogen and volatile fatty acids (VFAs), particularly for protein-rich wastes. For example, using hydrochloric acid increased VFA yields from waste-activated sludge (WAS) by 153%, while free nitrous acid achieved a 370% increase [110], [111]. This method is particularly effective compared to other pretreatments for protein-rich substrates [104], [105]. Acid pre-treatment of food waste (FW) has been conducted using HCl and H2SO4, with H2SO4 concentrations ranging from 0.1 to 1 M and HCl at 3 M. Optimal pH values for this process are typically between 1 and 4, as lower pH levels can lead to the formation of toxic compounds and inhibitors that disrupt FW management processes like anaerobic digestion (AD). Zhang et al., pre-treated FW using HCl to achieve a pH of 1.0 for 24 hours, followed by batch anaerobic fermentation at 108 rpm and 37°C, significantly enhancing butanol production. Similarly, treating FW with 98% (w/w) H_2SO_4 at pH 1 before anaerobic fermentation increased hydrogen production by 62.8% [112], [113], [114].

Alkali treatment targets lignocellulosic materials by dissolving lignin, increasing substrate solubility and buffering capacity, which helps maintain stable pH during acidogenesis. While studies focusing on VFA production are limited, alkaline pretreatment has shown promising results. For instance, a 19% solubilization rate of lignocellulosic feedstocks led to over a 40% increase in hydrogen production [115]. Optimizing the alkali concentration is crucial in pre-treatment, as excessive Na+ and K+ cations can inhibit microbial growth and cause toxicity in subsequent processes, while insufficient concentrations may fail to achieve the desired pretreatment outcomes. Studies have utilized NaOH concentrations ranging from 0.1 to 3 M at pH levels of 8 to 12, with varying exposure times and temperatures depending on the characteristics of the food waste (FW) and the process objectives [116]. These conditions have resulted in differing levels of improvement in methane or hydrogen production. NaOH pretreatment increased acetic and butyric acid production sixfold, although maximum VFA concentrations remained under 2 g/L [117]. In primary sludge, using sodium carbonate (Na2CO3) quadrupled VFA yields, with the success attributed to an initial pH of 10 and the breakdown of sludge flocs [118].

Ozonation employs ozone (O3) to oxidize and break down feedstocks, effectively delignifying substrates and sterilizing materials by damaging microbial cell walls. It is environmentally friendly, as ozone decomposes into oxygen, but it is energy-intensive, requiring approximately 12 kWh per kilogram of O3 produced [119], [120]. While ozonation increased hydrogen production by 158% for lignocellulosic wastes [121], it negatively impacted the dark fermentation of food waste by degrading proteins and carbohydrates [122].

2.5.3 Thermal methods

Thermal treatment enhances the hydrolysis phase of acidogenic fermentation by altering the structure of insoluble fractions, lowering viscosity, and increasing soluble chemical oxygen demand (sCOD). This adjustment favors acidogenesis while suppressing methanogens, making it ideal for volatile fatty acid (VFA) production [123]. Thermal pre-treatment of food waste (FW) was initially introduced as a conditioning process to enhance digestion [124]. This method relies on temperature as a key operating factor to accelerate the solubilization of FW compounds [125]. A wide temperature range, typically between 50 and 220°C, has been shown to improve the bioavailability of soluble organic substances, with exposure times ranging from 5 minutes to 48 hours [126].

Ali et al. observed that thermal pre-treatment increased propionic acid production from food waste (FW) by 38%. However, they noted that this method is not commercially viable for large-scale waste streams [127]. For waste-activated sludge, thermal treatment at 100 °C for 60 minutes resulted in a 680% increase in VFA yield at a fermentation pH of 9, whereas neutral pH yielded a smaller increase of approximately 300% [78]. However, the lack of control pH data makes it difficult to attribute these increases solely to the pretreatment method. Food waste exhibited a more modest improvement (~55%), but combining thermal treatment with enzymatic or pre-fermentation methods enhanced yields by 380% and 200%, respectively [79], [128].

Microwave irradiation integrates thermal and non-thermal effects by disrupting crystalline structures while heating the aqueous environment, effectively enhancing solubilization. However, this approach is energy-intensive and costly. For sludge, microwave treatment increased hydrogen production by 66% [129]. When combined with alkaline treatment, microwave irradiation achieved a 30% increase in solubilization and a 400% boost in VFA and hydrogen production from lignocellulosic waste [130]. Ortigueira et al. reported that microwaving FW has accelerated the H2 production rate by 62.8% [131].

Ultrasound is frequently identified as one of the most effective physical pretreatment techniques [125], [132]. Ultrasonication pre-treatment of food waste (FW) involves using sound energy to agitate and break down particles, enhancing the solubilization of the matrix. It combines physical and chemical degradation, utilizing cavitational bubble collapse and free radical generation to break down substrates. Ultrasound can enhance enzyme activity or promote enzyme production, depending on the application. However, the high energy demand and maintenance costs are significant limitations [105]. For waste-activated sludge, ultrasound pretreatment increased sCOD 28-fold, significantly boosting acidogenesis. Applied to food waste, ultrasound achieved a disintegration degree of 57% and a maximum VFA yield of 0.98 g COD/g VS [133]. This improves the digestion stability of FW and optimizes the overall process. Li et al. reported that ultrasonication significantly increased interactions with organic matter and enzymes by over 10%, highlighting its crucial role in maximizing waste utilization [125].

2.5.4 Physiochemical methods

Thermochemical treatment combines heat with chemical agents to improve substrate solubilization. For vegetable waste, using 1% sulfuric acid and autoclaving at 121 °C for 15 minutes increased solubilization 4.7-fold, yielding 0.62 g VFA per gram of reducing sugars at pH 6 [134]. However, a comparison to untreated substrates was not provided. Pretreatment with diluted nitric acid on lignocellulosic waste like corn stover showed partial success, acidifying less than 10% of soluble sugars [135].

Ionic liquids (ILs) dissolve cellulose or extract lignin, enhancing substrate biodegradability. This physiochemical method typically operates at 80–180 °C and has been extensively studied for bioethanol production from lignocellulosic materials [136]. For anaerobic digestion, IL pretreatment has improved biogas yields by 64–140% from lignocellulosic substrates [137]. However, in some cases, inhibitory compounds like melanoidins and n-derivative amides have negated benefits [138]. While there is potential for ILs to improve VFA production, direct studies are needed to confirm their efficacy.

2.5.5 Biological methods

Biological pre-treatment is an environmentally friendly method that avoids harmful environmental impacts, high investment costs, and energy consumption [125]. It uses biological agents to degrade food waste (FW), facilitating enzymatic hydrolysis—an advantage not offered by physical or chemical pre-treatment methods. Unlike other methods, biological pre-treatment does not require high temperatures, pressure, acids, alkalis, or reactive chemicals. However, it has a longer processing time. This approach includes enzymes (e.g., protease, amylase, Viscozyme, Flavourzyme, S. cerevisiae KA4, and Palatase) and fungi (e.g., *Aspergillus awamori*, *Aspergillus oryzae*, and *Monascus*). Enzymes primarily break down proteins and carbohydrates into amino acids and monosugars, while fungal pre-treatment targets the decomposition of complex FW compounds [139].

Fungi are adept at breaking down substrates not easily degraded by fermentation or anaerobic digestion species. Although fungal pretreatment for AF has not been explored, enzymes tailored to specific substrates are an alternative. For example, proteases like trypsin are effective for hydrolyzing protein-rich materials but can harm acidogenic bacteria by degrading their proteins (placido & zhang 2018). As a result, enzymatic pretreatment is generally discouraged for variable feedstocks like organic fraction municipal solid waste (OFMSW).

Enzymatic pretreatment can either precede or occur during AD/AF. However, when done concurrently, enzyme activity may decline due to endogenous proteases from AD microbes (Odnell 2016). Despite this limitation, biological treatments are advantageous because they are eco-friendly and do not generate additional waste streams, as enzymes and biological agents naturally degrade during fermentation [104], [105].

2.6. Tailoring microbial diversity for enhancing VFA production

Tailoring VFA production toward specific metabolites can substantially enhance the economic and functional value of fermentation processes. Among the different VFAs, butyric acid was selected as the principal target in this study owing to its dual significance as both a metabolic indicator of efficient carbon conversion and a high-value platform compound. Butyrate is a key precursor for biofuel (butanol) and bioplastic synthesis, and it can be further elongated to medium-chain fatty acids such as caproate, offering greater market potential than acetate or propionate [140], [141]. From a biochemical standpoint, the butyryl-CoA pathway maintains redox balance and energy conservation under anaerobic and psychrophilic conditions, making butyrate formation a thermodynamically favorable end point [142]. Focusing on butyric acid therefore enables a more targeted, energetically efficient, and value-driven approach to optimizing acidogenic fermentation.

2.6.1 Application of biosurfactants

To enhance the hydrolysis step, different pretreatment methods are utilized for the substrate such as thermal, chemical, ultrasonic etc. However, with respect to VFAs, the use of biosurfactants enhances organic solubilization and VFA accumulation in substrates such as primary and waste-activated sludge [143]. A study by Johnravindar et al., showed enhanced hydrolysis and acidogenesis in anaerobic digestion with increasing rhamnolipid dosages from 0.02 to 0.25 g/g TSS, with a maximum VFA concentration achieved at 9.53±0.53 g/L at the highest dosage. This was a more than three-fold increase compared to the control [144]. Using waste-activated sludge as substrate, Li et al. also observed a nearly three-fold increase in VFA production and accumulation at 2.6±0.3 g/L using rhamnolipids dosage of 0.04 g/g TSS [143]. A combined effect of rhamnolipids (0.003 g/g SS), alkali conditions (pH 10), and microwave on disintegration of dairy waste activated sludge showed a 55% increase in the SCOD [145]. Another similar study by He et al observed the positive combined effect of rhamnolipids (0.2 g/g TSS) and alkaline pH 10, yielding 4-fold increase in total VFA [146]. There are various other studies employing rhamnolipids in the mesophilic digestion of sludge, concluding at least a 3-fold increase in VFA generation [147], [148]. Other biosurfactants, such as surfactin and saponin have also been studied for their effect on solubilization enhancement in waste activated sludge. One such study by Huang et al., observed a 4-fold increase in VFA production at surfactin's dosage of 0.05 g/g of dissolved solids, while 0.10 g/g dosage of saponin. A combination of biosurfactants (saponins, surfactin, rhamnolipids) with alkaline conditions at pH 9 improved the activity of hydrolase enzyme, inhibited methane formation and increased VFA production. The saponin and use of pH 9 had the least negative effect on the metabolic potential and diversity. Generally, the degradation of polysaccharides and proteins is faster than fats due to the additional requirement of emulsification for them to be broken down as well as lipases that are not as abundant as glycosidases and proteases. Rhamnolipids essentially solubilize hydrophobic compounds and thus increase their solubility for uptake by microorganisms, thus assisting with the degradation of all three macro constituents of the substrate. The fat content in sludge ranges between 5-10%, while in food waste, it ranges between 10-30%, making food waste a richer substrate for fat solubilization by rhamnolipids, and offers more potential for enhanced microbial uptake and subsequent conversion into VFAs during fermentation. A study by Wang et al [149], studied the effect of rhamnolipid in treating mariculture solid waste. They also observed the enhanced accumulation of acetic acid with rhamnolipid treatment from 50% in control to 70% in treated set. Another mechanism of rhamnolipid's positive effect is due to protection of enzymes from metal ions via micelles formation [150]. Wang et al [149], also observed the difference in microbial diversity with rhamnolipid treatment and found similar increase in Firmicutes from 5.8% to 41.7%. However, Proteobacteria were reduced from 28.85 to 10.7%, which are known for enrichment of acetic, propionic and valeric acid. The metabolic pathways related to VFA synthesis (specifically lipid and amino acid metabolism) were observed to have been enriched during fermentation with rhamnolipids. The genes encoding glutamate, aspartate, L-cystine transporters (for transport of extracellular protein) were found to be enhanced by 9.4 times than control, while genes associated with carbohydrate transport also showed an increased expression.

2.6.2 Bioaugmentation to Support Microbial Enrichment for Targeted VFA Production

Bioaugmentation, the process of introducing specific microbial strains into a system, is an effective strategy to enhance volatile fatty acid (VFA) production. It can be particularly valuable under psychrophilic conditions (≤20°C), where lower temperatures typically reduce microbial activity, slowing down hydrolysis and acidogenesis—the key steps in VFA production. By introducing psychrotolerant or acidogenic microorganisms, bioaugmentation can overcome these limitations by enhancing enzymatic activity and shifting the microbial community towards acidogenesis, thereby improving substrate utilization and VFA yields.

For example, species like *Clostridium butyricum* and *Clostridium tyrobutyricum* are notable for their ability to sustain metabolic activity under a broad range of temperature from 8-40ºC. Ortigueira et al., observed improved butyric acid production along with hydrogen after bioaugmentation, reaching a peak of 2.5 g/L concentration, albeit under mesophilic temperatures [151]. Similarly, Zhou et al. (2018) highlighted that the introduction of cold-adapted acidogenic microbes improved substrate hydrolysis by producing cold-active enzymes, which addressed the bottleneck of reduced hydrolysis rates under psychrophilic conditions [24]. Study by Atasoy et al., assessed bioaugmentation as a strategy for tailored production of VFA [152]. *Propionibacterium acidipropionici* was used to bioaugment mixed microbial cultures in anaerobic sequencing batch reactors treating cheese wastewater under alkaline pH. Bioaugmentation increased propionic acid production nearly fourfold (3779 ± 201 mgCODeq/L in the bioaugmented reactor vs. 942 ± 172 mgCODeq/L in the control) without significantly altering VFA composition. The gene copy number of *P. acidipropionici* increased 20-fold and positively correlated with total VFA and isovaleric acid concentrations. Additionally, the abundance of *Flavobacteriaceae* increased, likely due to syntrophic interactions with *P. acidipropionici*. Another study investigated bioaugmentation with homoacetogenic bacteria to enhance volatile fatty acid (VFA) production during lignocellulose fermentation. Methanogenesis in wet-exploded corn stover fermentation was inhibited using 10 mM 2-bromoethanesulfonate (BES), which reduced acetic acid yield by 24% but increased headspace hydrogen from 1% to 60%. Bioaugmentation with *Acetitomaculum ruminis* and *Acetobacterium woodii* resulted in hydrogen consumption and increased acetic acid production by 45% and 70%, respectively.

One of the critical benefits of bioaugmentation is its potential to alter the microbial community structure. By selectively increasing the population of acidogenic bacteria, bioaugmentation can suppress competing methanogenic pathways, leading to higher VFA accumulation. Bioaugmentation could effectively balance microbial consortia by fostering acidogenic bacteria while minimizing methanogen activity, ensuring VFAs are the predominant metabolites. Furthermore, bioaugmentation can optimize specific VFA profiles by selecting strains with metabolic pathways tailored to produce specific VFAs, such as acetic or butyric acids. Correlation analysis of *Clostridium butyricum* and individual VFAs performed by Atasoy et al., showed the enhancement of acetic, butyric and valeric acid in their study by a syntrophic relationship [153]. Such syntrophy is likely mediated through metabolite cross-feeding, where hydrolytic and fermentative bacteria produce short-chain substrates (e.g., lactate, ethanol, or acetate) that *C. butyricum* can further convert into butyric acid.

Operational parameters such as inoculum dosage, timing of augmentation, and the substrate's compatibility with the bioaugmented strains significantly influence the success of this strategy. For instance, bioaugmentation with *C. tyrobutyricum* at the onset of fermentation of lignocellulosic biomass led to enhanced butyric acid production due to its competitive growth advantage over native microbes during the early acidogenesis phase [154]. However, timing must be carefully optimized; adding strains too early or late may result in poor integration or reduced efficacy due to unfavorable community dynamics.

Despite its promise, bioaugmentation faces challenges in practical applications. Ensuring the survival, activity, and dominance of the introduced strains in complex microbial communities remains a critical hurdle. Additionally, the cost of culturing and maintaining specific microbial strains at scale can be significant. Addressing these challenges involves optimizing inoculum size, improving the resilience of bioaugmented strains, and exploring synthetic consortia that mimic naturally occurring microbial interactions.

Future advancements in synthetic biology and microbial engineering could further refine bioaugmentation for psychrophilic VFA production. Engineered microbes could be tailored for specific substrates, environmental conditions, or desired VFA profiles. Coupled with metagenomic and metabolomic analyses, these approaches can provide insights into microbial interactions, enabling the design of more effective bioaugmentation strategies. As global interest in sustainable waste-to-resource technologies grows, bioaugmentation offers a promising avenue to maximize the efficiency of psychrophilic fermentation systems for VFA production.

2.7 Extraction methods of VFA

The extraction of VFA from complex matrices requires specialized techniques to ensure efficient recovery and high purity due to their volatile nature and high solubility in water. The choice of extraction method depends on multiple factors, such as the polarity and volatility of VFAs, the composition of the matrix (solid, liquid, or gas), the required concentration and purity levels, and whether the scale of operation. There are several methods available for extracting VFAs, including solvent-based, adsorption-based, membrane-based, and electrochemical techniques. Each method varies in efficiency, selectivity, scalability, and cost-effectiveness (Table 3).

2.7.1 Solvent-based extraction methods

Liquid-liquid extraction is a traditional and effective method for separating VFAs based on their solubility differences between two immiscible liquid phases. Typically, the aqueous solution containing VFAs is mixed with an organic solvent, such as trioctylamine, tributylphosphate, octanol [155], diethyl ether [156], chloroform, ethyl butyrate [157], and dodecane [158]. As the VFAs partition into the solvent phase, the two phases are separated, usually through the use of a separatory funnel or centrifugation. The organic phase is then subjected to solvent evaporation to recover the extracted VFAs.

Solid-phase extraction is another solvent-based method that utilizes a solid sorbent to selectively retain VFAs while allowing other unwanted components to be eluted. In this process, the sample is loaded onto a column packed with a suitable sorbent, such as C18 silica [159], polymeric resins like Oasis HLB [160] and Strata-X [161], or anion-exchange resins. The VFAs adsorb onto the sorbent, while non-target compounds are washed away. Subsequently, VFAs are desorbed using a solvent such as methanol or acetonitrile.

2.7.2 Adsorption-based extraction methods

Ion-exchange resins are commonly used for the selective adsorption of VFAs. These resins work by exchanging ions with VFAs in solution, effectively concentrating them on the resin surface. Anion-exchange resins, such as Amberlite IRA-67 [162] and Dowex 1X8 [163], are particularly effective for extracting negatively charged VFAs, whereas cation-exchange resins like Amberlite IR-120 [164] can be used under certain conditions. The bound VFAs are eluted using a regenerating agent, typically a strong acid or base, such as NaOH or HCl. Activated carbon is another effective adsorbent for extracting VFAs. It utilizes hydrophobic interactions and π-π stacking to capture VFAs from aqueous solutions. In this process, the sample is passed through an activated carbon column, allowing VFAs to be adsorbed onto the porous surface. The adsorbed VFAs are then desorbed using an organic solvent [165].

2.7.3 Membrane-based extraction methods

Membrane-based methods rely on diffusion through a membrane to achieve separation. Membrane contactors use hydrophobic membranes to separate VFAs from aqueous solutions into an organic phase. The process involves passing an aqueous solution containing VFAs through one side of the membrane, while an organic solvent is circulated on the opposite side. VFAs diffuse across the membrane and accumulate in the organic phase. This method allows continuous operation and prevents emulsification issues associated with traditional solvent extraction techniques [166]. Another technique called pervaporation removes VFA from aqueous solutions by phase transition. VFAs permeate a selective membrane as vapor under vacuum conditions, after which the vapor is condensed and collected [167].

2.7.4 Electrochemical extraction methods

Electrochemical techniques leverage the charge properties of VFAs for their selective extraction. Electrodialysis employs ion-exchange membranes to selectively transport VFAs under an electric field. The process involves the movement of VFAs through anion-selective and cation-selective membranes, concentrating them in separate compartments [167]. It is one of the most advanced techniques for VFA recovery and is widely used in wastewater treatment and biorefinery applications.

Table 3: Comparison between different VFA extraction methods

Method	**Efficiency**	**Selectivity**	**Scalability**	**Limitation**
Liquid-liquid extraction	High	Medium	High	Solvent toxicity
Solid-phase extraction	High	High	Medium	Small sample volume
Ion-exchange resins	High	High	Medium	pH-sensitive
Membrane contactors	High	High	High	Membrane fouling
Electrodialysis	Very high	High	High	High costs

2.8 Applications of VFA

The generated VFA, both individually and in mixed-form have a range applications across different industries. Acetic acid is widely used in several industries, including the polymer, chemical, electronics, and food sectors. A major global application of acetic acid is in the production of terephthalic acid, which is essential for manufacturing polyethylene terephthalate used in packaging, fibers, clothing, plastic bottles, and films. Acetic acid is also a key component in the formation of acetate esters, which serve as solvents for inks, paints, and coatings. In the food industry, acetic acid is used as a solvent and in product preparation. It is the main compound in vinegar, which contains 5–20% acetic acid, and functions as a preservative, acidity regulator, and flavoring agent [2], [168], [169].

Propionic acid is used as a building block chemical in various industries, a preservative in the food sector, and as a component in animal feed and grain preservation. It is also used as a flavoring agent, ester, and herbicide, and plays a role in plastics and petrochemical production. Additionally, propionic acid is utilized in the pharmaceutical industry. The C4 VFA, butyric acid and its derivatives, have various applications in the food, pharmaceutical, perfume, and polymer industries [2], [26], [42]. Butyric acid is also used as a precursor for biofuels, such as ethyl and butyl butyrate. Its derivatives include salts and esters, with potassium butyrate, calcium butyrate, and magnesium butyrate being the primary salts, alongside sodium butyrate. Among these, sodium and calcium butyrate are in high demand, particularly for use in animal feed products. In the polymer industry, butyric acid is used to synthesize cellulose acetate butyrate (CAB), a butyryl polymer known for its desirable properties [170]. Low-molecular-weight esters of butyric acid, like methyl butyrate, are valued for their pleasant aromas or flavors. In the healthcare sector, butyric acid is also utilized as a component in anticancer prodrugs [14]. Caproic acid is primarily used to produce esters for artificial flavors and hexyl derivatives like hexylphenols. It is also used in parenteral nutrition for individuals needing supplemental nutrition and is increasingly applied in foods, drugs, and cosmetics due to its nontoxic nature [171], [172].

Apart from their individual applications, the mixed VFA solutions have unique applications such as in production of biofuels, bioplastics, and in biological nutrient removal. Polyhydroxyalkanoates, biodegradable plastics made from renewable resources, can be synthesized using VFAs derived from waste materials, making their production more cost-effective. VFAs, especially acetic and butyric acids, are favored as they are direct precursors to

Polyhydroxyalkanoates monomers such as hydroxybutyrate and hydroxyvalerate [173], [174]. In biofuel production, VFAs are used to produce microbial oils, hydrogen, biobutanol, and methane. VFAs like acetic acid are important for microbial oil synthesis, as they are converted into lipids suitable for biodiesel production [37], [175]. Additionally, VFAs can be utilized in hydrogen production through anaerobic fermentation and can be converted into biobutanol, a higher energy-density alternative to ethanol. Furthermore, VFAs serve as carbon sources in wastewater treatment processes for biological nutrient removal [176], improving the efficiency of nitrogen and phosphorus removal, thus contributing to sustainable environmental practices.

CHAPTER 3

Research Gaps, Hypotheses, and Objectives

3.1 RESEARCH GAPS

The literature on the production of VFAs suggests the need for further research to optimise operational parameters and strategies for enhancing VFA production under psychrophilic temperatures as well as devising methods for a targeted VFA production from mixed cultures.

3.1.1 Variability of operational parameters under psychrophilic and mesophilic temperatures

Limited studies have systematically investigated the effects of different operational parameters at psychrophilic temperatures in food waste fermentation. Most studies focus on mesophilic and thermophilic conditions, while psychrophilic conditions have remained underexplored, especially in terms of optimising operational parameters for VFA production. The microbial dynamics and their adaptation mechanisms to low temperatures remain unclear, particularly in relation to VFA composition and yield. Furthermore, with mixed culture fermentation, the variability in feedstock composition leads to fluctuations in microbial activity and VFA generation, thus leading to process inconsistency and optimization. A systematic assessment of the effect of varying operational parameters such as pH, temperature, organic load among others, on VFA production under psychrophilic temperatures is necessary.

3.1.2 Enhancement of hydrolysis step under psychrophilic temperatures

The hydrolysis step, critical for breaking down complex substrates, often becomes the first rate-limiting factor, particularly due to the varying conditions such as pH and enzyme activity needed to efficiently process different major constituents like carbohydrates, proteins, and lipids in waste-stream substrates. Furthermore, as hydrolysis progresses and VFA concentrations increase, the resulting acid accumulation can lower the pH, further inhibiting enzymatic activity and microbial function. This feedback effect intensifies the hydrolysis bottleneck, making it necessary to employ buffering agents or process modifications that can maintain pH stability and selectively influence VFA composition to reduce acid toxicity and sustain efficient conversion. .The impact of different pretreatment strategies on microbial community shifts and their ability to enhance VFA production at low temperature is not well understood. Furthermore, there is insufficient research on the combined effect of pretreatment and varying pH conditions on the hydrolysis efficiency at psychrophilic temperature to increase VFA production. This makes it difficult to determine the most effective pretreatment approach for enhancing low temperature acidogenic fermentation.

Furthermore, under psychrophilic conditions, the degradation of the lipid fraction in organic waste, especially food waste, poses a particular challenge due to the reduced solubility of fats and oils at low temperatures. This leads to poor bioavailability of lipid substrates for hydrolytic and acidogenic microbes, limiting the overall efficiency of substrate utilization and VFA production. The solidification or phase separation of fats under cold conditions further hinders enzymatic access, thereby intensifying the hydrolysis bottleneck. In this context, the use of rhamnolipid biosurfactant offers a promising approach to improve emulsification and enhance the solubilization of hydrophobic compounds, particularly long-chain fatty acids. However, there remains a critical research gap in evaluating the effectiveness of rhamnolipids under psychrophilic conditions, where both microbial activity and surfactant performance may differ significantly from mesophilic systems. Furthermore, little is known about how rhamnolipid addition influences microbial community dynamics, lipid hydrolysis pathways, and downstream VFA composition in psychrophilic fermentation systems. The potential interactions with operational factors such as pH and organic load also remain underexplored. Addressing these gaps is essential to determine whether biosurfactant-assisted strategies can reliably improve hydrolysis and lipid utilization under low-temperature fermentation systems.

3.1.3 Supporting microbial activity for psychrophilic fermentation and targeted VFA production

Current research lacks systematic strategies for supporting key microbial population in the mixed culture systems that drive selective VFA production, especially under psychrophilic fermentation. Maintaining a stable and efficient microbial consortium can be difficult due to the dynamic nature of mixed cultures where certain species may be more involved in different stages of the fermentation process, meanwhile exhibiting complex syntrophic interplay. For instance, in food waste fermentation, fast-growing lactic acid producers such as *Lactobacillus* can dominate early stages, lowering the pH rapidly and suppressing the activity of butyric acid producers such as *Clostridium* spp. The addition of selected strains through bioaugmentation can address these limitations by enhancing specific metabolic pathways or outcompeting inhibitory or inefficient microorganisms. Use of bioaugmentation strategies has been studied to enhance the VFA production using different substrates such as cheese industry wastewater, rice straw, and cellulosic substrates. However, with waste streams such as food waste and wastewater sludge, there is a significant variability in their composition and inherent bacterial diversity that interferes with specific VFA production. Bioaugmentation can help stabilize microbial communities during process fluctuation. By carefully selecting and introducing beneficial microbial strains, bioaugmentation has the potential to optimize fermentation processes and improve VFA production rates. However, there is a need for a microbiome-informed bioaugmentation strategy that is tailored to specific waste-inoculum and psychrophilic conditions to target a specific VFA, such as butyric acid. There is a lack of studies assessing whether bioaugmentation leads to sustainable shifts in microbial community structure, or if the introduced strains are outcompeted by native psychrophilic bacteria over time.

3.2 HYPOTHESES

The present research study comprises the following hypotheses:

Hypothesis 1: The interaction of operational parameters, such as pH, and initial volatile solids concentration influences microbial adaptation and VFA composition differently under psychrophilic and mesophilic temperatures leading to variations in fermentation efficiency.

Hypothesis 2: The application of substrate pretreatment, particularly thermal-alkaline treatment, enhances hydrolysis efficiency at psychrophilic temperatures by solubilization of organic matter, thereby increasing VFA production and modifying its composition to mitigate acid toxicity effects. Rhamnolipid biosurfactants further enhance this hydrolysis efficiency by improving the emulsification and solubilization of the fat fraction, in turn promoting higher VFA production and offering greater efficiency than conventional chemical surfactants.

Hypothesis 3: Bioaugmentation with specific microbial strains, such as *Clostridium butyricum*, enhances microbial stability and metabolic activity under psychrophilic conditions, promoting targeted butyric acid production while reducing competition from native microbial communities.

3.3 OBJECTIVES

The overarching objective of this study is to enhance the VFA concentration by acidogenic fermentation of food waste at psychrophilic temperatures. Furthermore, it aims to understand the microbial diversity shift supporting a targeted VFA production by employing metagenomic tools.

The following objectives were carried out, based on the reported research gaps and the aforementioned hypotheses:

Objective 1: Assessing the impacts of different operational parameters on the fermentation process to enhance VFA production under psychrophilic temperature by:

- Analysis of the effect of low temperature on the concentration and composition of VFA produced by acidogenic fermentation of food waste and assessing the changes in the microbial community.
- Conducting meta-analysis of literature to understand the effects of varying food waste characterization and operational parameters in VFA production.
- Investigating the effect of different volatile solids content on the VFA production and composition.

Objective 2: Enhancing VFA production under psychrophilic temperature using pretreated substrate by:

- Comparing the effect of thermal alkaline substrate pretreatment on hydrolysis and VFA production at psychrophilic and mesophilic temperatures, and analyzing changes in the microbial diversity.
- Enhancing VFA production using rhamnolipid biosurfactants.

Objective 3: Enhancement of targeted VFA production through bioaugmentation by:

- Performing bioaugmentation to enrich specific microbial species based on previous microbial diversity analyses to promote targeted butyric acid production and investigating the microbial community shifts resulting from bioaugmentation

3.4 ORIGINALITY

“Provide a targeted approach for enhanced and selective VFA production (butyric acid) under psychrophilic temperature by enhancing the hydrolysis of food waste and bioaugmentation of selective species in a mixed culture system, providing novel insights into psychrophilic fermentation and microbial community dynamics.”

CHAPTER 4

Materials and Methodology

This chapter provides a comprehensive overview of the materials, experimental protocols, analytical and statistical techniques employed throughout this study. It outlines the standard methodologies used to conduct the core experimental work. All common procedures, including substrate preparation, inoculum sourcing, fermentation setup, microbial diversity analysis and analytical measurements, are described in detail here. While the foundational methods remain consistent across experiments, specific modifications or condition-dependent variations relevant to individual objectives are noted within the methodology in the respective chapters.

4.1 Characterization of the feedstock and inoculum

Anaerobically digested sludge was collected from Clarkson wastewater treatment plant and Humber wastewater treatment plant. To arrest methanogens, it was heated to 95°C for 1 h [177]. This treatment selectively inactivated methanogenic archaea and other heat-sensitive microorganisms, while heat-tolerant and spore-forming bacteria (e.g., *Clostridium, Bacillus, Paenibacillus*) survived. As a result, the overall microbial richness decreased, but functional diversity relevant to hydrolysis and acidogenesis was retained, effectively enriching fermentative bacteria over methanogens. Food waste used as the substrate was collected from the restaurants' generated organic waste at York Lanes, York University in Toronto, Canada. Its composition was post-consumption, uncooked and cooked food comprising vegetables, fruits, meat, noodles, rice, and bread. A homogenous slurry was made upon grinding the food waste using a blender. Characterization was performed for both the inoculum and substrate collected. Total solids (TS) and volatile solids (VS) were measured using a standard method [178]. Measurement of total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) was performed by following the manufacturer's protocol using the Hach COD Low Range (LR) kit. Similarly, total nitrogen (TN) and total Kjeldahl nitrogen (TKN) were measured following the manufacturer's protocol using the Hach TN LR and the Hach TKN kits (Loveland, USA).

The macronutrient composition of the food waste was analyzed to quantify carbohydrates, proteins, and lipids. These analyses were performed to characterize the substrate more comprehensively and to assess correlations between nutrient composition and VFA production potential. Total carbohydrates were determined using the phenol–sulfuric acid method (Dubois et al., 1956), with glucose as the calibration standard. Absorbance was measured at 490 nm using a UV–visible spectrophotometer, and results were expressed as grams of glucose equivalent per gram of dry sample. Protein concentration was estimated by the Lowry method (Lowry et al., 1951), using bovine serum albumin (BSA) as the standard. Absorbance was measured at 750 nm after reaction with Folin–Ciocalteu reagent. Values were expressed as grams of BSA equivalent per gram of dry sample. Total lipids were measured following the Bligh and Dyer method (1959) using a chloroform–methanol extraction (2:1 v/v). The solvent-extracted lipid fraction was recovered by evaporation and quantified gravimetrically on a dry-weight basis. All analyses were performed in triplicate, and results were reported as mean ± standard deviation on a dry-weight basis.

4.2 Experimental setup for comparing VFA production under mesophilic and psychrophilic temperatures

For studying the comparative VFA production under mesophilic and psychrophilic temperatures, a batch-type experiment was set up with each condition in duplicate. Serum bottles of 120 mL were used with a working volume of 80 mL. The initial organic concentration was 1.2 g VS/L based on the observed stability while initiating fermentation and digestion reactions in the literature [179]. The inoculum-substrate ratio was 1:3 v/v based on VS. Upon mixing the sludge and food waste, the initial pH was found to be 6.7 which was left uncontrolled for the duration of the experiment. Serum bottles were then sealed using rubber septa. To create anaerobic conditions, each bottle was purged with nitrogen gas for 5 minutes. The bottles were then incubated at 37°C, 27°C, and 17°C in a shaker incubator for 10 days.

4.3 Experimental setup for VFA production using pretreated food waste under psychrophilic conditions

Food waste was subjected to thermal-alkaline pretreatment. Based on the literature, two temperatures, 100°C and 120°C were selected at pH 9 due to previously observed enhanced VFA production under such conditions [81], [180]. A higher temperature than 120°C was not studied as it is reported that excessive temperature damages the physicochemical characteristics of food waste and negatively impacts the C/N ratio available for fermentation [181]. The blended food slurry was set to pH 9, followed by heating at 100°C and 120°C respectively for 1 hour inside an oven with manual shaking in the intervals of 10 minutes. The control set had untreated food waste. Serum bottles of 500 mL were used with a working volume of 200 mL. Based on volatile solids, the inoculum-to-substrate ratio of 1:3 v/v was used. The initial organic load was 1.2 g VS/L. Before starting the fermentation process, all treatment media were brought to a common initial pH for a clear comparison of the pretreated and untreated food waste on VFA produced. The initial pH for each of the treatments was set to 9 with 6 M NaOH which was the closest alkaline pH that could be set for each condition without adding excess NaOH. The alkaline condition was set up to provide initial buffering as a preventive measure against acid inhibition in the batch system [182]. The reactor bottles were closed with rubber septa. The reactors were then purged for 5 min with nitrogen gas, creating anaerobic conditions followed by incubation at 17°C and 37°C.

4.4 Experimental setup for assessing different initial VS concentrations for VFA production under alkaline and psychrophilic conditions

For assessing the effect of different concentrations of initial VS in the fermentation system and controlled alkaline pH, batch reactions were performed in 500 mL serum bottles with working volume of 300 mL. Four initial VS concentrations were selected – 1.2 g, 2 g, 3 g, and 3.5 g based on the reported VS values in literature, albeit in the lower spectrum to avoid organic overload for the microorganisms, especially under psychrophilic conditions. The pretreated food waste and sludge were combined in the inoculum-to-substrate ratio of 1:3 v/v in accordance with the respective four VS concentration. The pH was set to 9 and maintained routinely with the addition of 1M NaOH during fermentation period. The sealed bottles were purged with nitrogen gas for 5 minutes to provide an anaerobic condition and incubated on a shaker incubator at 17°C For 14 days.

4.5 Production and characterization of rhamnolipids

Burkholderia thailandensis E264, a non-pathogenic microorganism, was utilized for rhamnolipid synthesis due to its safe rhamnolipid-producing capability. To preserve the strain, 50% (v/v) glycerol stocks were stored at –20°C. Cultures were initially grown on nutrient agar plates to obtain single isolated colonies, which were subsequently transferred to nutrient broth for inoculum preparation. Skim milk was used to simulate the solids-not-fat (SNF) arising as a side (waste) stream from dairy processing and employed as a fermentation medium for rhamnolipids production. Fermentation was performed in 500 mL flasks containing 150 mL of skim milk and incubated at 30°C with shaking at 150 rpm for four days, as per our previously published method [3]. All productions were carried out in triplicate for consistency. Following the cultivation, the rhamnolipids were extracted using our previously reported method of liquid-liquid extraction, and following cell and protein removal from the broth [183], [184].

Structural analysis of rhamnolipids involved Fourier Transform Infrared (FTIR) spectroscopy using a diamond high-refractive-index prism to detect the key functional groups of rhamnolipids as in Zhu et al. (2022) [4]. Liquid Chromatography-Tandem mass Spectrometry (LC-MS/MS) in negative electron ionization mode was used to provide a detailed composition and identification of rhamnolipids, as per the methods detailed in Zhu et al. (2022) [184].

4.6 Experimental setup for VFA production with rhamnolipids and chemical surfactant addition

The experiments on VFA production were performed in serum bottles of 500 mL total volume with a working volume of 300 mL. The initial organic concentration of 3 g VS/L was selected based on previous lab experiments supporting VFA production (Section 4.4). The inoculum-substrate ratio was 1:3 v/v based on VS. Three triplicate sets of fermentation were performed – control set without rhamnolipid addition, set 1 (RL-0.2) with rhamnolipid addition at 0.2 g/g TSS, and set 2 (RL-0.3) with rhamnolipid addition at 0.3 g/g TSS. The pH was set at 9 after mixing the sludge, food waste and rhamnolipids, which was adjusted to 9 throughout the fermentation. Sealed serum bottles were purged with nitrogen gas for 5 minutes and incubated at 17°C in a shaker incubator for 14 days, based on our previous studies (Kumar et al., 2024).

The chemical surfactant selected in this study was SDS. Sodium dodecyl sulfate (SDS) was selected over other chemical surfactants due to its strong solubilizing ability, cost-effectiveness, and well-documented performance in enhancing substrate availability in fermentation systems. Its anionic nature allows it to effectively disrupt hydrophobic interactions, making complex organic matter more accessible for microbial degradation. The experimental setup for chemical surfactant addition was done similarly to Section 2.2. Exact dosages were tested for SDS for comparison under the same conditions.

4.7 Preparation of a pure culture of *Clostridium butyricum*

To obtain a pure culture of *Clostridium butyricum*, Reinforced Clostridial Medium (RCM) was employed in both liquid and solid (agar supplemented) forms, prepared under aseptic conditions. Initially, the dietary supplement containing *C butyricum* (Vitamatic, Raritan, New Jersey, USA) was rehydrated by suspending it in 25 mL of sterile liquid RCM. The suspension was gently mixed to ensure complete dissolution and subsequently allowed to rest at room temperature for 10–15 min to facilitate bacterial recovery and adaptation. The rehydrated culture was streaked onto RCM agar plates using a sterile inoculating loop. Both plates and flasks were incubated anaerobically at 37 °C for 48 h using an anaerobic gas jar system (Millipore Sigma, St Louis, Missouri, USA). Characteristic circular, glossy, and grey colonies were observed which were indicative of *C. butyricum*. To ensure culture purity, several distinct colonies were picked and restreaked onto fresh RCM agar plates. These plates were incubated under identical anaerobic conditions until morphologically uniform colonies appeared. The species were confirmed using 16S rRNA sequencing (Section 2.5). Subsequently, well-isolated colonies were selected and inoculated into sterile 120 mL serum bottles containing 50 mL of liquid RCM broth. Cultures were incubated at 37 °C for 72–96 h to generate an activated bacterial suspension. Bacterial growth was monitored spectrophotometrically, and an optical density (OD_{600}) of approximately 1.0 was used as the benchmark for activation. The resulting cell suspension was either stored at 4 °C for short-term use or preserved as glycerol stocks (final glycerol concentration 20%) at –20 °C for long-term storage.

4.8 Experimental setup for enhancing butyric acid by bioaugmentation

The fermentation experiments were conducted using 500 mL serum bottles, each containing a working volume of 300 mL. An initial organic loading of 3 g VS/L was selected based on prior experiments. The inoculum-substrate ratio was 1:3 v/v based on VS. Two sets of experiments were prepared in triplicate: a control group without any bioaugmentation and a test group to be bioaugmented with *Clostridium butyricum* on day 3 of fermentation. The time point of day 3 for bioaugmentation was selected based on the acetic acid trend in our previous work, close to the exponential phase of hydrolytic bacteria. Following the mixing of sludge with food waste, the pH was initially adjusted to 9 and subsequently maintained at this level throughout the fermentation process. The serum bottles were sealed and flushed with nitrogen gas for 5 mins to ensure anaerobic conditions, then incubated at 17 °C in a shaker incubator for a period of 14 days.

4.9 Analytical methods and calculations

Liquid samples were collected routinely to analyze pH, SCOD, and VFA analysis. To remove turbid materials, samples were centrifuged at 1956 x g, followed by filtration using 0.45 µm syringe filters and dilution as necessary.

4.9.1 pH

The pH of the samples was measured using a Thermo Scientific Orion Star A211 pH meter (Thermo Fisher Scientific, USA). The meter was calibrated before each experimental work using standard buffer solutions of pH 4.00, 7.00, and 10.00 prior to measurement to ensure accuracy

4.9.2 Soluble Chemical Oxygen Demand

SCOD was analyzed using the Hach 430 COD LR kit as per the manufacturer's protocol.

4.9.3 Volatile Fatty Acids

VFA analysis was performed using gas chromatography (GC) (Agilent 7890B) with a hydrogen flame ionization detector and capillary column (Nukol, 15 m × 0.53 mm × 0.5 µm, Sigma Aldrich, St Louis, Missouri, USA). Butanol of 1 g/L was used as an internal standard.

4.9.4 Ammonia

Ammonia was measured using Hach TNT 832 kit (Loveland, USA) for day 1, 4, 7, and 12 to gather an overview of ammonia trends at different stages of the experiment.

4.9.5 Lysine

To assess protein degradation during VFA fermentation, lysine concentration in the fermentation samples was determined using a lysine oxidase-based colorimetric assay as described by Matsuda & Asano [185].

4.9.6 Calculation for VFA yield and degree of acidification

The calculation for the yield of VFA was done according to the following equation 1:

$$VFA\ Yield\ \left(\frac{gVFA}{\frac{gVS}{L}}\right) = \frac{\left[Highest\ VFA\ concentration\ \left(\frac{g}{L}\right)\right]}{[Initial\ VS\ added\ (g)]}\ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

The degree of acidification (DA) representing the amount of total VFA produced from the SCOD was calculated by the following equation:

DA% = [VFA (g/L) / SCOD (g/L)] X 100…………………………………………….........2

4.7.7 Calculation for energy consumption during fermentation

Calculation of energy consumption by the shaker incubator (Infors HT Multitron Triple Incubator Shaker) at two operational temperatures of 37°C and 17°C was performed for two different ambient temperatures of 25°C and 20°C. According to the user manual of the incubator, shaking power consumption, and heat load for cabinet and top cooler were noted. Thermal power coefficient k of the instrument was estimated based on a standard operating temperature of 37°C using the following equation:

Thermal power coefficient, k = Total heat load / (operating temperature – outside ambient temperature) 3

The heating or cooling power for duration of 11 days was then calculated as per the following equation:

Heating/cooling power = [k (Operating temperature – ambient temperature)] + shaker power consumption 4

4.10 Microbial community analysis

The microbial community present in the system were characterized to assess the diversity changes using 16S rRNA gene amplicon sequencing. Total genomic DNA was extracted using Soil DNA Kit (GenBio Systems, Inc., Canada) according to the manufacturer's protocol. The extracted DNA was analysed for its purity and concentration using Nanodrop spectrophotometer. The V3–V4 hypervariable region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) by using universal primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′), 1492 R (5′-GGTTACCTTGTTACGACTT-3′). Following this, Sanger sequencing was performed using the same primers. Raw sequences were merged, filtered, and assigned taxonomically using DADA2 pipeline to obtain high-resolution amplicon sequence variants (ASVs). Sequences were filtered for quality (Phred ≥ 25), denoised, merged, and taxonomically classified using the SILVA v138 reference database. Microbial diversity was assessed by calculating alpha diversity indices (Shannon, Simpson) to describe species richness and evenness within samples, and beta diversity (Bray–Curtis distance) to evaluate compositional differences between treatments. The abundance of bacteria diversity was analyzed and the graphical plots were made using MicrobiomeAnalyst 2.0 (Quebec, Canada).

4.11 Statistical analysis

To analyze the statistical significance of the VFA concentrations achieved under different conditions, the PAST 4 software (PAST 4.0, University of Oslo, Norway) was utilized for statistical analysis. Both one-factor and two-factor ANOVA were employed to test the statistical difference.

4.12 Systematic review and meta-analysis

4.12.1 Study protocol and search strategy

A comprehensive review of research related to VFA and biogas production using food waste was carried out as per guidelines mentioned in the Recommended Standards for Systematic Reviews and Meta-Analyses (PRISMA) [186]. Studies involving biogas production were investigated only when the data provided information on VFA production. To collect the required data, two global databases were systematically queried – Scopus, Springer, and Web of Science. The search timeframe selected was from January 1, 2000 to March 31, 2024. The objective was to compile all research articles published during this time in from North America. Following keywords were used using a combination of Boolean operators: [(‘Acidogenic fermentation’) OR (‘anaerobic digestion’) OR (‘fermentation’)] AND [(‘food waste’) OR (‘kitchen waste’) OR (‘OFMSW’)] AND [(‘volatile fatty acids’) OR (‘VFA’)].

4.12.2 Inclusion criteria

For the search, specific inclusion criteria were defined: a) Type of substrate – Focused on studies addressing food waste; b) Process type – Research articles were included that focused on acidogenic fermentation and anaerobic digestion process for producing VFAs, biogas, hydrogen; c) Period of publication – Articles between the time period of 2000-2024 were included since key research this domain were conducted in this timeline; d) Language – Articles written in English language were selected; e) Type of article – Original research articles were included.

4.12.3 Exclusion criteria

For the search, specific exclusion criteria were defined: a) Period of publication – Articles before the year 2000 were excluded; b) Type of article - Review articles and book chapters were excluded;

c) Substrate type – Food waste comprising of agricultural and other cellulosic organic waste were excluded; d) Region – Research articles were restricted to North American region. Employing the exclusion criteria ensured selectivity of research articles to meet the objective of the study. The selected article record was maintained in Mendeley 2.110.0 reference management software.

4.12.4 Generation of bibliometric network

All articles were exported in .ris format to VOSviewer 1.6.20 software (Leiden University, Netherlands) to create visual representation of research and keyword network map during the selected time period.

4.12.5 Data extraction from research articles

Based on the inclusion and exclusion criteria, the extraction of the data was conducted from the selected research articles. The database was built in MS Excel software and covered the following aspects: Author details, DOI, type of substrate, substrate composition, substrate characterization, operational factors, and VFA concentration. Necessary unit conversions were made to ensure consistency. Reported VFA concentrations were normalized to their equivalent COD values using theoretical conversion factors, expressed as g COD g^{-1} VFA. These factors represent the stoichiometric oxygen requirement for the complete oxidation of each acid to CO_2 and H_2O and increase with carbon chain length due to the higher degree of reduction in longer-chain VFAs. Accordingly, conversion factors of 1.07, 1.51, 1.82, 2.04, and 2.20 were used for acetic, propionic, butyric, valeric, and caproic acids, respectively [187].

4.12.6 Data analysis

To perform data analysis, studies were shortlisted which provided all necessary inputs such as pH, temperature, initial substrate, and VFA concentration. Furthermore, for food waste characteristics, a subset of studies was selected that had information on total solids (TS), volatile solids (VS), chemical oxygen demand (COD), and carbon:nitrogen (CN) ratio. The data was processed using meta-essentials [188] in MS Excel. For statistical values including standard error while assessing correlation between variables, the values used were mean of reported standard error in literature.

CHAPTER 5

Impacts of Operational Parameters on the Fermentation Process for Enhancing VFA Production

5.1 - Tailored production of butyric acid from mixed culture fermentation of food waste

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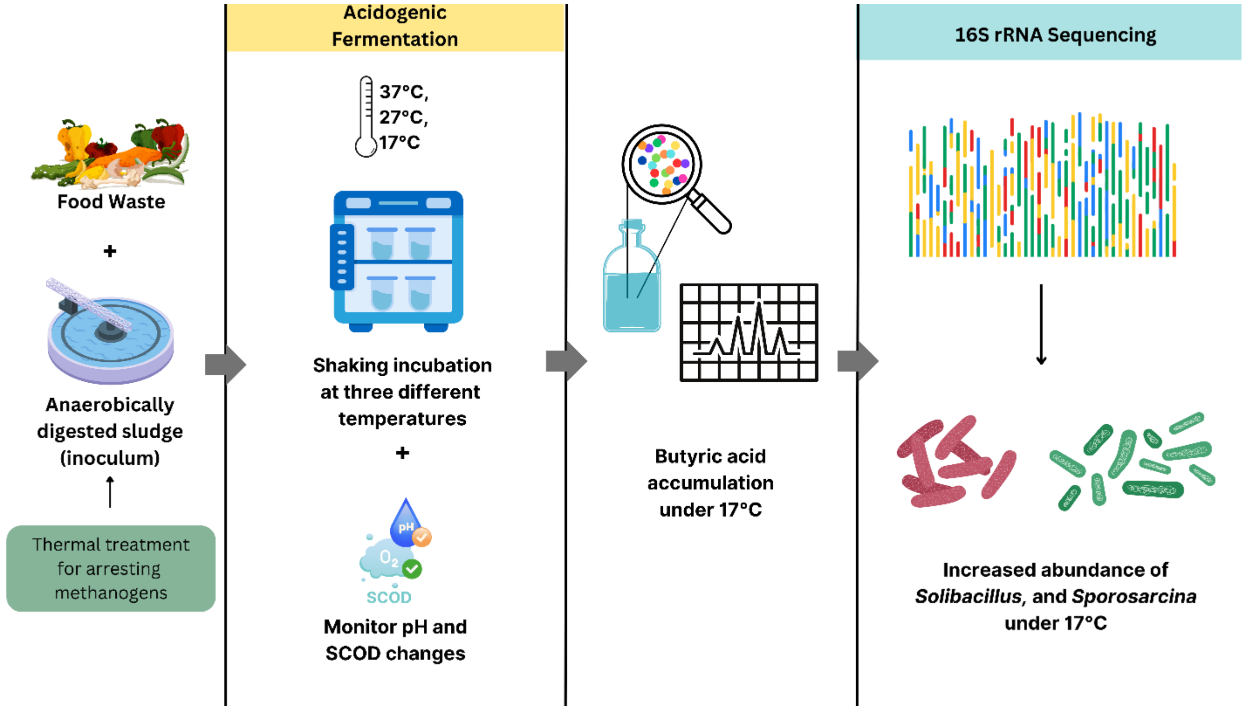
Contribution Statement: I conducted the background literature review; designed the experiments; performed all laboratory work, data collection, and primary analyses; created figures/tables; and wrote the manuscript. This chapter is adapted from the published article, with experimental setup concisely summarized here and full methodological details provided in Chapter 4, and formatting aligned with the thesis.

5.1.1 Abstract

Volatile fatty acids (VFA) are high-value-added products obtained from the fermentation of waste feedstock. These are generally produced as a VFA mixture of C2 - C6 acids through mixed culture fermentation. The prospect of tailoring the process conditions in mixed culture fermentation is attractive since it can produce a predominance of a target VFA without the need for a sterile, pure culture-based process. Among VFAs, butyric acid has a wide range of industrial applications which are currently met by chemical synthesis. This study showed a targeted production of butyric acid in a mixed culture VFA fermentation under psychrophilic temperature. Compared to mesophilic conditions, the butyric acid produced at 17°C accumulated to up to 7 days at 0.5 g/L compared to in 37°C where it was not detected after day 1. The microbial community study showed the increased abundance of *Sporosarcina* and *Solibacillus* genus which degrades proteins, aiding the *Clostridium_sensu_stricto* spp. in producing butyric acid potentially through protein degradation. Within the detected bacterial diversity showing a lower Shannon index of 0.84 at 17ºC, these genera also showed a higher abundance. For further enhancing the hydrolysis, thermal-alkaline pretreatment of food waste was performed. However, it reduced the subsequent production of butyric acid as compared to untreated food waste. Under 17ºC, butyric acid's concentration was 0.06 g/L, while at 37ºC, it was 0.12 g/L. This showed that intrinsic microflora of food waste was essential for its production. This selective accumulation of butyric acid over other VFAs offers a means of targeted VFA production using mixed culture fermentation under psychrophilic temperature.

Keywords**:** Butyric acid, psychrophilic temperature, acidogenic fermentation, volatile fatty acids, food waste

5.1.2 Graphical Abstract



5.1.3 Introduction

Conventionally, the organic fraction of municipal solid waste ends up in landfills while it can be diverted for value-addition to produce volatile fatty acids (VFAs) through fermentation. Fermentation reduces the chemical oxygen demand (COD) of the waste, thus cutting its environmental impact [189]. The value-added platform chemicals, VFAs, generated in this process are C2-C6 short-chain fatty acids, namely, acetic acid, propionic acid, butyric acid, valeric acid, and caproic acid. These VFAs have a broad range of applications such as in the textile industry, bioplastics, biofuels, flavouring agents etc [2].

Butyric acid is a C4 VFA with an industrial demand of 80,000 metric tons annually at approximately $1.8 rate per kg [14]. Its applications vary across diverse industries, such as food, pharmaceutical, perfume, chemical, and animal feed. Currently, it is produced industrially via chemical synthesis using petroleum-based feedstocks. With a shift towards bio-based production of butyric acid, there is more focus on metabolic engineering and strain development while using lower-cost feedstock. Employing pure-culture inoculum is subject to sterile conditions throughout the process to ensure optimum fermentation and is also more sensitive to changes in environmental conditions. Using mixed-culture fermentation voids the need for sterile conditions, thus reducing the steps required for sterility maintenance. Furthermore, the syntrophy among various species present in a mixed culture also provides increased tolerance to a wider range of environmental parameters and substrate requirements. In fermentation processes, current challenges include low concentrations of below 25 g/L, difficult recovery and purification of VFAs [14]. Furthermore, in mixed culture fermentation and complex feedstock, each operational parameter has a distinct effect on the microflora, the resultant metabolites, and associated maintenance costs. The temperature has a strong impact on microbial activity targeting their metabolism at a biochemical level, altering the interchangeable shift between glycolysis and pentose-phosphate pathway [65]. Even with an environmental microbiome, each microbe is affected at the species level along with its syntrophic relationships as was observed by Dijkstra et al., who used metabolic tracers in environmental microbial samples to study the effect of temperature at a biochemical level [65] Gong et al., observed the difference in the fermentation type between mesophilic and thermophilic temperatures with similar substrate and inoculum, where 35°C resulted in a mixed-type fermentation while 55°C favoured butyric acid [190]. Secondly, the alkaline conditions decrease the Bacteroidetes and Actinobacteria abundance thus reducing propionate and acetate as end products, leading to more butyric acid accumulation. Most of these fermentation studies have been performed at mesophilic and thermophilic temperatures, while temperature maintenance is a high operational cost, especially in colder countries where average ambient temperatures stay in the psychrophilic range [191]. Due to this reason, studies have been undertaken to perform anaerobic digestion at psychrophilic temperatures for assessing biogas production. A digestion study notably showed the increased accumulation of VFAs at 2.1 g/L under a low temperature of 20°C [63]. The targeted enhancement of butyric acid under mesophilic conditions has been studied by Atasoy et al., by bioaugmentation of mixed culture using *Clostridium butyricum* with a reported increase of 0.2 gCOD/L to 2.8 gCOD/L [168]. However, there is not yet much information about enhancing the overall and targeted VFA production at psychrophilic temperatures. By focusing on optimizing the conditions favouring the active microflora, this targeted approach can result in specific VFA accumulating more over the others. One key bottleneck behind the low VFA production is slow hydrolysis at low temperatures due to the lower activity of hydrolytic bacteria [192]. The hydrolysis step is enhanced to promote acidogenesis by the use of substrate pretreatments that convert the complex compounds in organic waste to simpler compounds, readily available to the microorganisms. Among physicochemical pretreatment methods, thermal-alkaline pretreatment has been shown to increase the hydrolysis by 20-30% [112], [123], [193] by depolymerization of complex carbohydrates, proteins, and saponification of fats. This study compares the production of VFAs between mesophilic and psychrophilic temperatures, focusing on butyric acid production. Furthermore, the effect of enhanced hydrolysis using thermal-alkaline pretreatment on butyric acid production under psychrophilic conditions is also explored.

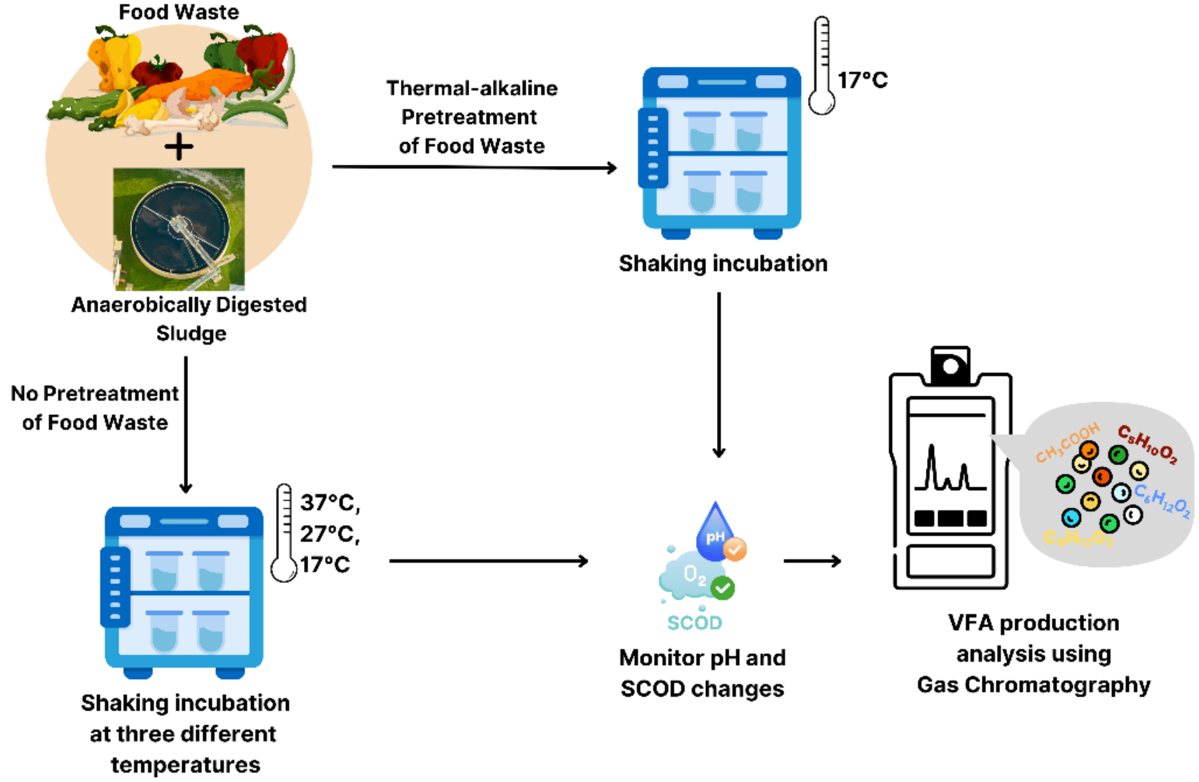


Figure 4. Workflow of comparative fermentation at mesophilic and psychrophilic temperatures followed by fermentation at psychrophilic temperature with substrate pretreatment.

5.1.4 Materials and Methods

Anaerobically digested sludge was collected from the Clarkson wastewater treatment plant in Ontario, Canada, to be used as an inoculum. Food waste used as the substrate was collected from the restaurants' generated organic waste at York Lanes, York University in Toronto, Canada. The characterization was performed for both the inoculum and substrate collected five separate times (n=5) and mean values were calculated due to the variability of waste characteristics (Table 1). The parameters and methods of the characterization are described in Chapter 4, Section 4.1.

Table 4. Characterization of substrate (food waste) and inoculum (anaerobically digested sludge) with mean values for n=5

Characteristics	**Study on temperature effect on VFA**	
	Food Waste	Anaerobically Digested Sludge
pH	5.1 ± 0.09	7.16 ± 0.01

Total Solids (TS) (g/L)	89 ± 3.3	11.5 ± 0.5
Volatile solids (VS) (g/L)	83.8 ± 2.7	8.04 ± 0.3
Total Chemical Oxygen Demand (TCOD) (g/L)	82 ± 0.6	12.2 ± 0.03
Soluble Chemical Oxygen Demand (SCOD) (g/L)	70.3 ± 0.5	9.8 ± 0.06
Total Nitrogen (TN) (g/L)	4.15 ± 0.2	1.85 ± 0.05
Total Kjeldahl Nitrogen (TKN) (g/L)	4.6 ± 0.02	1.79 ± 0.03

The experimental setup for comparing VFA production under mesophilic and psychrophilic temperatures, as well as after substrate pretreatment, is described under Chapter 4, Section 4.2 and 4.3 respectively. All experimental batches were set in duplicate and the resulting data was presented as the average of two samples along with their standard deviation. The analytical methods, microbial community analysis, and statistical analysis are described under Chapter 4, Section 4.9, 4.10, and 4.11 respectively.

5.1.5 Results and Discussion

pH and Chemical Oxygen Demand conditions during comparative fermentation at mesophilic and psychrophilic temperature

Acidogenic fermentation leads to a decrease in the pH due to the production of volatile fatty acids, their undissociated forms, and other acidic intermediates. The decline in pH over time from an initial value of 6.67 was observed faster at 37°C, followed by 27°C, and lowest at 17°C from day 2 onwards (Figure 5a). This correlated to a comparatively slower hydrolysis rate at psychrophilic temperature and thus delayed acidogenesis. The pH values increased at 37°C on day 6 and at 27°C on day 5 and then declined again. This could be due to the conversion of volatile fatty acids further into gases like carbon dioxide and hydrogen as is observed with the decrease in VFAs' concentration similar to other studies, for instance, Bolzonella et al., observed a similar pH increase coinciding with an increase in biogas under uncontrolled pH conditions and temperature range of 14-22ºC [194]. However, at 17°C, the pH values stayed stable around 6 after day 2 as well as the total VFAs' concentration. The pH maintenance around 6 selectively supported the activity of specific microbes and thereby the VFA composition and also prevented its further oxidation. Moreover, with the prevention of acid toxicity, hydrolysis can be completed over time to obtain maximum VFAs [195].

The soluble COD peaks around days 2 and 3 (Figure 5b), coincided with the decline in pH and the maximum VFA values. However, the VFA concentration comprised 8-10% of the sCOD values. The observed second peaks could be associated with the oxidation of remaining nitrogenous compounds such as generation of ammonia from protein degradation. The presence of nitrogenous compounds, including ammonia and organic nitrogen, can interfere with COD measurements, since these compounds may also undergo partial oxidation during the dichromate digestion process. This analytical overlap can lead to a slight overestimation of sCOD, particularly in protein-rich substrates such as food waste. Moreover, the generation of ammonia helped in stabilizing the pH under uncontrolled conditions by offering a buffering effect from ammoniacal nitrogen as was observed in a fermentation study at mesophilic and thermophilic temperatures using food waste [196]. At the end of 12 days, the concentration of ammonia in this study was found to increase from 3.4 g/L to 3.9 g/L at 17ºC (14.7% increase), from 4 g/L to 4.6 g/L at 27ºC (15%), and from 4.1 g/L to 5.1 g/L at 37ºC (25%), while the concentration of ammonia under each condition dropped to 0 in the middle of fermentation period. The *p*-value was <0.05 for ammonia concentration under all temperature conditions.

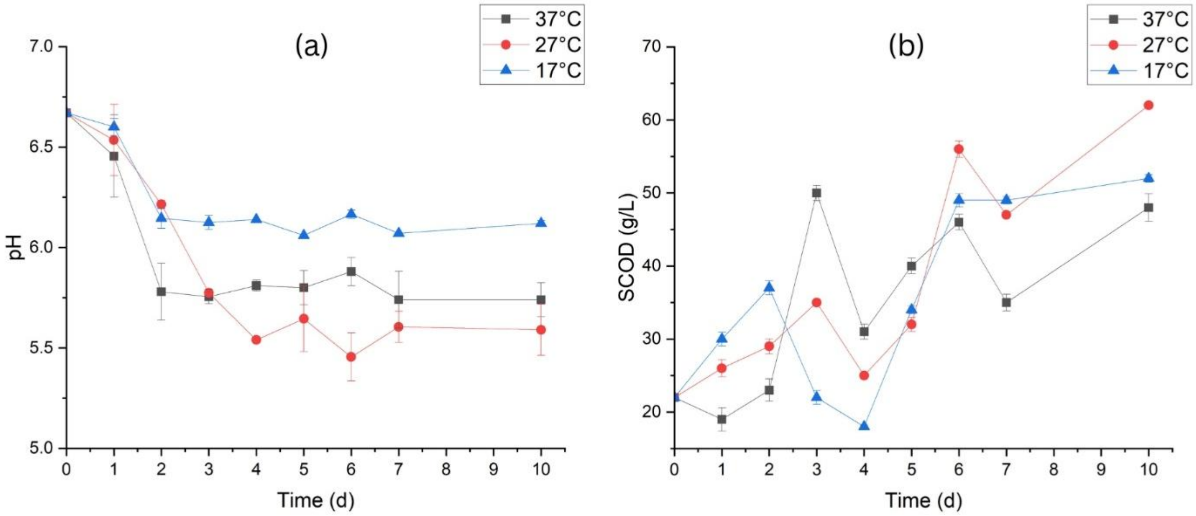
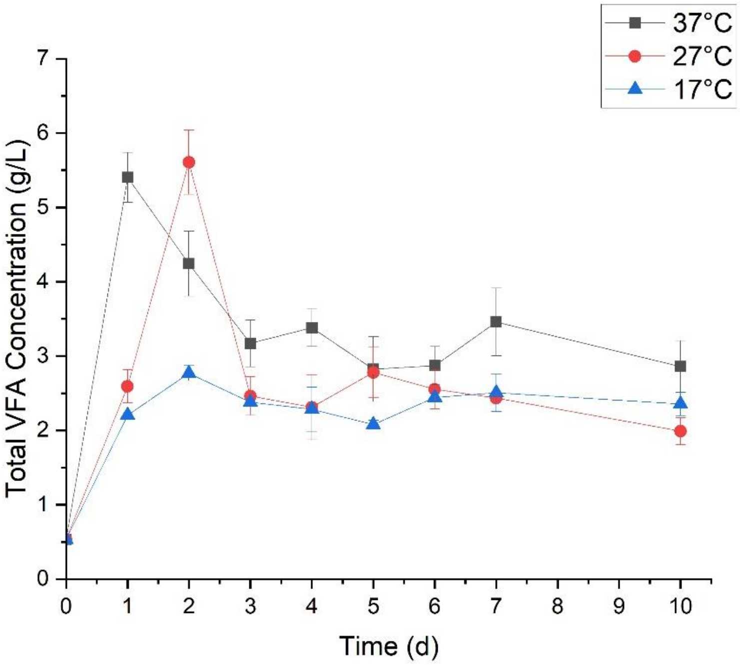


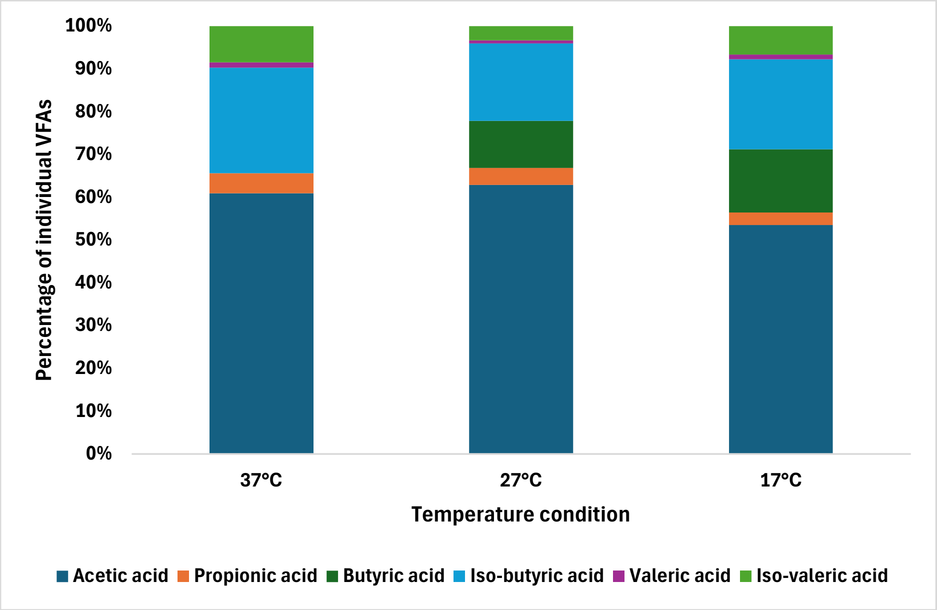
Figure 5. (a) pH and (b) SCOD values across 10 days at 37°C, 27°C, and 17°C.

Volatile fatty acid production

The concentration and composition of total volatile fatty acids produced at the three different temperatures were detected using gas chromatography (Figure 6). The initial VS concentration added to the serum bottles was 1.2 g. The highest concentration of total VFA was obtained at 27°C with 5.6 g/L on day 3, followed by 5.4 g/L at 37°C on day 2. At 17°C, the highest concentration obtained was 2.7 g/L on day 3. In terms of yield, the VFAs produced per gram of VS added were 4.6 g/gVS L^{-1} at 37 ºC, 4.5 g/gVS L^{-1} at 27ºC, and 2.25 g/gVS L^{-1} at 17ºC. These values corresponded to the maximum COD values obtained around days 2 and 3 at all three temperatures as well as stability of pH afterwards. The results were statistically significant (*p*-value<0.05) for concentrations of VFA under three different temperatures.



(a)



(b)

Figure 6. (a) Concentration and (b) composition of total volatile fatty acids produced at 37°C, 27°C, and 17°C

Shorter chain VFA like acetic acid was found to be the most predominant VFA (approximately 60% of total VFA) at all three temperatures, followed by iso-butyric acid (Figure 7a and 7c). Medium chain VFA like valeric and iso-valeric acid were present at a low concentration of 0.065 g/L and 0.460 g/L respectively at 37°C on day 1, while at 27°C and 17°C maximum concentration obtained was 0.040 g/L on day 12. This indicated that the major metabolic pathway involved in this fermentation was the acetyl-CoA pathway and oxidation of longer-chain or medium-chain VFA such as propionic acid and butyric acid [24]. Moreover, with the reduction in temperature, the concentration of propionic acid was found to decline with a maximum of 0.251 g/L at 37°C on day 1, 0.209 g/L at 27°C on day 2, and 0.144 g/L at 17°C on day 12. Lower propionic acid concentrations, thus, could not have shown a significant inhibitory effect on the fermentation. This observation was, however, in contrast to digestion studies using other organic wastes like paunch, under psychrophilic temperatures, where propionic acid generated during acidogenesis is found to be higher than in mesophilic conditions [55].

An interesting observation was made with butyric acid concentration (Figure 4b). At 37°C, there was no butyric acid detected on day 1 and onwards. The initially present butyric acid was consumed. This could be due to its utilization as a substrate for microbial growth and metabolism or further oxidation to carbon dioxide and hydrogen depending on the microbial community. Another reason for its disappearance could be its isomerization to form iso-butyric acid as is observed by its increased concentration, also supported by a previous study [197]. The authors in the study showed that the generation of iso-butyric acid increased in the presence of acetic acid and butyric acid under pH 5.5, similar to the one eventually obtained in our study. *Clostridium luticellarii,* a chain-elongating bacteria, could oxidize acetic acid to butyric acid and its isomerization to iso-butyric acid. Although this study utilized pure culture fermentation, but food waste has been reported to inherently provide *Clostridium luticellarii.* It was reported in another fermentation study by Li et al., where the intrinsic microflora of food waste was acclimatized for chain elongation to produce long-chain VFAs (Li et al., 2024). Other species reported for this mechanism were *Caproiciproducens galactitolivorans, Megasphaera elsdenii, Ruminococcaceae bacterium* etc.

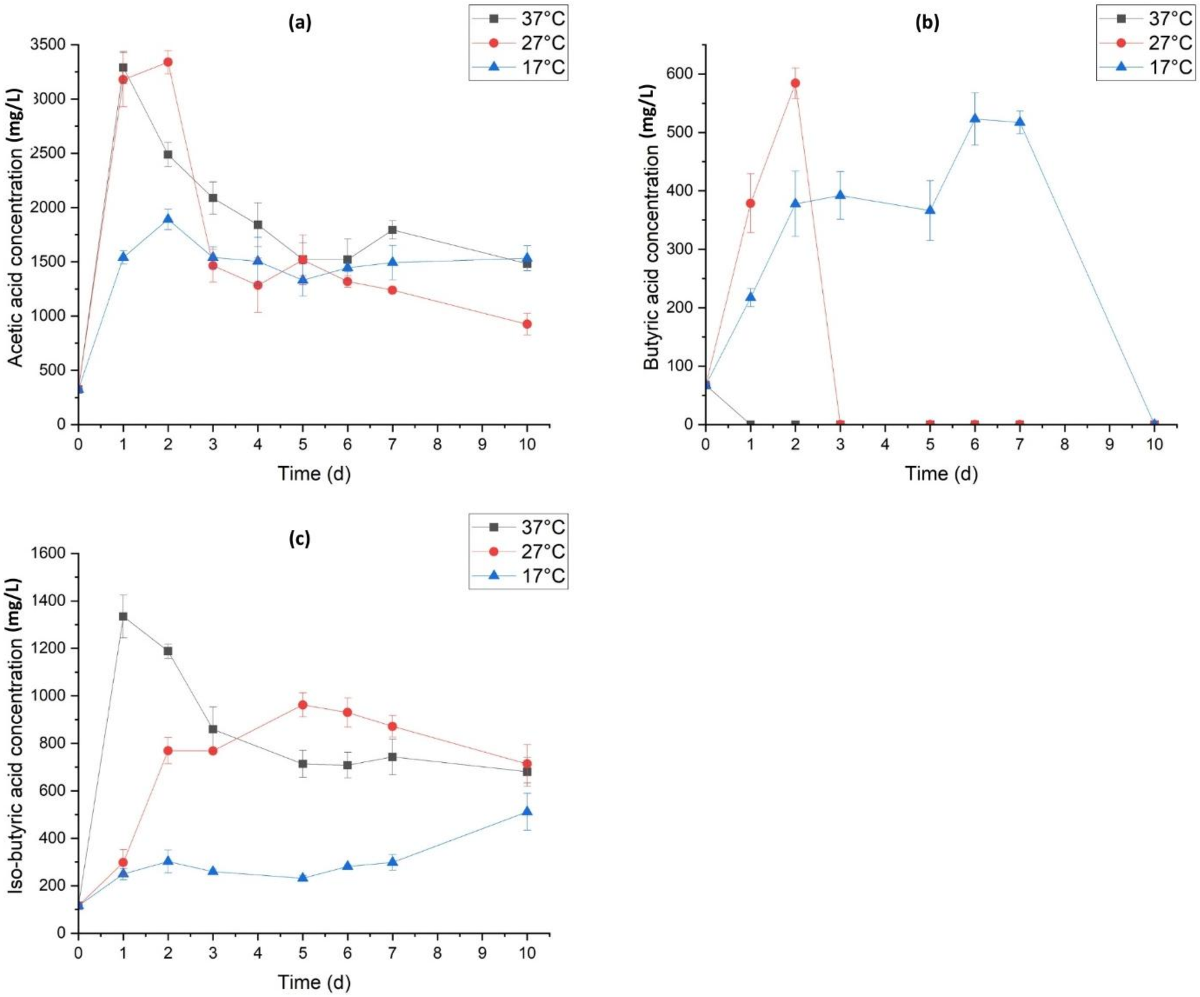


Figure 7. Concentration of different VFAs across 10 days at 37°C, 27°C, and 17°C: (a) Acetic acid, (b) Butyric acid, and (c) Iso-butyric acid.

At 27°C, there was approximately an 8-fold increase in its amount on day 2 from 0.067 g/L to 0.6 g/L which was then not detected on day 3. In the case of 17°C, approximately a 7-8 fold increase was seen on day 6 from 0.067 g/L to 0.52 g/L with butyric acid being detected until day 7 and then abruptly reaching a zero value afterwards. These results were found to be statistically significant (p-value<0.05). The reported titers of butyric acid production using mixed-culture fermentation have been found in the range of 0.5-10 g/L under mesophilic temperatures (Table 5). It is noted that under psychrophilic temperatures, the reported studies using mixed culture fermentation for butyric acid or other VFA production are scarce. This longer persistence of butyric acid at 17°C could be correlated to maintenance of a favourable pH around 6 as opposed to comparatively lower pH at higher temperatures on the same days [199]. This selectively maintained the activity of butyric acid-producing microbes. The low concentration of butyric acid at acidic conditions was also supported by literature where in mixed culture fermentation, the pH values near neutral were optimum for butyric acid production [24]. Low temperatures also reduced the activity or metabolism of butyric acid consumers or microbial species competing with butyric acid producers, for instance, the acetogens and homoacetogens. Thus, sustaining butyric acid in the fermentation broth as these microorganisms have optimal temperature requirements in mesophilic and thermophilic ranges [200], [201]. A study on hydrogen production by fermentation of activated sludge also reported an increase in butyric acid production at low temperatures [202]. Major butyric acid producers belong to the genera *Clostridium, Butyribacterium, Butyrivibrio, Eubacterium, Megasphera, Fusobacterium,* and *Sarcina* [203]. Of these, *Clostridium* spp. was extensively studied for industrial applications, including *C. butyricum, C. tyobutyricum, C. populeti* etc. The minimum temperature for growth of *Clostridium butyricum* is reported to be 8°C [204]. The delay in butyric acid oxidation can also be correlated with the stable concentration of acetic acid which does not increase after day 2. Moreover, it is known that there is a shift towards the production of medium-chain and longer-chain VFAs with increasing time availability of electron donors and acceptors [205] due to chain elongation which is seen to be facilitated at a lower temperature of 17°C after day 7 with increasing concentration of iso-valeric and valeric acid.

Table 5. Butyric acid production using mixed-culture fermentation reported in the literature.

Substrate	**Temperature**	**pH**	**Butyric acid concentration**	**Reference**
Food waste	17ºC	5.5-6	0.52 g/L	This study
Synthetic wastewater and sludge	20ºC	9	<0.1 g/L	[72]
Wastewater sludge	20ºC	4.5	0.05 g/L	(Andreides et al., 2024)
Food waste	37ºC	9	8.52 ± 0.10 g/L	[206]
Food waste and granular sludge	37ºC	6.9-5.5	0.8 g/L	[207]
Food waste	37ºC	-	6.5 g/L	[208]
Food waste	35ºC	4-6	0.25 g/gVS	[209]
Food waste and sludge	55ºC	5-6	5.26 ± 0.22 g/L	[124]

Bacterial community analysis

The bacterial diversity was analyzed to evaluate its effect on the VFA production and composition. In this study, the dominant taxa at the phylum level were *Firmicutes,* followed by *Proteobacteria, Chloroflexi,* and *Bacteroidetes*. At the genus level (Figure 8), the dominant taxa differed under different temperatures with time.

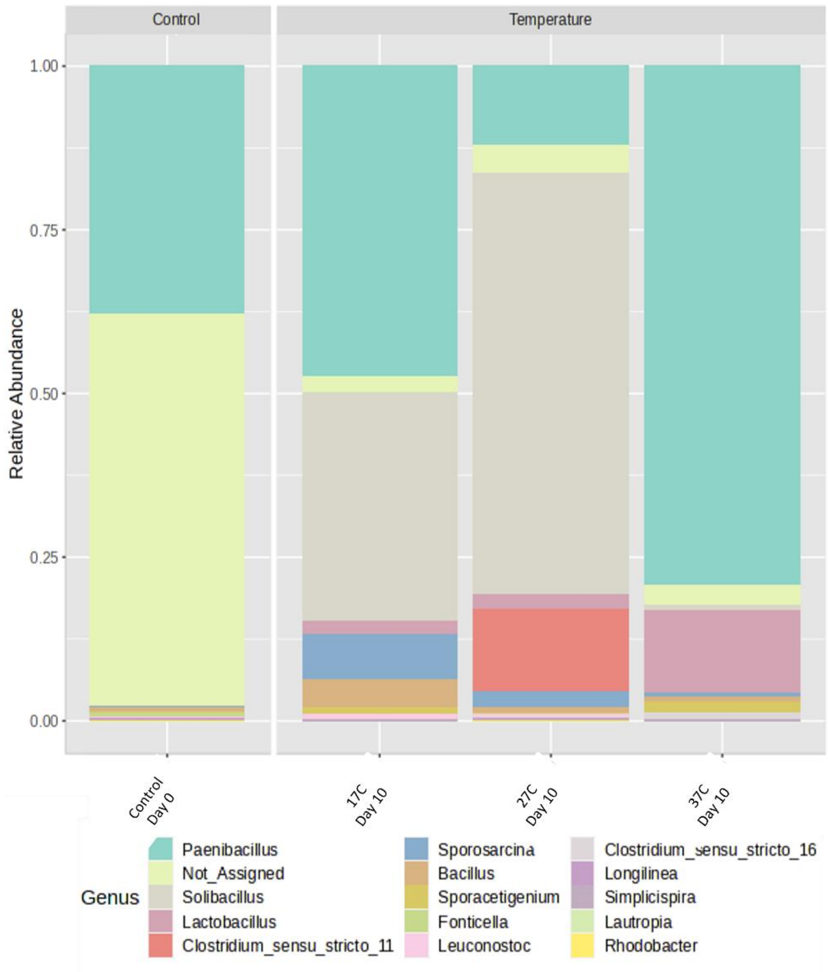


Figure 8. Relative abundance of bacterial community at genus level on day 0 (C-Day0) and day 10 at temperatures 17°C, 27°C, and 37°C

The bacterial composition at the genus level showed a dominance of *Paenibacillus* and unassigned bacteria on day 0. Over time, with resource utilization and the colonization dynamics of bacteria, the diversity increased. As shown in Table 6, the Shannon Index for Alpha Diversity shows that the overall diversity reduced under 17°C at the end of fermentation and certain genera dominated unevenly over the others.

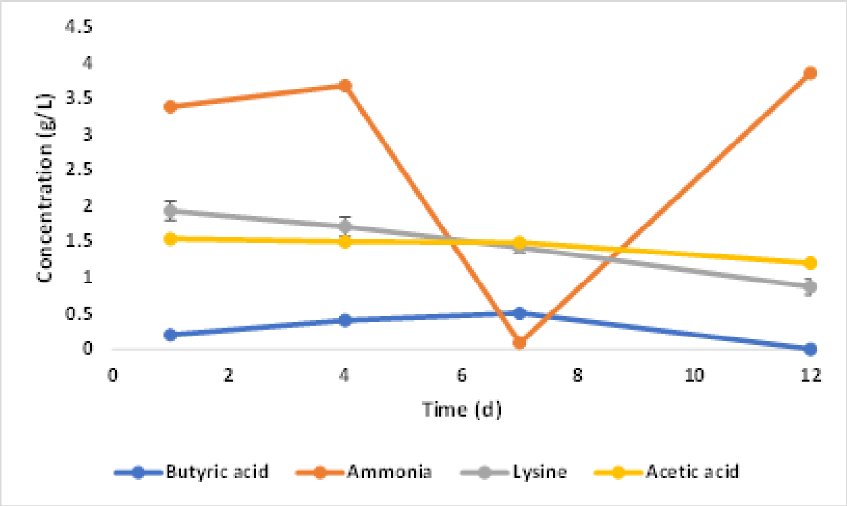
Table 6. Alpha Diversity Shannon Index for day 0 and day 10 at 37ºC, 27ºC and 17ºC.

Experiment Condition	**Alpha Diversity Shannon Index**

Control – Day 0	1.25
17°C – Day 10	0.84
27°C – Day 10	1.26
37°C – Day 10	1.35

Under the temperatures of 17°C and 27°C, an increasing dominance of genera *Solibacillus, Bacillus,* and *Sporosarcina* was observed as opposed to 37°C. While there are limited studies involving the genera *Solibacillus* and *Sporosarcina* concerning VFA production, they have been recognized for their potential in biotechnological applications due to their robust enzymatic activities, especially proteolytic enzymes [210], [211]. Related to *Bacillus* genus, *Solibacillus* and *Paenibacillus* spp. can be found together in clusters under fermentative conditions such as in the rumen of goats [212]. As observed in the heat map (Figure 7), the genera *Bacillus, Solibacillus, Sporosarcina,* and *Leuconostoc* occur close together in a cluster with higher abundance over other genera at 17ºC. At 37ºC, the genera *Clostridium_sensu_stricto_16, Lactobacillus, Sporacetigenium,* and *Paenibacillus* were found in a cluster. When occurring together in the presence of a sugar-rich substrate, the first two fermentative bacteria are found to work in syntrophy to produce acetic acid and hydrogen [213]. Furthermore, at 17°C, the increasing abundance of *Sporosarcina* indicates protein degradation. *Sporosarcina* are reported to utilize nitrogenous compounds to release ammonia, for instance, in liquor fermentation, as was also observed with increasing ammonia near the end of fermentation (Li et al., 2024). In a phenotypic characterization study by Keshri *et al*., *Sporosarcina* bacteria were found to be related to *Bacillus* and *Clostridium* through a common GerA spore germination protein. They also identified a urease accessory protein UreG which has a role in the decomposition of urea to produce ammonia and carbon dioxide [215]. The related *Sporosarcina* species identified in Keshri *et al*'s study were from the fecal matter of a Canadian goose. This explains their increased abundance in this study due to their occurrence in the used sludge (inoculum) which was collected from a Canadian wastewater treatment plant likely receiving the geese's fecal matter through stormwater. Furthermore, the species under this genus are endospore-forming bacteria that can tolerate low temperatures, for instance in fish meat as observed by Tsuda *et al*. at 10°C [216]. Among others, *Paenbacillus* spp. were also reported to proliferate at low temperatures, however, *Sporosarcina* spp. were more adaptive as was also observed in this study between the three temperatures. Among these genera, the major species observed in this study were *Solibacillus silvestris, Sporosarcina ureae,* and *Sporosarcina psychrophile*. *Sporosarcina psychrophile*, specifically, is a psychrophilic fermentative bacteria used as a poultry probiotic [215]. With close relation to the *Sarcina* genus, *Sporosarcina* also shows the capability of butyric acid production [41]. Given the simultaneous increase in butyric acid and ammonia, this indicates the possibility of butyric acid generation through protein degradation in addition to glucose oxidation. Enhanced proteolytic activity by *Solibacillus* and *Sporosarcina* under 17ºC can allow for *Clostridium* spp., to generate butyric acid from amino acids like glutamate, lysine and threonine as is studied to occur in the human intestinal tract under the presence of proteolytic enzymes [217]. The dominant *Clostridium* genus identified in this study were *Clostridium_sensu_stricto_11* and *Clostridium_sensu_stricto_16*. In food waste fermentation studies by Zhao *et al.,* and Yu *et al.,* these genera were also shown to increase butyric acid production through ion substitution electrodialysis and varying pH conditions respectively [218], [219]. As with Yu *et al.,* the pH close to 6 favoured butyric acid production, similar to this study. While these studies were performed in mesophilic and thermophilic conditions, the presence of *Clostridium_sensu_stricto* genera selectively favours butyric acid production. Thus, under psyrchophilic conditions, the psychro-tolerant bacteria *Sporosarcina* and *Solibacillus* degrades the nitrogenous compounds to ammonia, and the *Clostridium* spp. likely produce butyric acid via protein degradation under low-temperature conditions. A comprehensive metagenomic analysis by Vital et al. identified key bacterial pathways involved in butyrate synthesis. The study revealed that butyric acid production in microbial communities occurs via four primary metabolic routes: the acetyl-CoA pathway, the glutarate pathway, the 4-aminobutyrate pathway, and the lysine pathway. All four pathways converge at a vital energy-generating step where crotonyl-CoA is converted to butyryl-CoA. This reaction, catalyzed by the butyryl-CoA dehydrogenase electron-transferring flavoprotein complex (Bcd-Etfαβ), is a pivotal point in butyrate synthesis, facilitating electron transfer to drive the production of butyryl-CoA, the direct precursor to butyric acid. Of these four pathways, the acetyl-CoA pathway was the most dominant accounting for an average of 79.7% of all butyrate-producing activity, followed by the lysine pathway at 11.2% [220]. To assess this correlation of lysine and butyric acid, the concentration of lysine was analyzed using the activity of lysine oxidase in the present study (Figure 9). At 17°C, lysine degradation was slower, likely due to a reduced enzymatic activity at lower temperatures, leading to a gradual release of ammonia. This suggested that the protein degradation pathways, including lysine deamination, were active but slower at psychrophilic temperatures, providing a steady supply of substrates for butyric acid-producing bacteria. The release of ammonia further supported the hypothesis of active proteolytic metabolism, as amino acid degradation typically leads to ammonia production. These findings align with the proposed mechanism of protein-derived amino acids contributing to a sustained butyric acid production in psychrophilic fermentation systems, as observed in our study. A schematic representation of the lysine-to-butyric acid metabolic pathway has been included in Appendix 6 for reference. Similar trend between lysine, ammonia and butyric acid was also observed by Vital et al, where enterobacteria produced butyric acid, thereby providing further support to our findings [220].

(a)



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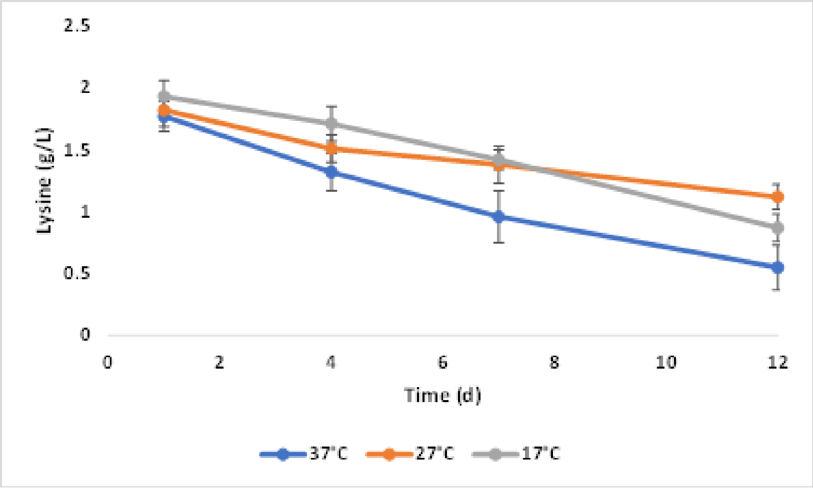

Figure 9. Concentration of (a) lysine, ammonia, acetic and butyric acid for 12 days of fermentation at 17ºC and (b) Comparison of lysine concentration at 37ºC¸27ºC, and 17ºC.

Furthermore, the decreasing presence of *Lactobacillus* genera also favours the butyric acid generation as lactic production shows a negative correlation with butyric acid producers. This is also due to its utilization in the chain-elongation process, generating butyric acid from acetic acid as studied by food waste fermentation by Li *et al* (Li et al., 2024).

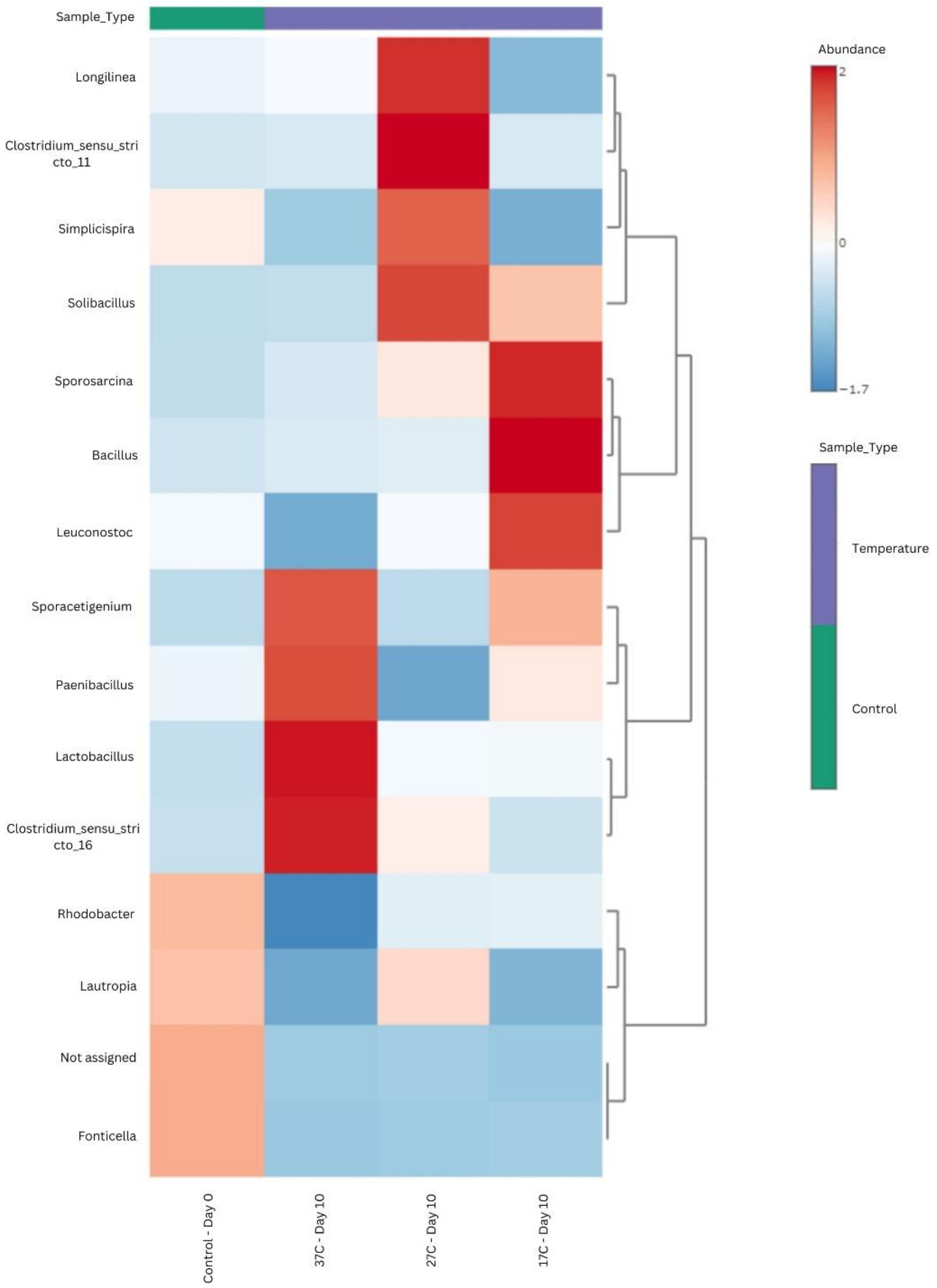

Figure 10. Heat map of major bacterial genera found under different temperatures.

Power consumption during incubation at two temperatures

Psychrophilic VFA production, while energy-efficient in certain respects, does introduce specific energy considerations. Operating at lower temperatures (<20°C) slows microbial metabolism, extending reaction times and potentially requiring longer fermentation periods compared to mesophilic or thermophilic processes. This can lead to additional energy demands for system maintenance, such as mixing and monitoring, as well as insulation or occasional cooling to maintain stable conditions, especially if ambient temperatures fluctuate above target levels [192]. However, psychrophilic conditions also foster microbial communities that activate specific metabolic pathways, enhancing specific substrate utilization efficiency and potentially improving targeted VFA yield and quality despite slower kinetics, as was seen in the present study. These specific microbial activities may partially offset energy input through more efficient substrate conversion over time. For instance, in this study, the sustained butyric acid accumulation observed via protein degradation. Additionally, in colder climates, psychrophilic fermentation aligns well with the ambient environment, reducing the need for cooling and supporting a low-energy, sustainable approach where hydrolysis can be enhanced for subsequent VFA increase, offsetting energy costs.

As a preliminary assessment, the energy consumption by the shaker incubator was assessed in the present study for two target temperatures, reflecting typical laboratory conditions in this range. For maintaining 37°C over 12 days, the heating power required was 159 kWh and 158 kWh at ambient temperatures of 25°C and 20°C, respectively. In contrast, maintaining 17°C over the same period required a significantly less power at 20°C, 70 kWh at 25°C and only 2.5 kWh. This indicated that operating at 37°C consumed over twice as much energy as at 17°C at 25°C ambient temperature, with an even greater disparity at 20°C. The total energy costs for both temperatures, calculated using Ontario's non-residential tiered electricity pricing (Ontario Energy Board), are summarized in Table 7. These results highlight the potential for significant cost savings through reduced temperature maintenance, though costs may vary regionally based on energy pricing. To extend these findings, a detailed techno-economic analysis of the fermentation process, including additional pretreatment steps, could provide a more comprehensive evaluation of associated costs, and is the topic of our future studies.

Table 7. Costs for temperature maintenance at 37°C and 17°C in a shaker incubator for 12 days.

Operating temperature	**Ambient temperature outside the incubator**	**Heating Power (kWh)**	**Unit cost (CAD/kWh)**	**Total cost (CAD/kWh)**
37°C	25°C	159	12.5	1987.5
37°C	20°C	158	12.5	1975
17°C	25°C	70	12.5	875
17°C	20°C	2.5	12.5	31.25

Effect of substrate pretreatment on butyric acid and iso-butyric acid production at psychrophilic temperature

Under psychrophilic temperatures, hydrolysis is the first bottleneck in fermentation due to slow microbial activity and complex organic compounds. Among different substrate pretreatments, thermal-alkaline pretreatment enhances the hydrolysis step by breaking down the complex compounds. This reduces the lag period for the microorganisms under psychrophilic temperature in hydrolyzing the substrate. In this study, the thermal alkaline pretreatment was performed on food waste to assess its effect on butyric acid and iso-butyric acid production with increased hydrolysis at 17°C. The TCOD and SCOD of the food waste increased to 93±0.1 g/L and 78±0.2 g/L respectively after the pretreatment, thus exhibiting more substrate availability for enhanced hydrolysis. While TCOD generally remains constant, the thermal-alkaline treatment can cause loss of volatile compounds and precipitation of some compounds due to chemical changes, thus changing the TCOD [221]. This assisted with covering the initial lag period for microbial growth under psychrophilic temperatures [222]. The pH trend was found to be similar to the previous experiments under 17°C. The enhanced hydrolysis resulted in increased total VFA concentration at 17°C with 0.080 g/L in the control, 1.6 g/L in pretreatment at 100°C, and 1.8 g/L in pretreatment at 120°C. However, the pretreatment conditions were not conducive for butyric acid production. Untreated food waste had a maximum of 0.18 g/L butyric acid, while pretreated sets had 0.07 mg/L (pretreatment at 100°C) and 0.05 mg/L (pretreatment at 120°C).

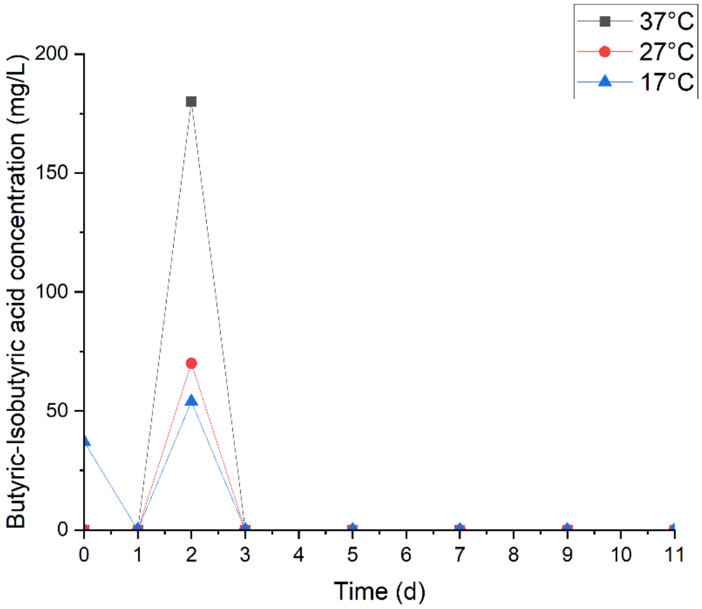

Figure 11. Concentration of butyric acid produced at 17°C for C (control with untreated food waste), P100 (pretreatment of food waste at 100°C and pH 9), and P120 (pretreatment of food waste at 120°C and pH 9)

Butyric acid is a valuable platform chemical and has a high market price of 2000-2500 USD/t with broad applications in the food and pharmaceutical industry [223]. With reduced input cost for maintaining mesophilic temperature in the reactor at cold climate regions, butyric acid production can thus be targeted for enhancement at a psychrophilic temperature. With pH and retention time being observed here as key factors altering the fermentation reaction's production and VFA composition, these can be further studied concerning psychrophilic temperature. Since hydrolysis is delayed at low temperatures, this can be enhanced by optimizing parameters like initial substrate concentration, pH maintenance and longer retention time to achieve higher butyric acid concentration. The reaction can also be run for a longer time to allow chain elongation reaction to produce medium-chain VFAs. Moreover, studying microbial diversity changes could provide more insight into changes in respective metabolic pathways leading to sustained butyric acid production at low temperatures. More insight into the effect of thermal-alkaline pretreatment on butyric acid producers in food waste can allow for optimizing the temperature and pH for such pretreatment to enhance hydrolysis. The active bacterial community observed in this study were tolerant to psychrophilic temperatures, their increased abundance favoured the butyric acid production through protein degradation. Studying the metabolic pathways behind this correlation would provide more insights into targeted butyric acid production at psychrophilic temperatures.

5.1.6 Conclusion

The VFA production at mesophilic and psychrophilic temperatures showed sustained production of butyric acid at 17ºC at 0.5 g/L for up to 7 days. At 27ºC, butyric acid was detected at a concentration of 0.5 g/L until 2 days, while at 37ºC, it was not detected at all. The maintenance of pH near 6 allowed for *Clostridium spp.* to selectively produce butyric acid, likely through protein degradation via psychrotolerant bacteria *Sporosarcina* and *Solibacillu*s. For enhancing the hydrolysis, using thermal-alkaline pretreatment of food waste, however, did not favour similar results due to alteration of the inherent microorganisms in food waste. More insights into the metabolic pathway behind the butyric acid production via protein degradation would aid in further enhancement in targeted production of butyric acid under psychrophilic temperatures.

5.2 Meta-analysis of Production of Volatile Fatty Acids from Waste Streams: Towards Creating Decision Support Tools for Process Optimization

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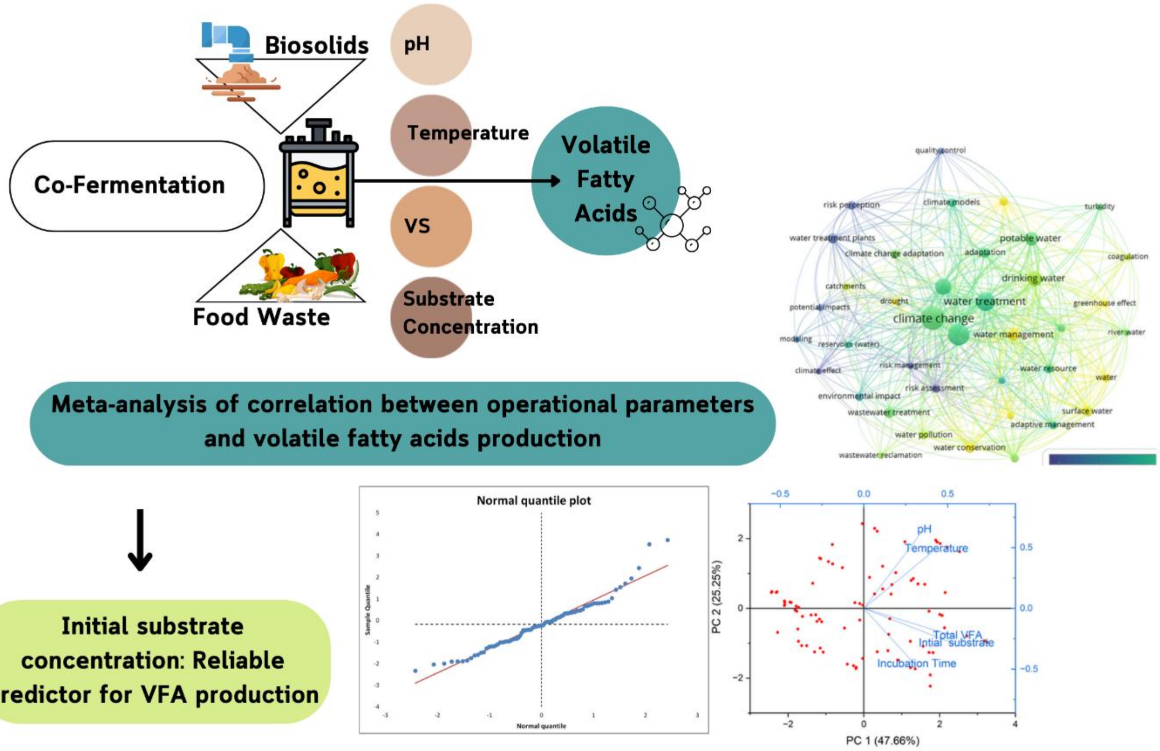
Kumar *et al*., "Meta-analysis of production of Volatile Fatty Acids from waste streams: Towards creating decision support tools for process optimization" *Water Environment Research*, Volume 97, Issue7, July 2025, e70137. https://doi.org/10.1002/wer.70137

Contribution Statement: I conducted the background literature review; designed the experiments; performed all laboratory work, data collection, and primary analyses; created figures/tables; and wrote the manuscript. This chapter is adapted from the published article, with experimental setup concisely summarized here and full methodological details provided in Chapter 4, and formatting aligned with the thesis

5.2.1 Abstract

The sludge in wastewater treatment plants encompasses both biosolids and food waste entering the sewer systems through food waste grinders in the kitchen sinks, especially in North America. The digested biosolids are discarded to landfills or incinerated. Production of volatile fatty acids (VFA) through fermentation of this waste stream is a lucrative value-chain to biosolids and waste management. The co-fermentation of sludge and food waste enhances microbial diversity, and provides optimal carbon:nitrogen ratio for VFA generation. However, variation in source and composition of the food waste significantly impacts the fermentation efficiency. In this study, a meta-analysis of 107 studies from North America was performed to understand the correlation between operational parameters and their effects on VFA production with an aim to use it as a tool for process optimisation. Initial substrate concentration was found to be a reliable predictor for VFA production, followed by temperature and pH. Initial substrate concentrations between 9-20 gCOD/L at temperatures around 25ºC or lower increased VFA production in the assessed studies.

5.2.2 Graphical Abstract



5.2.3 Introduction

The global human population is estimated to reach about 9.8 billion by the year 2050 [224]. This exponentially increasing growth simultaneously generates approximately 2 billion tonnes of municipal solid waste [1], consumes more than 4 trillion cubic metre water [225], and 460 terrawatt hours (TWh) energy [226] every year. Depletion of the natural resources has forced the society to re-evaluate the linear economy approach of consumption. Current research actively focuses on enhancing technologies involving renewable energy sources and global concerns related to solid waste and wastewater management through the lens of resource recovery and circular economy.

To address this issue, the concept of biorefinery has gained prominence in recent years for valorization of waste streams. In wastewater treatment treatments plants, the wastewater brings an influx of biowaste and food waste from residential and commercial sources. For instance, in North America, food waste grinders are installed in kitchen sinks that grinds food waste and mixes with water to create a slurry that gets disposed off into the sewer system [227]. The wastewater is received in treatment plants from various sources and during the treatment, sludge is generated via primary, secondary treatment, and anaerobic digestion. According to US EPA, the biosolids are generally used in land application such as agriculture, landscaping etc (56%) and the remaining 27% and 16% are disposed in landfilling sites and is incinerated respectively [228]. The diversion of 43% of biosolids from incineration and landfills to biorefinery systems thus offers a value-addition approach. The biogas and digestate through digestion process have limited applications and market value when compared to intermediate compounds, such as, volatile fatty acids (VFAs) [229]. Acidogenic fermentation of organic waste allows its valorization to generate VFAs which play a role as platform chemicals in the generation of biofuels and several other industries such as fragrance, pharmaceuticals, bioplastics etc. Furthermore, VFAs have attained a market value of more than 25 Billion USD, surpassing that of biomethane at 6.6 Billion USD, thus making their production a luctrative approach. [2]. In the North American context, acidogenic fermentation is gaining momentum as wastewater utilities and municipalities shift toward low-energy, resource-recovery approaches compatible with existing treatment infrastructure. The region's cold-to-temperate climate supports the development of psychrophilic or ambient-temperature processes, while its organic waste streams—rich in food residues, fats, oils, and greases—provide ideal substrates for VFA generation. In addition, evolving circular-economy policies increasingly favor the recovery of high-value intermediates over conventional biogas production, positioning VFA-oriented biorefineries as a strategic pathway for sustainable waste management.

The sludge and digestate are a rich nutrient source containing suspended solids, organic matter, high nitrogen and phosphorus [230]. While the rich composition of food waste enhances the valorization by increasing the available organic matter along with microbial-diversity rich inoculum in the form of sludge This combination of sludge and food waste, where the sludge amount is more by weight, creates optimal C:N ratio [229]. The co-fermentation also reduces the acid toxicity due to the buffering capacity of the sludge, given its high nitrogen content.

Food waste as a substrate has been variably termed as kitchen waste, organic retail waste, household food waste, organic fraction of municipal solid waste etc. which is based on its source of generation. However, with the varying sourcing, the composition and characterization of these waste streams varies considerably. For instance, the ratio of carbohydrates, protein, and lipids can differ significantly depending on the source and region of the waste generation. This affects the performance of bioreactors and shows different operational requirements. This, in turn, significantly impacts the effectiveness and yield of the process [231]. Numerous laboratory-scale studies are restricted to specific food waste types and locations, limited or varying inoculum sources which presents a limitation for a global valuation of valorization potential of food waste-sludge co-fermentation to VFAs [232], [233]. Furthermore, this poses more challenges in the scale-up possibility of sludge and food waste valorization due to inconsistency in the existing research with respect to substrate composition and characterization which is not always a correct representation of the region. Further research on these aspects is needed to develop robust fermentation processes for the VFA production.

Although it is not practical to establish a standard composition of generated food waste, more quantifiable information regarding regional differences (of substrate characterization) and respectively studied optimum operational factors yielding in higher efficiency of fermentation systems can be useful in the scale-up of existing research. In contrast to biogas [234], there is a significant gap in literature that shows such quantifiable information concerning VFAs.

This study focuses on a meta-analysis to understand the effects of varying substrate characterization and use of wastewater sludge as inoculum across North America in terms of its contribution to VFA production. The study also evaluates the differences in operational factors of the fermentation process. The objective of this study is to quantify the existing research in terms of operational factors and substrate characterization resulting in higher production of VFA from food waste fermentation.

5.2.4 Methodology

The detailed methodology for performing this meta-analysis is described in detail under Chapter 4, Section 4.12. Overall workflow of this study is shown in Figure 12.

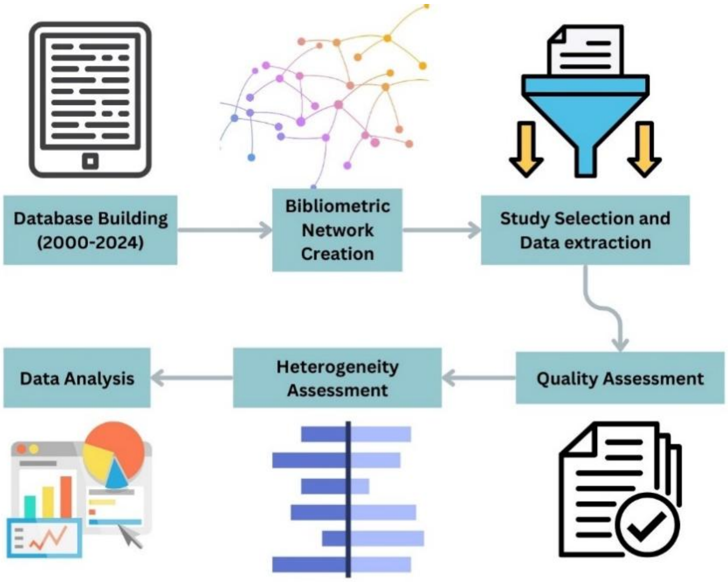


Figure 12. Workflow of the meta-analysis study

5.2.5 Results and discussion

Systematic review of literature on food waste fermentation in North America

In total, 303 articles were identified using Scopus and Web of Science database. After including more filters and removing duplicates, 165 articles were screened. Based on the inclusion and exclusion criteria, final 107 articles were selected for review in this study.

Figure 13 shows the keyword map of research focus from 2016 onwards. There was an increase of interest in the field of fermentation and/or digestion using food waste after 2015 when the concept of circular economy and sustainability started gaining attention [235]. The use of organic wastes offered an avenue for resource recovery and value-addition to a source which otherwise ends up in landfills, creating environmental problems.

The given timeline indicated an increased focus shift towards in-depth research around microbial communities, the interspecies-electron transfer, co-digestion, and biohydrogen production in addition to volatile fatty acids. The size of each circle corresponds to how often the keyword appears in the research articles included in the review. Essentially, larger circles indicate a higher occurrence of that keyword in the articles. Furthermore, the color of the circles indicates the distribution of the keywords over time, while the lines connecting them represent the relationships between the keywords. The closer two words are positioned to each other, the stronger their relationship; in essence, proximity signifies the intensity of their connection. This analysis of keywords offers an additional and enriching perspective for comprehending the main themes in the reviewed research, providing valuable insights to identify trends and potential areas of interest for future studies. In the initial stages of valorization research during 2000-2017, the focus was primarily on biogas and biohydrogen production as an alternative fuel. The parameters studied rigorously included COD, mesophilic and thermophilic temperature ranges, acidic pH etc. for designing the optimal conditions for this bioprocess. Additionally, there was an increasing interest in understanding the role of microorganisms at a biochemical level to harness their full potential for maximum efficiency of digestion and fermentation process [236]. Further ahead, near 2020s, more information was being gathered on addressing the restrictions of these processes, the relation between operational parameters and use of -omics technologies to delve deeper into the functionality of microbes [131], [237].

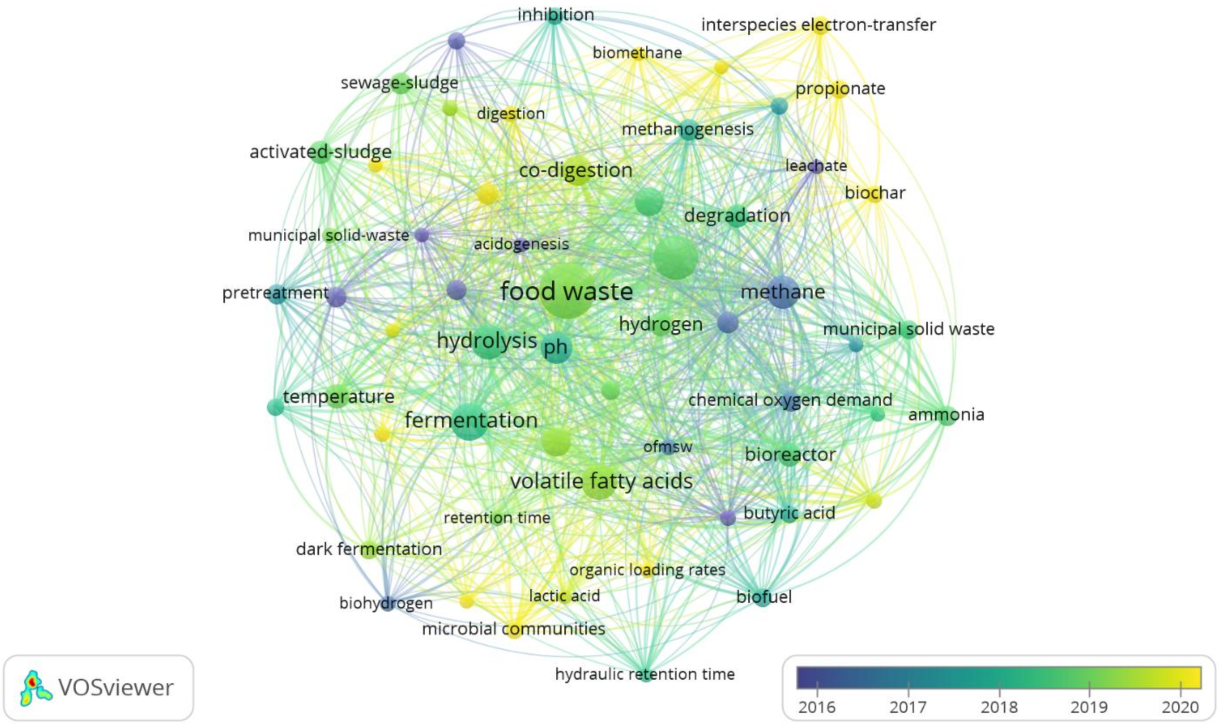
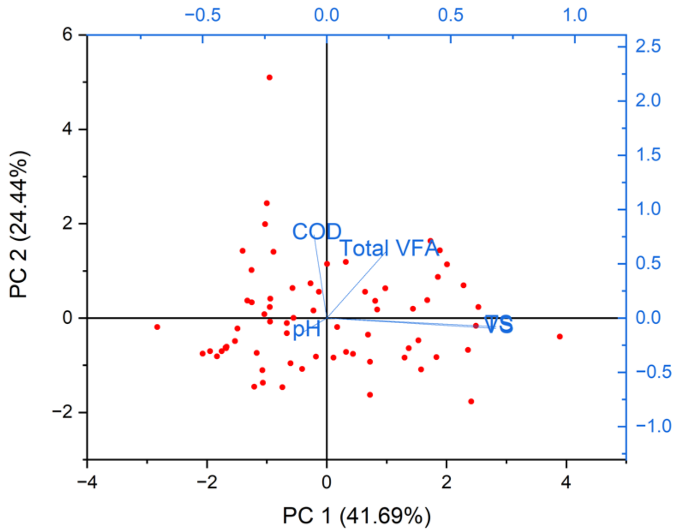


Figure 13. Keyword map for bibliographic network

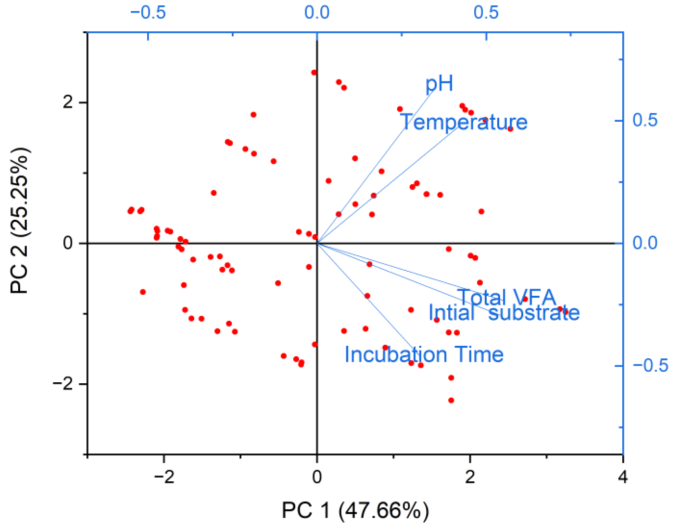
Meta-analysis of food waste characteristics, operational parameters and correlation with VFA production

The extracted information from the research articles was organized in an Excel file. Upon initial observation, the majority of these studies were carried out under mesophilic temperature conditions, with only four articles investigating psychrophilic conditions. A noteworthy discovery from bibliometric analysis is the growing trend in the utilization of psychrophilic temperatures over the last decade. This trend indicates a rising interest in exploring the feasibility of fermentation or digestion at lower temperatures. The shift towards psychrophilic temperatures presents promising prospects for advancing research in the field of organic solid waste management, especially in colder countries. Moreover, it provides more selectivity towards VFA production due to the impact of psychrophilic temperature on microbial activity and syntrophy at a biochemical level [65].

To undertake meta-analysis, certain assumptions were made to fulfill calculations for studies missing one necessary input variable. Few studies did not explicitly show the sample size, which was assumed to be 3 i.e. in triplicate. The correlation coefficient ‘r’ was also not mentioned in several articles. To resolve this problem, r values were assumed based on the average of values mentioned in the literature depending on the two variables for which the r value was required. The assumed values of r used were 0.3 for pH and VFA, 0.9 for temperature and VFA, 0.7 for initial substrate concentration and VFA, and 0.8 for VS in food waste and VFA.



(a)



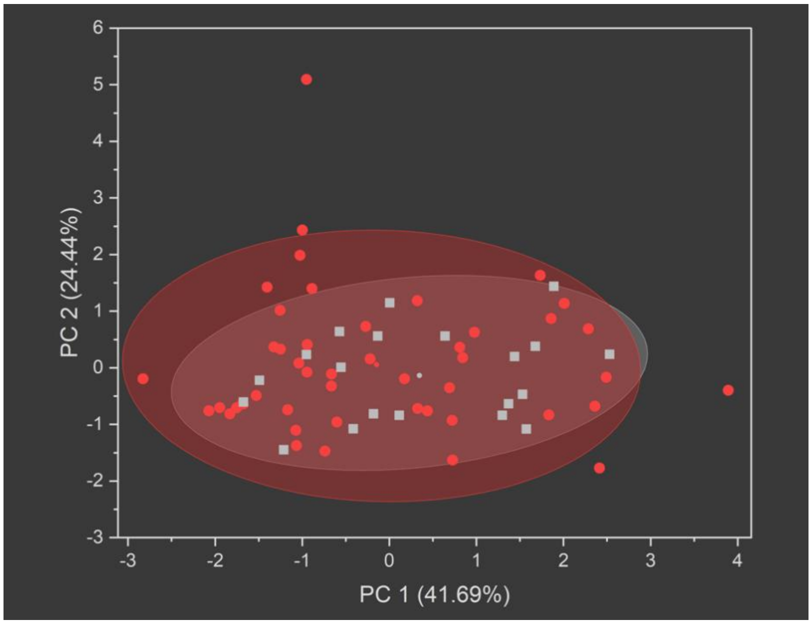
(b)

Figure 14. Biplot for multivariate analysis (a) Characteristics of food waste and total VFA produced (b) Operational parameters of fermentation and total VFA produced

On conducting a principal component analysis (PCA) of the food waste characteristics and operational parameters with total VFA, the biplot (Figure 14) showed the data points scattered across the quadrants for operational parameters effect which were later analyzed using cluster analysis. As for food waste characteristics, the data points were scattered across quadrants but close together. Among food waste characteristics, similarly positive correlation was obtained between COD and VS to VFA. Thus, indicating that these variables contribute similarly to VFA production. Among operational parameters, there was a strong positive correlation between variables pH and temperature; between total VFA and initial substrate concentration and close positive correlation with incubation time. However, with respect to total VFA production, temperature was found to be more influencing as compared to pH. Among other parameters, both and pH and temperature are crucial for microbial growth and its metabolic activity that eventually results in the production of VFA. This is because both impact the microbes at a biochemical level. Since metabolic enzymes are sensitive to pH and temperature changes and function within their tolerance range, any change to these two factors affects the enzymatic activity within the organisms. Temperature change can induce a stress response in the microorganisms, shift the preferred metabolic pathways, affect their growth and reproduction, and thus the overall community interactions [65]. This effect was evidently studied in an anaerobic digestion process by Gomes *et al.*, where temperature showed more significant impact on methane generation than substrate concentration while performing statistical optimization of methane generation [238]. Garcia-Aguirre *et al.,* tested different substrate types for VFA producing potential under different pH and temperature ranges, and found a consensus of temperature and pH effect across all substrates. Under the tested conditions, the authors concluded mesophilic and acidic conditions to support VFA production over thermophilic ranges. Furthermore, the composition of VFA also varied by varying the pH and with different carbohydrate and protein content in the substrate [239]. Thus, directing towards the need of appropriate control of process parameters with respect to the substrate and inoculum conditions.

The lack of systematic comparisons between different initial substrate concentrations limits the understanding of what could be the ideal concentration range for maximum VFA production. Given its high positive correlation, it is critical to make progress in this regard with studies exploring the initial substrate concentration, also focusing on key characteristics such as CN ratio, carbohydrate-lipid-protein ratio etc which are extremely variable from region to region [240]. These properties of substrates affect VFA production as well as its composition by influencing the existing metabolic pathways and degree of acidification in the fermentation [241]. For instance, Wang *et al.,* observed difference in VFA composition with varying initial substrate concentration. VFAs at 200 g/L substrate showed lesser acetic acid and more potential for chain elongation by producing butyric acid than at a higher substrate concentration of 400 g/L [114]. Another study by Owusu-Agyeman *et al.,* observed an increase in valeric and caproic acid with proportion of organic waste co-digested with sludge. Furthermore, over time the generated VFAs showed higher capability of denitirifcation which holds significance in wastewater treatment plants for biological nitrogen removal process [241]. Substrate rich in carbohydrates tend to result in more propionic acid and butyric acid production while a higher protein content results in valeric and iso-valeric acid production [239]. Owing to the complex characteristics of food waste, especially with the inclusion of an inoculum, the initial substrate concentration is a parameter requiring further research and its effect in sync with other operational parameters. This increased heterogeneity was observed by generating a cluster plot of the data.

(a)



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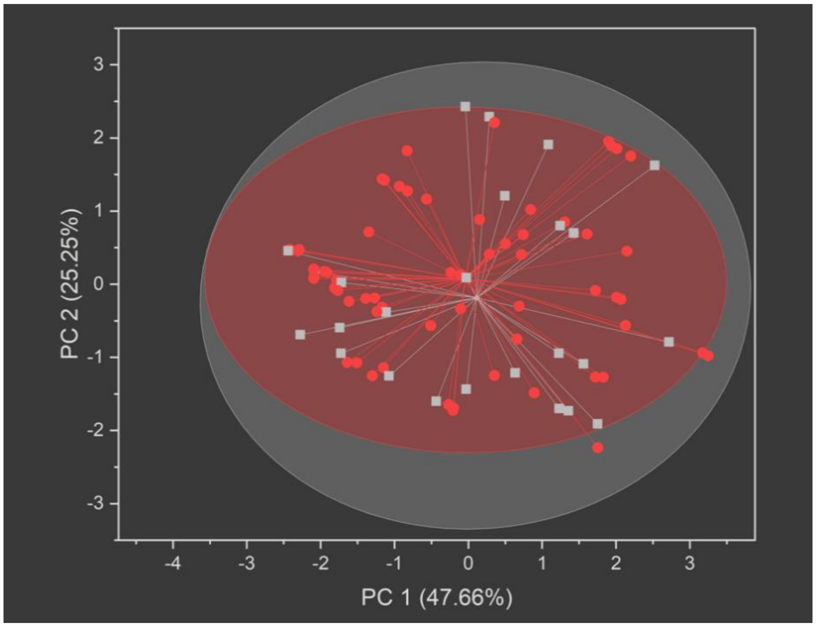


Figure 15. Cluster plot for multivariate analysis (a) Characteristics of food waste and total VFA produced (b) Operational parameters of fermentation and total VFA produced

The studies evaluated in this review either had sludge use or with the substrate alone. Thus, to obtain clusters of the data points in the biplot, two clusters were selected showing the presence or absence of inoculum use along with food waste. On obtaining the cluster plot (Figure 15), the studies with use of additional inoculum showed higher variance. The studies using just food waste as the substrate without additional inoculum showed more concentrated distribution and homogeneity. Furthermore, the inoculum that were reported across studies had a lot of variation in terms of type, source, and microflora. Thus, indicated through the high variance observed in the data. To reduce the heterogeneity, there is a need of systematic studies of inoculum effect as well. The inoculum used ranges from sources such as digested sludge from wastewater treatment plants, cow manure, pig manure, etc.

However, there are certain challenges which include, infrequent reporting of correlation coefficient of operational factors and resultant products. Furthermore, the sample size is often a point of concern, especially where single reactors are run.

Heterogeneity assessment for correlation between operational parameters, volatile solids in food waste and VFA production

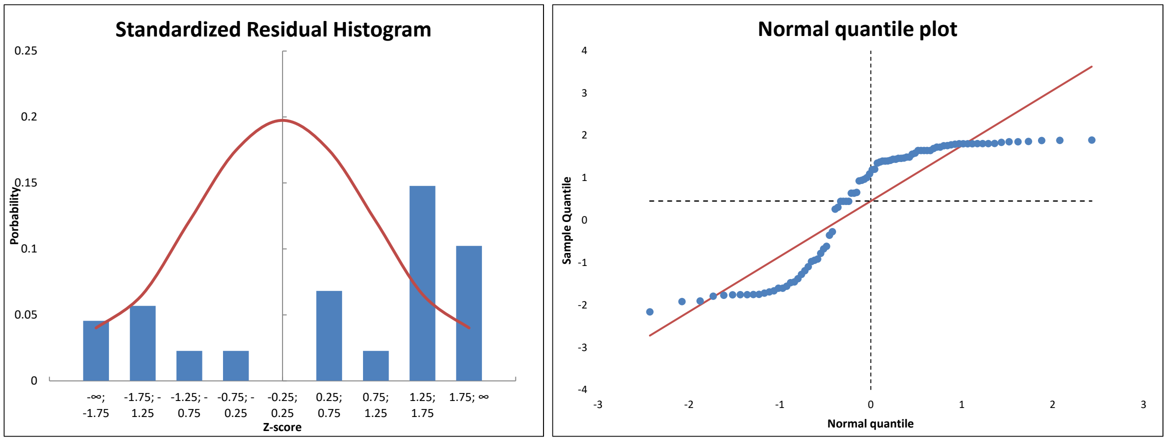
Table 8. Results of heterogeneity assessment for correlation between operational parameters, volatile solids in food waste and VFA production

	pH	**Temperature**	**Initial Substrate Concentration**	**Volatile Solids in Food Waste**
Q	334.59	2329.83	492.68	1086.37
pQ	0.000	0.000	0.000	0.000
I^2	74%	96.27%	82.54%	92.73%
T^2	12.30	17.46	19.05	33.46
T	3.51	4.18	4.37	5.78

The heterogeneity assessment for different operational parameters and volatile solids in food waste with VFA production was performed. The parameters tested for this against VFA were pH, temperature, and initial substrate concentration. As shown in Table 8, the Cochran's coefficient Q obtained for all these parameters was in order pH < initial substrate concentration < temperature. This sequence suggests that varying temperatures had the most significant influence on VFA production, with substrate concentration and pH playing lesser roles in affecting outcomes in comparison. This further strengthens the PCA plot results. I^2 represents the proportion of total variation in effect estimates that is due to heterogeneity rather than chance. This too followed the same ascending pattern as that of Cochran's coefficient, further confirming the presence of significant heterogeneity in the dataset. Likewise, volatile solids in food waste showed high heterogeneity among studies. An unstructured mathematical model by Moguel-Castaneda *et al.,* tested simultaneous effect of temperature and pH on the kinetics of digestion process and showed that these parameters exhibit most effect on the maximum growth rate of microorganisms. Higher pH values reduced the cellular activity of methanogenic bacteria, thus promoting VFA production [242]. However, with biowaste alone, contradicting observations have also been made by Fernandez-Dominguez *et al.,* at mechanical-biological treatment plants where VFA profile was not affected by temperature changes and VFA yielf differences were less than 10% [243].

Homoscedasticity and normal distribution analysis between operational parameters, volatile solids in food waste and VFA production

For assessing homoscedasticity, two diagnostic tools, a standardized residual histogram and a normal quantile plot were generated (Figure 16). Homoscedasticity assumes that the variance of errors is constant across all predictor variables. It provides an estimate of bias in the parameters tested. In the standardized residual histogram, homoscedasticity holds when the residuals are consistent and bell-shaped across all predictor values. Thus, predictors for initial substrate concentration for fermentation showed more symmetric spread, followed by temperature and pH. As for food waste characteristics, volatile solids had the least normal distribution. Using a normal quantile plot, normal distribution was observed in the order: initial substrate concentration > temperature > pH > volatile solids.



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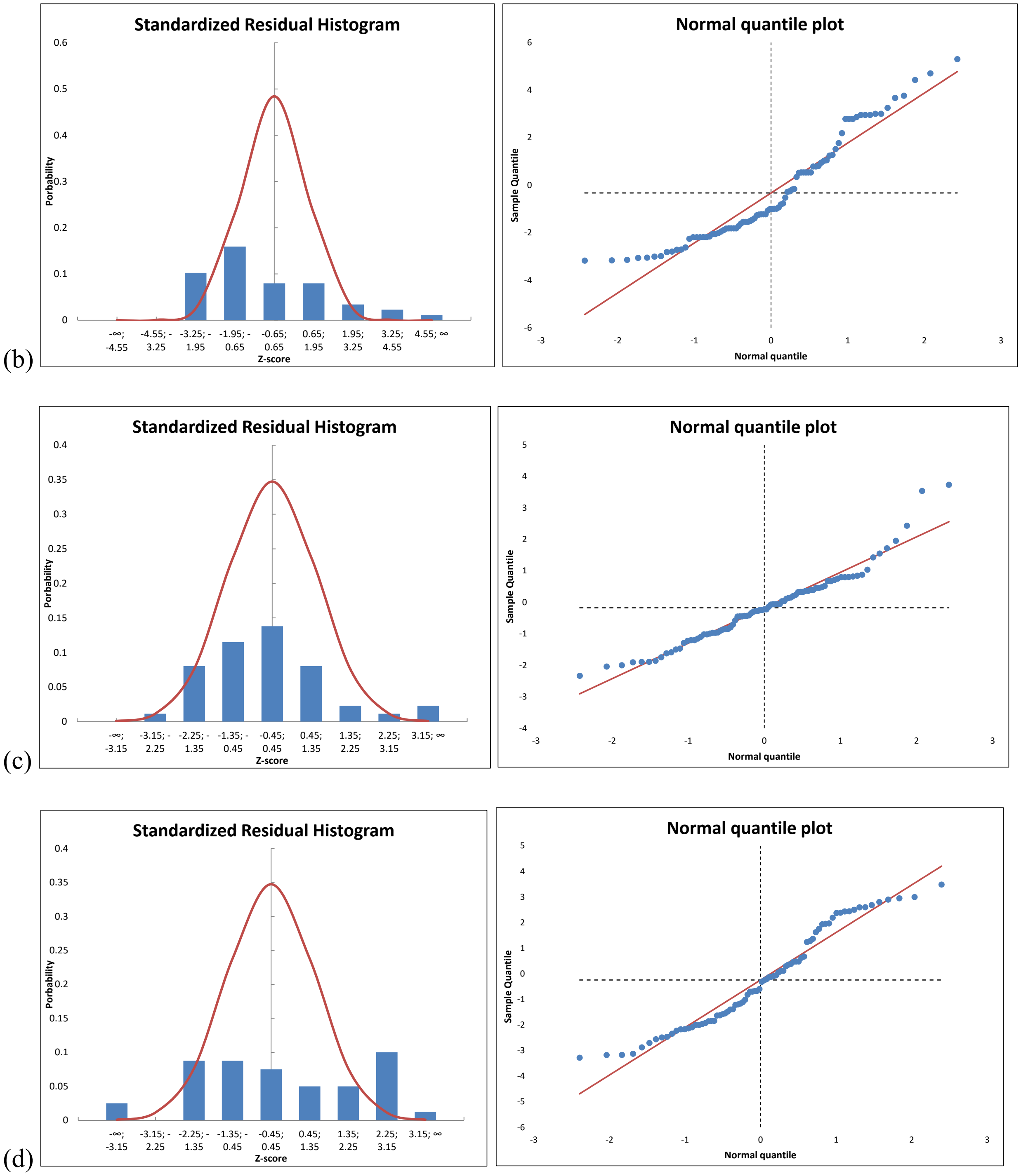

Figure 16. Standardized residual histogram and normal quantile plot between (a) pH and VFA, (b) temperature and VFA, (c) initial substrate concentration and VFA, and (d) volatile solids in food waste and VFA

The findings suggest that high heterogeneity among operational parameters and volatile solids in food waste have high variability in their effects on VFA production. Studies exhibiting statistically more homoscedasticity were with the parameter of initial substrate concentration for VFA production. A maximum positive correlation between the two was also observed with the PCA. This suggests that higher initial substrate concentrations tend to result in increased VFA production. Thus, indicating that it has a more predictable and consistent impact on VFA production. With a crucial effect of substrate concentration on VFA production as well as the composition, there is a lack of reported information on VFA composition. Majorly reported VFAs are acetic acid, butyric acid, and propionic acid. Valeric acid and caproic acid are also observed to increase in steady long-term fermentation processes due to chain-elongation [241]. This inconsistency in reported literature brings forth a partial conclusion. This makes it ideal for targeted optimization and process control for efficient and consistent VFA production especially when considering a specific VFA to be enhanced the most. The reported range for initial substrate concentration which was nearest to the mean value of 11.45 gCOD/L was 9-20 gCOD/L. This range also reported the use of operational temperature on the lower spectrum of the mesophilic temperature of 25°C and below. Thus showing the potential of exploring the lower temperature ranges by optimizing the initial substrate concentration. Additionally, with its positive correlation to incubation time, this is another important aspect that requires further research with respect to substrate concentration. The substrate composition and process temperature together have been assessed to regulate the metabolic pathway using stastical analysis by Greses *et al.,* that showed specific VFA production with substrate features [244].

Two distinct ranges of pH were observed to be primarily reported. On one higher end, was the pH range of 4-5.5, and on the other lower end, was the pH range of 7-9. pH was also uncontrolled during the fermentation in most studies. The two distinct pH ranges have specific effects on VFA production, both in terms of its overall concentration and individual VFAs being produced [245].

As for food waste characteristics, total volatile solids concentration showed systematic bias. With a lack of normal distribution, it is not a reliable predictor for VFA production. The practical implication of this observation is that substrate characteristics are used as a starting point for further optimizing the initial substrate concentration for the fermentation process based on either volatile solids or COD of substrate. This was evident throughout the studies where the characteristic volatile solids and initial substrate concentration did not correspond to each other.

5.2.6 Conclusion

This study critically evaluated multiple fermentation studies across the North American region to statistically identify relationships between food waste characteristics, and operational parameters with resultant VFA production. Among the screened 107 articles, predominant research has been performed under mesophilic conditions due to higher microbial activity. However, there is high variability among reported operational parameters like pH, temperature, initial VS concentration and COD, use of inoculum etc. With positive correlation and normal distribution, initial substrate concentration was found to be a reliable predictor for targeted optimization to enhance VFA production. The range of this variable found nearest to the mean was 9-20 gCOD/L, also corresponds to the lower spectrum of the mesophilic temperature of 25°C and below. This implicates the need for further research on optimizing the substrate concentration and exploring it in sync with lower temperatures.

5.3 Effect of different volatile solids content on the VFA production and composition

5.3.1 Introduction

Anaerobic fermentation of food waste to VFA holds considerable promise for sustainable bioconversion, yet most research has focused on mesophilic or thermophilic regimes. Our recent meta-analysis confirmed that initial VS loading, pH, and temperature are the primary factors influencing VFA yield, but nearly all of the supporting studies were conducted at mesophilic temperatures [246]. In mesophilic systems, increasing the organic load generally enhances acidogenesis. For instance, a thermophilic dark fermentation study reported ~5.3 g L^{-1} butyrate from an initial VS of approximately 26.4 g L^{-1} [247]. Similarly, semi-continuous mesophilic fermentation of municipal solid and food waste achieved total VFA concentrations of 9.8–11.5 g L^{-1}, with butyrate comprising 17–21% of the total, at VS contents of ~4–5% (w/w) [248]. These findings underscore the positive correlation between VS and VFA output under conventional temperature regimes.

However, psychrophilic fermentation deviates from these patterns. At ~17 °C, butyric acid has been observed to persist at ~0.5 g L^{-1} over several days, whereas under mesophilic conditions (37 °C), it dissipates rapidly [6]. This behavior suggests slower microbial kinetics at low temperatures may favor selective VFA accumulation by delaying downstream conversion or consumption processes. It was also noted that while propionic acid concentrations decreased in cold fermentations, butyrate remained detectable and at significantly higher levels than in mesophilic controls, indicating that low temperature may suppress competing metabolic pathways and shift VFA profiles.

Alkaline pH control is known to suppress methanogenesis and steer fermentation toward VFA production, while also supporting hydrolysis. In mesophilic food-waste systems, adjustments to neutral or alkaline pH improved both total VFA yield and acid composition, typically increasing the proportion of butyrate and acetate [249]. Maintaining pH at higher levels also buffers the system against acid-induced inhibition, potentially counterbalancing the reduced activity typical of psychrophilic microbes.

Together, these observations frame the rationale for the current experiment. The present experimental investigation thus assesses VFA production under psychrophilic (17 °C) and alkaline conditions. By exploring different initial VS loadings under simultaneous psychrophilic and alkaline conditions, this work seeks to determine whether the positive relationships between VS loading and VFA yield observed in warmer systems can be replicated or optimized in colder environments. This knowledge is essential for designing energy-efficient fermentation processes tailored to cold climates, facilitating VFA recovery from food waste without the high operational costs of heating. Through systematic variation of initial VS, selected to avoid organic overload under low-temperature conditions, this approach aims to map the response of VFA formation (and especially butyrate accumulation) to substrate concentration in the underexplored psychrophilic-alkaline domain.

5.3.2 Materials and methods

Anaerobically digested sludge was done from the Humber wastewater treatment plant in Ontario, Canada. The collection of food waste was done from organic waste generated from different restaurants at York Lanes, York University in Toronto, Canada. Characterization for both food waste and sludge was performed as described under Chapter 4, Section 4.1 (Table 9). Thermal-alkaline pretreatment was performed on the food waste according to Banu *et al.* 2020 [180] as described under Chapter 4, Section 4.3.

Table 9. Characterization of food waste and anaerobically digested sludge

Characteristics	**Food Waste**	**Anaerobically Digested Sludge**
pH	5.7 ± 0.01	7.1 ± 0.03
TS* (g/L)	181.83 ± 2.5	7.9 ± 0.20
VS* (g/L)	174.9 ± 2.3	4.9 ± 0.13
TCOD* (g/L)	131 ± 0.2	22 ± 0.09
SCOD* (g/L)	120 ± 0.3	17 ± 0.06
TN* (g/L)	9.2 ± 0.01	2.1 ± 0.03
TKN* (g/L)	8.8 ± 0.01	1.9 ± 0.01

*SCOD – Soluble Chemical Oxygen Demand, TCOD – Total Chemical Oxygen Demand, TKN – Total Kjeldahl Nitrogen, TN – Total Nitrogen, TS – Total Solids, VS – Volatile Solids

Batch experiments were set up as described under Chapter 4, Section 4.4. Analytical methods, bacterial community diversity, and statistical analysis are described under Chapter 4 Section 4.9, 4.10, and 4.11 respectively.

5.3.3 Results and discussion

The temporal profiles of SCOD exhibited a clear dependence on the initial VS concentration, with all treatments showing a gradual increase during the early stages of fermentation, followed by a progressive decline after reaching a maximum between days 5 and 6 (Figure 17). The highest SCOD was obtained at 3.5 g VS (32.4 ± 0.6 g/L), closely followed by 3.0 g VS (30.9 ± 0.5 g/L). In contrast, the 2.0 g and 1.2 g VS sets reached lower peaks of 27.3 ± 0.7 g/L and 26.2 ± 0.8 g/L, respectively. The positive correlation between organic loading and SCOD during the hydrolytic phase is consistent with findings from mesophilic fermentations of food waste and organic fraction of municipal solid waste, where higher VS inputs enhanced the extent of particulate solubilization through increased substrate availability for hydrolytic enzymes [81], [250]. Under alkaline conditions, the elevated solubilization can be further attributed to the chemical hydrolysis of complex polymers such as proteins, lipids, and carbohydrates, as the deprotonation of functional groups facilitates polymer chain cleavage and cell wall disruption [75], [83].

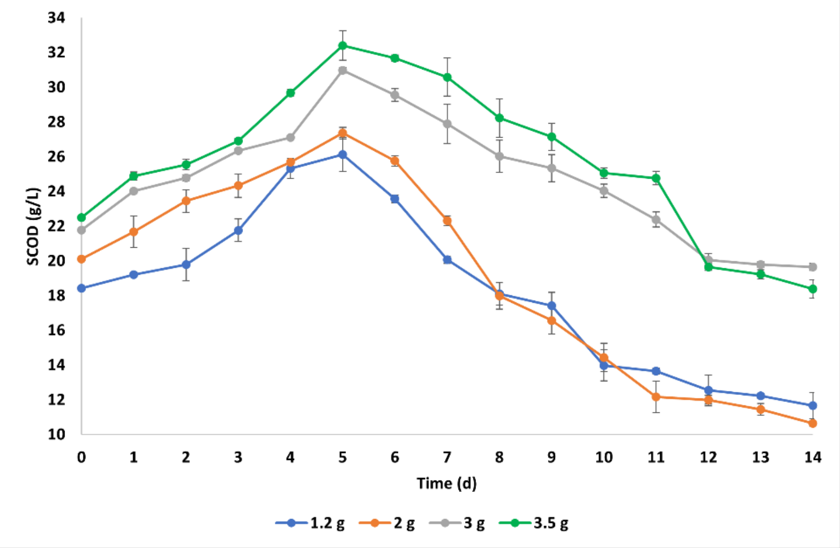

Figure 17. SCOD trend for pretreated food waste fermentation with different initial VS concentrations

The subsequent decline in SCOD after day 6 reflects the microbial consumption of solubilized intermediates during acidogenesis. The relatively smaller difference in SCOD between 3.0 g and 3.5 g VS (~5.5%) suggests that hydrolytic activity approached saturation, where additional particulate matter at higher loading did not translate proportionally into solubilized COD. Similar inhibitory effects have been observed in mesophilic anaerobic systems when organic loading surpasses the microbial processing capacity, resulting in VFA accumulation, pH depression, and process instability [251]. Under psychrophilic conditions, this limitation is exacerbated by slower enzymatic kinetics, reduced microbial growth rates, and the possibility of mass transfer constraints at elevated solids content.

Total VFA production coincided with SCOD trends, with concentrations increasing during the first 5–6 days, reaching a maximum, and then declining gradually (Figure 18). The highest total VFA concentration was recorded at 3.5 g VS (2.85 ± 0.13 g/L), followed by 3.0 g VS (2.12 ± 0.11 g/L), 2.0 g VS (1.73 ± 0.09 g/L), and 1.2 g VS (1.34 ± 0.08 g/L). The strong positive relationship between VS and VFA yield in the initial phase aligns with earlier mesophilic studies reporting that higher organic loadings increase the supply of fermentable substrates, thereby promoting acidogenesis [252]. A study using microalgal biomass in continuous reactors found that VFA conversion efficiency rose from 0.30 ± 0.02 at 3 g COD L^{-1} d^{-1} to 0.37 ± 0.02 at 12 g COD L^{-1} d^{-1}, but did not increase further at 15 g COD L^{-1} d^{-1}—indicating possible acidogenesis inhibition at the highest loading [253]. However, the relative gain from increasing VS from 3.0 g to 3.5 g was modest (≈34% higher VFA concentration), indicating that at this temperature and pH, the fermentative capacity may be approaching its optimum. Moreover, the difference found between the two VS concentrations was found to be not significant with *p* value >0.05. Beyond this point, further increases in VS could risk organic overloading, leading to localized accumulation of acids and inhibitory conditions that are particularly detrimental under low-temperature operation [254].

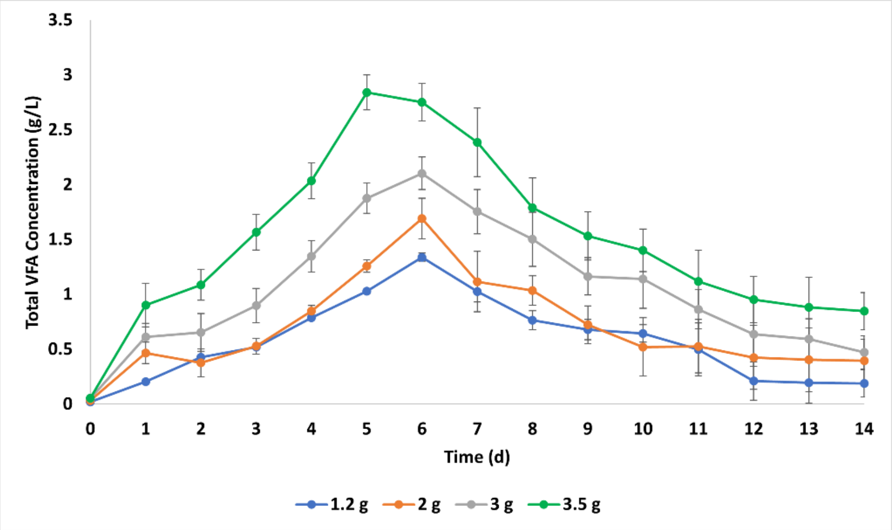

Figure 18. Production trend of total VFA from pretreated food waste fermentation with different initial VS concentrations

The decline in VFAs post-peak may be attributed to factors such as: (i) consumption by syntrophic acetate-oxidizing bacteria, which can survive even under alkaline stress [255]; and (ii) depletion of readily fermentable substrates, as indicated by the concurrent SCOD decrease [256]. The sharper decline in the 1.2 g and 2.0 g VS sets suggests faster exhaustion of the soluble substrate pool when the total organic load is limited.

Analysis of individual acid profiles revealed acetate as the dominant product, followed by butyrate. Higher VS loadings resulted in a marginally greater proportion of butyrate, suggesting a shift towards more reduced metabolic pathways when substrate availability increases. This shift may be attributable to greater release of fermentable sugars and long-chain fatty acids, supplying surplus electron donors that facilitate chain-elongation pathways toward C_4 products—a phenomenon well-described in mixed-culture fermentations [257]. Under alkaline conditions, thermodynamic favorability for butyrate production increases, particularly under elevated partial pressures of hydrogen, thereby lowering the barriers for conversion from acetyl-CoA to butyryl-CoA [258]. Cold conditions slow enzymatic turnover and reduce microbial growth rates, impeding the efficiency of chain-elongation toward butyrate—a trend supported by studies showing butyrate persistence under psychrophilic fermentation due to inhibited β-oxidative activity [81].

From a process optimization standpoint, although the highest SCOD and total VFA concentrations were achieved at 3.5 g VS, the differences relative to 3.0 g VS were not statistically significant ($p > 0.05$). In such cases, selecting the lower VS loading is advantageous to minimize the risk of organic matter accumulation and potential process instability during extended operation, particularly under psychrophilic conditions where hydrolysis and fermentation kinetics are inherently slower [81]. Operating near the threshold of hydrolytic and fermentative capacity can lead to excessive residual solids, localized acid build-up, and inhibitory effects on microbial activity, a phenomena well documented in mesophilic systems at high organic loadings [252], [254]. By contrast, moderate organic loading levels have been shown to maintain stable acidification, prevent accumulation of refractory organics, and sustain higher microbial activity over longer retention periods [249], [250]. Therefore, the selection of 3.0 g VS as the working loading offered a statistically supported initial VS concentration to maximize VFA under psychrophilic temperatures for further experiments.

5.3.4 Conclusion

This experiment demonstrates that under pH 9 and 17 °C, initial VS loading exerts a strong influence on substrate solubilization and VFA production from food waste. Both SCOD and total VFA increased with organic loading, but gains diminished at higher concentrations, with no statistically significant difference between 3.0 and 3.5 g VS. Acetic acid remained the dominant product, while higher VS loadings slightly favored butyrate accumulation, reflecting shifts toward more reduced metabolic pathways under abundant substrate conditions. The selection of 3.0 g VS as the optimal loading balances VFA productivity with process stability, avoiding the potential risks of organic overload in low-temperature systems. These findings help identify suitable substrate loading for subsequent experiments aimed at improving VFA production under cold-climate fermentation conditions.

CHAPTER 6

Enhancement of Hydrolysis for VFA Production Under Psychrophilic Temperature

6.1 - Acidogenic fermentation of pretreated food waste under psychrophilic conditions for volatile fatty acids production

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Kumar *et al*., "Acidogenic fermentation of pretreated food waste under psychrophilic conditions for volatile fatty acids production" *Biofuels, Bioproducts, and Biorefining* (Under review post revision since November 2025)

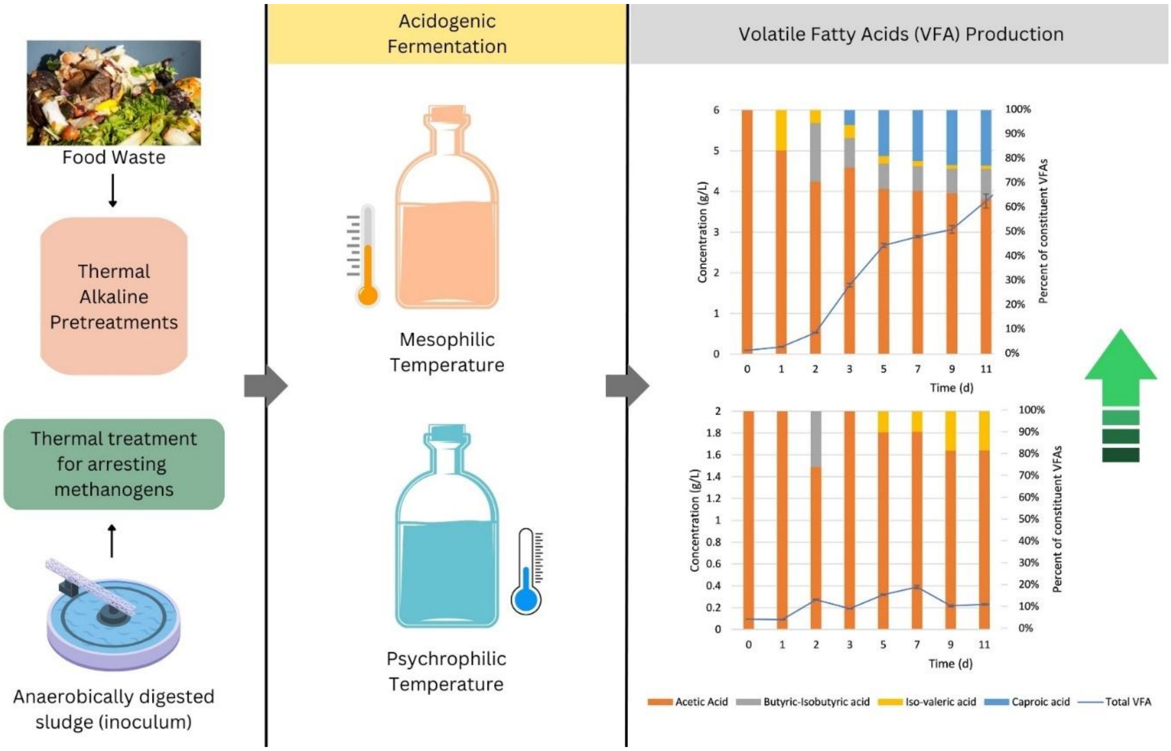
Contribution Statement: I conducted the background literature review; designed the experiments; performed all laboratory work, data collection, and primary analyses; created figures/tables; and wrote the manuscript. This chapter is adapted from the submitted article, with experimental setup concisely summarized here and full methodological details provided in Chapter 4, and formatting aligned with the thesis.

6.1.1 Abstract

Mixed culture fermentation generally produces volatile fatty acids (VFAs) as a mixture of C2-C6 acids. Among operational parameters, temperature has a distinct effect on the microorganisms' metabolic activity, which can shift the composition of the metabolites. Performing food waste fermentation at mesophilic or thermophilic temperatures results in a rich mixture of metabolites where further extraction of targeted VFAs can be challenging. Moreover, in colder countries, it is energy-intensive when the average temperatures remain under psychrophilic ranges for most of the year. This study evaluated the production of VFAs under the psychrophilic temperature of 17°C in comparison to the mesophilic temperature of 37°C after thermal-alkaline pretreatment of the substrate to enhance hydrolysis. With pretreatment at 120°C and pH 9, the concentration of total VFAs was 1 g/L under psychrophilic temperature. This also resulted in a less complex VFA mixture with acetic acid as the major constituent. This enhanced acetic acid production was due to the increased abundance of *Hafnia* and *Fonticella* species, mostly responsible for acetic acid production. The estimated power consumption also highlighted the mesophilic operation to be more than twice as intensive as the psychrophilic operation. The use of psychrophilic temperature and substrate pretreatment selectively promotes the abundance and activity of microorganisms towards a more tailored generation of VFAs.

Keywords: Mixed culture fermentation, psychrophilic temperature, thermal-alkaline pretreatment, acetic acid, chain elongation

6.1.2 Graphical Abstract



6.1.3 Introduction

Food waste is an appealing source for producing value-added products such as volatile fatty acids (VFAs) through fermentation. The VFAs are important building block chemicals with increasing market demand at 3% each year with a projected demand of around 19,000 kilotons by the year 2023 [259]. VFAs have a wide range of potential applications, varying with their carbon chain length (C2-C6) for producing bioplastics, biofuels, textiles and flavouring agents [2].

In the acidogenic fermentation process, different operational parameters such as temperature, pH, and solid and liquid retention time regulate the complex metabolic reactions and conversion of substrates into target metabolites [237]. Fermentation processes can be psychrophilic (10 to 30°C), mesophilic (30 to 40°C), and thermophilic (50 to 60°C) [260]. Multiple studies have been undertaken to enhance VFA production under mesophilic and thermophilic temperatures. However, a relatively high amount of energy is needed to maintain the system at these temperatures [191]. At industrial scale, VFAs have been produced from sludge fermentation between 20-25°C for downstream applications such as removing nitrogen from wastewater [176]. There is still limited research on acidogenic fermentation below 30°C, or psychrophilic ranges, primarily due to slow microbial activity and resultant low VFA production even though the capital and operational costs can be 20-40% lower than the processes run at thermophilic temperatures [261]. Moreover, the complexity of the metabolite mixture obtained in mesophilic and thermophilic temperatures is challenging for single VFA extraction. Concentrating and separating VFAs individually either suffers from low yield or high expenses in terms of chemical requirements [262]. The prospect of obtaining a predominance of a single VFA by mixed culture fermentation is therefore lucrative. Pressure-driven extraction techniques are also energy-intensive [263]. A targeted approach towards producing all or specific VFAs using mixed-culture fermentation by optimizing parameters towards selective metabolic activity can thus reduce the complexity of the mixture.

Temperature significantly influences the efficiency of acidogenic fermentation by exerting a strong impact on the activity of acid-forming microorganisms. It affects the metabolism of these microorganisms at a biochemical level by altering the changeable shift between glycolysis and the pentose-phosphate pathway [65]. *Enterobacter* and *Bacillus* species have been observed to show increased pentose-phosphate pathway activity at lower temperatures and increased glycolysis activity at higher temperatures [66]. To the knowledge of the authors, with respect to food waste, studies at psychrophilic temperatures are scarce and due to the difference in composition, the fermentation process is not directly comparable to other substrates like sludge and manure [264], [265]. The slower metabolic rate extends the time required for the complete breakdown of organic matter in the food waste. Moreover, the active microbial diversity is limited which may require additional process optimization. Psychrophilic conditions can result in incomplete degradation along with the accumulation of other metabolites such as alcohols and lactic acid which can be inhibitory for eventual VFA generation. This happens because the syntrophy between hydrolytic and acidogenic bacteria gets affected when interspecies electron transfer gets affected [67]. Pretreatment could be used to increase the rate of hydrolysis since it is known to promote substrate hydrolysis by increasing the surface area of food waste constituents for effective biodegradation and lowering the degree of polymerization [68], [69]. This, in turn, increases VFA production by increasing the availability of simpler or smaller-sized molecules in the aqueous phase in less process time. Amongst different pretreatment methods, a combination of thermal-alkaline pretreatment can offer an efficient method of enhancing the hydrolysis of the substrate. It is a simple method which can easily be incorporated into the system industrially without significant infrastructural modifications [266], [267].

In this study, food waste was subjected to fermentation under psychrophilic conditions after thermal-alkaline pretreatment to enhance VFA production and assess the effect on VFA composition in comparison to mesophilic temperature. Furthermore, the bacterial diversity changes were observed under psychrophilic temperature for the initial time period of 3 days.

6.1.4 Materials and Methods

Collection of the anaerobically digested sludge was done from the Clarkson wastewater treatment plant in Ontario, Canada. The collection of food waste was done from organic waste generated from different restaurants at York Lanes, York University in Toronto, Canada. Characterization for both food waste and sludge was performed as described under Chapter 4, Section 4.1 (Table 10). Thermal-alkaline pretreatment was performed on the food waste according to Banu *et al.* 2020 [180] as described under Chapter 4, Section 4.3. In the control set, there was no pretreatment performed, i.e. untreated food waste was used.

Table 10. Characterization of food waste and anaerobically digested sludge

Characteristics	**Food Waste**	**Anaerobically Digested Sludge**
pH	5.8 ± 0.1	7.1 ± 0.1
TS* (g/L)	205.5 ± 0.8	7.05 ± 0.06
VS* (g/L)	198.1 ± 0.7	4.8 ± 0.1
TCOD* (g/L)	88 ± 0.2	29 ± 0.09
SCOD* (g/L)	71 ± 0.3	18 ± 0.06
TN* (g/L)	7.9 ± 0.02	2.4 ± 0.01
TKN* (g/L)	7.8 ± 0.01	2.3 ± 0.01

*SCOD – Soluble Chemical Oxygen Demand, TCOD – Total Chemical Oxygen Demand, TKN – Total Kjeldahl Nitrogen, TN – Total Nitrogen, TS – Total Solids, VS – Volatile Solids

Batch experiments were set up in duplicate with a working volume of 200 mL in 500 mL serum bottles as described under Chapter 4, Section 4.3. Analytical methods, bacterial community diversity, and statistical analysis are described under Chapter 4.

6.1.5 Results and Discussion

Effect of substrate preparation on pH during fermentation

The initial pH set for this experiment was 9 which declined over time and only minor fluctuations were observed from day 5 to day 11. A steep drop in pH was observed under the mesophilic temperature of 37°C (Fig.19). This correlates with the increasing total VFA concentration (Fig. 20a). Meanwhile, the drop in pH for the control under the psychrophilic temperature of 17°C could be mainly attributed to lactic acid accumulation owing to the dairy-based constituents of food waste [268]. This observation was made in a preliminarily performed experiment under similar conditions where lactic acid accumulated more under low temperature by about 4 times at 4 g/L as opposed to mesophilic temperature at less than 0.5 g/L, resulting in a more acidic pH. Other studies have also reported higher lactic acid as opposed to VFAs under psychrophilic temperature. For instance, Komemoto *et al.,* observed the generation of lactic acid, VFAs, and biogas using 300 g wet weight of 94% VS comprising food waste under pH 7 and different temperature conditions. They observed that at 15°C and 25°C, lactic acid accumulated longer in the system as opposed to mesophilic and thermophilic temperatures [269]. Furthermore, an abundance of lactic acid bacteria is observed to deviate fermentation under low temperatures and compete with other microorganisms such as hydrogen-producing bacteria as well, as observed by Rodríguez-Valderrama *et al* [270].

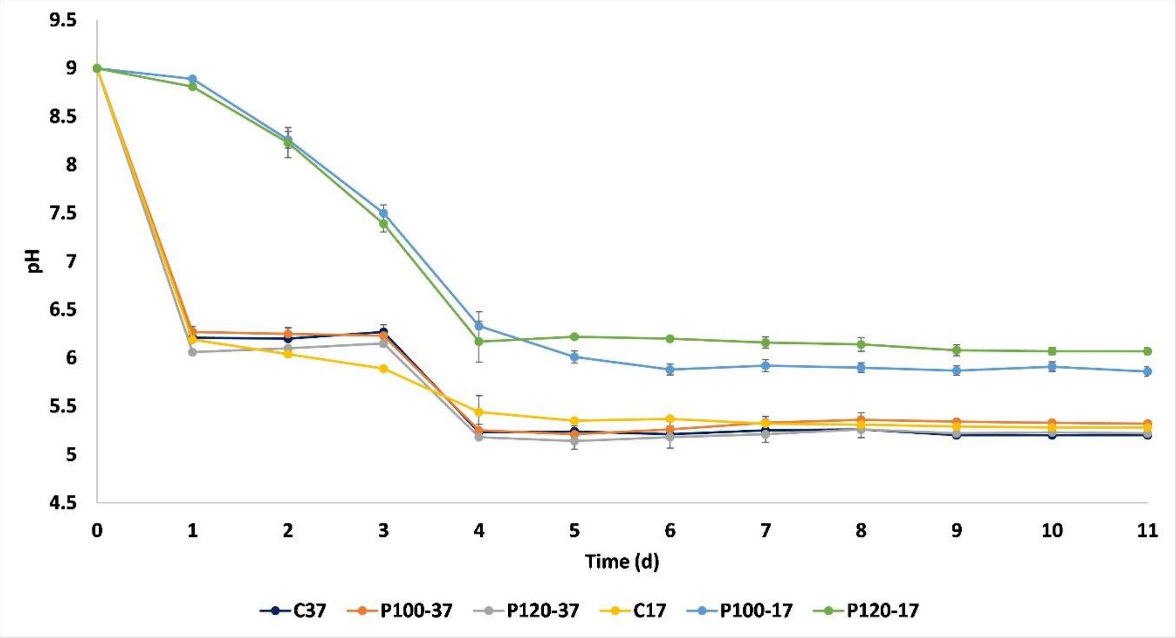


Figure 19. Trend of pH across 11 days for C37 (control at 37°C), P100-37 (100 °C pretreatment, 37°C fermentation), P120-37 (120°C pretreatment, 37°C fermentation), C17 (control at 17°C), P100-17 (100°C pretreatment, 17°C fermentation), and P120-17 (120°C pretreatment, 17°C fermentation).

In comparison, pH drop in mesophilic conditions corresponded to increasing VFA concentration. Under psychrophilic temperatures, when the microorganisms are in their adaptation phase, they accumulate more lactic acid and TCA cycle intermediates which promote cell growth at low temperatures. This happens because of down-regulation of primary metabolic pathways such as glycolysis, pentose phosphate pathway, and TCA cycle to reduce the generation of reactive oxygen species as a stress response until they adapt. [222]. The pretreated substrate conditions under psychrophilic temperature showed a delayed drop in pH corresponding to the total VFA concentration. While lactic acid accumulates under control conditions at 17°C, the thermal-alkaline pretreatment can be conducive to lactic acid production by eliminating lactic acid-producing bacterial species under *Lactobacillus* and *Clostridiceae*. Li *et al.,* observed in their study for lactic acid production from food waste, that thermal pretreatment temperature around 110°C resulted in reduced lactic acid compared to the untreated substrate [181]. The microbial diversity in the mixed culture thus allowed for VFA production over lactic acid accumulation. The self-stabilization of pH occurred over time in all the conditions could be due to the buffer effect from ammoniacal nitrogen produced by protein degradation [271]. Our preliminary experiments showed that the initial ammonia concentration decreased with time and this correlated with a stable pH during the food waste fermentation (data not shown). Moreover, the ammonia concentration is reported to be lower under psychrophilic temperatures which also prevents its inhibitory activity against VFA generation. This was observed by Ortner *et al.,* where the total VFA generated using slaughterhouse waste at 25°C increased with a simultaneous decrease in free ammonia nitrogen concentration. While at 37°C, the free ammonia nitrogen was higher in concentration and was inhibitory to the system [272]. Likewise, the decrease in ammonia concentration at 17°C was favourable for both pH stabilization and subsequent VFA generation.

Changes in VFA concentration and composition

The SCOD of the food waste increased after pretreatment from 63.4±0.3 g/L to 78±0.2 g/L respectively. This supported hydrolysis by increasing the availability of simpler compounds in the substrate for the microorganisms to assimilate. The increasing SCOD values (Fig.20b) during fermentation corresponded to increasing VFAs and decreasing pH. However, under a closed batch system, further VFA generation from SCOD conversion is inhibited due to nutrient limitation and the presence of long-chain fatty acids (LCFAs) in food waste. Xu *et al.,* reported that the activity of certain enzymes in VFA generation such as α-glucosidase, lipase, F420 etc., is reduced by the presence of LCFAs in food waste which adheres to the bacteria and restrains the enzyme-substrate interaction [273].

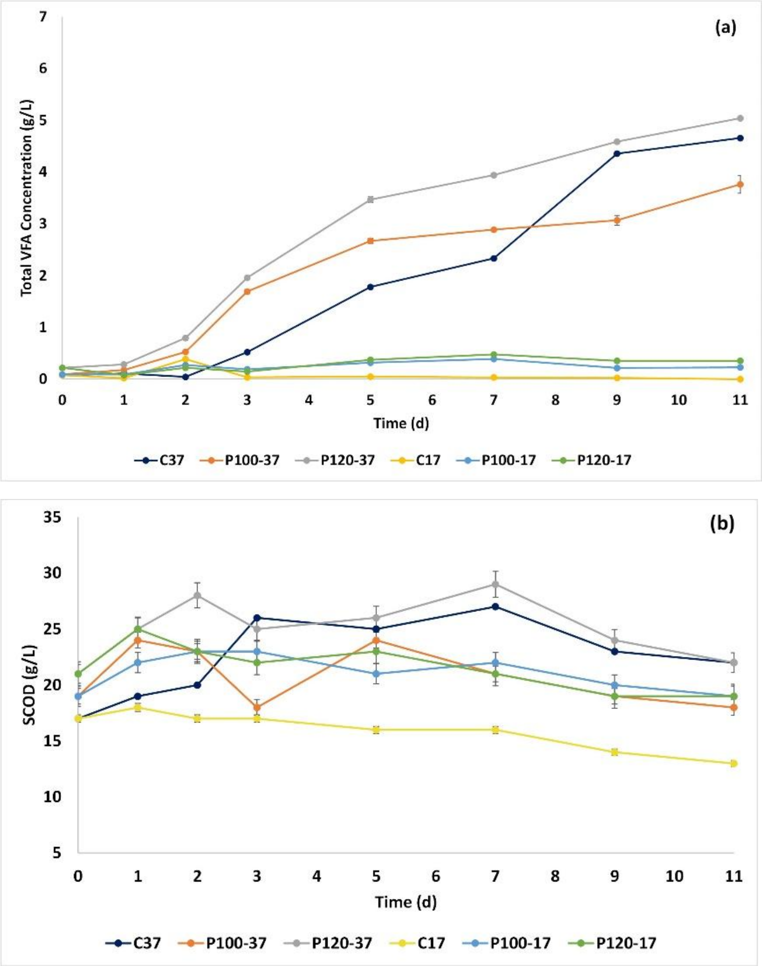


Figure 20. (a) Concentration of total VFA (g/L) generated and (b) SCOD across 11 days for C37 (control at 37°C), P100-37 (100 °C pretreatment, 37°C fermentation), P120-37 (120°C pretreatment, 37°C fermentation), C17 (control at 17°C), P100-17 (100°C pretreatment, 17°C fermentation), and P120-17 (120°C pretreatment, 17°C fermentation).

Under both mesophilic and psychrophilic conditions, the DA was higher for the assays using substrate pretreated at 120°C (Table 11). This shows the enhanced acidogenesis in addition to hydrolysis. The observed DA was higher than the reported 6-48% in other studies using food waste as substrate [274], [275], [276]. For psychrophilic operational temperature, an improved DA due to substrate pretreatment has not been reported earlier.

Table 11. Degree of acidification (DA) under various conditions tested in this study.

Temperature	**Pretreatment condition**	**DA**

37°C	Control - Untreated FW	38±0.1%
	Pretreatment 1 - Alkaline pH set to 9 followed by thermal treatment at 100°C for 1 hour	35±1.3%
	Pretreatment 2 - Alkaline pH set to 9 followed by thermal treatment at 120°C for 1 hour	58±0.5%
17°C	Control - Untreated FW	1±0.01%
	Pretreatment 1 - Alkaline pH set to 9 followed by thermal treatment at 100°C for 1 hour	14±0.7%
	Pretreatment 2 - Alkaline pH set to 9 followed by thermal treatment at 120°C for 1 hour	17±0.9%

The highest concentration of total VFA (5 g/L) was obtained for the treatment using substrate pretreated at 120°C fermented under mesophilic conditions, while the control also showed comparable amount of VFA. These values correspond to the total VFA concentrations reported in the literature ranging from 3 to 7 g/L under similar mesophilic temperatures and pH as well as anaerobically digested sludge as an inoculum source [277], [278], [279]. While the pretreatment was performed for both temperature conditions to compare its effect on the production of VFAs, it was targeted especially for psychrophilic temperature to increase hydrolysis. The control group under psychrophilic temperature showed an undetectable amount of total VFAs and pretreated conditions showed increased VFA amount. The extensive lag observed under psychrophilic temperature is often associated with the inactivity of the mesophilic microbes at low temperatures [280]. Moreover, with the accumulation of lactic acid and low pH, the conditions become unsuitable and inhibitory for further VFA production (Appendix 8). However, the substrate pretreatment allowed increased VFA production with maximum values on day 7 at 1 g/L with both pretreatments under psychrophilic temperature. On performing ANOVA to test the statistical difference of VFA concentrations achieved under different pretreatment conditions and temperatures, the p-value was found to be <0.05, thus showing the data to be significant. Under two pretreatment conditions, pretreatment 2 at 120°C and pH 9 showed higher significance with respect to total VFAs ($p < 0.01$).

Table 12 shows a few examples of studies undertaken at psychrophilic temperatures. The VFA value obtained in this study is comparable to the values found in the literature at low temperatures. However, the use of psychrophilic temperatures has been assessed for the anaerobic digestion process for biogas generation as the primary objective while monitoring VFAs as intermediates. With VFA production as the primary objective, the fermentation process can be optimized further to bring VFA production from the pretreated substrate up to the range of mesophilic temperature. Furthermore, since VFAs were observed to increase later, the initial lag phase can further be reduced by acclimatizing the inoculum in the future or understanding the microbial diversity to better adjust the operational parameters. With reduced lag phase, the adapted microorganisms can perform acidogenesis earlier.

Table 12. VFA production reported in fermentation/digestion studies performed under psychrophilic temperature.

Feedstock & inoculum	Operational conditions	VFAs	Reference
Food Waste and digested sludge	Batch reaction, 17°C, initial pH 9	1 g/L after pretreatment	This study
Food waste	Batch reactor, 15°C, pH 7	<1 g/L acetic acid	[269]
Wetland plant litter	Batch reaction, 10 & 15°C, pH 7 & 12	1.1 g/L at 15°C & pH12	[281]
Corn straw & cattle manure	Batch bioreactor, 20°C	3-4 g/L	[282]
Dairy manure & biochar	Batch reaction, 20°C, initial pH 7.7	<1 g/L	[62]
Paunch	Leach bed dry digestion, 22°C	7 g/L	[55]
Food waste	Batch reaction, 20°C	6.6 g/L	(Li et al., 2017)
Food waste	Continuous Stirred-Tank reactor, 25°C	3 g/L	[283]

Amongst the produced VFAs under all conditions, acetic acid was predominant, forming about 50% of total VFA (Fig.21). With food waste as substrate, acetic acid has been previously reported to form 50-70% of the total VFAs produced due to the higher content of carbohydrates that serve as the carbon source during its breakdown [29], [55], [269], [284], [285]. The remaining VFAs, including propionic acid, butyric acid, and valeric acid, were found to be very low in concentration. Normally, the concentration of propionic acid is observed to be higher with the digestion process under psychrophilic temperature, the results obtained with this fermentation process were found to be in contrast [55]. As reviewed by Chen *et al.,* most studies have shown propionic acid generation within acidic pH range of 5-5.5, [26] however, the initial pH for this study was alkaline. Furthermore, lactic acid is a key substrate for predominantly found *Propionibacteria* spp., to produce propionic acid. Thus, reduced lactic acid due to pretreatment would eventually be conducive for *Propionibacteria* activity. The accumulated values obtained for propionic acid at 17°C were approximately 0.01 g/L for pretreated substrates. Both pretreatment at 100°C and 120°C showed more acetic acid generation compared to control sets under psychrophilic and mesophilic conditions. This shows that the major metabolic pathway in play initially was acetate-type, taking precedence over the mixed-type pathway due to low concentrations of other VFAs in the beginning and the nature of the substrate [24].

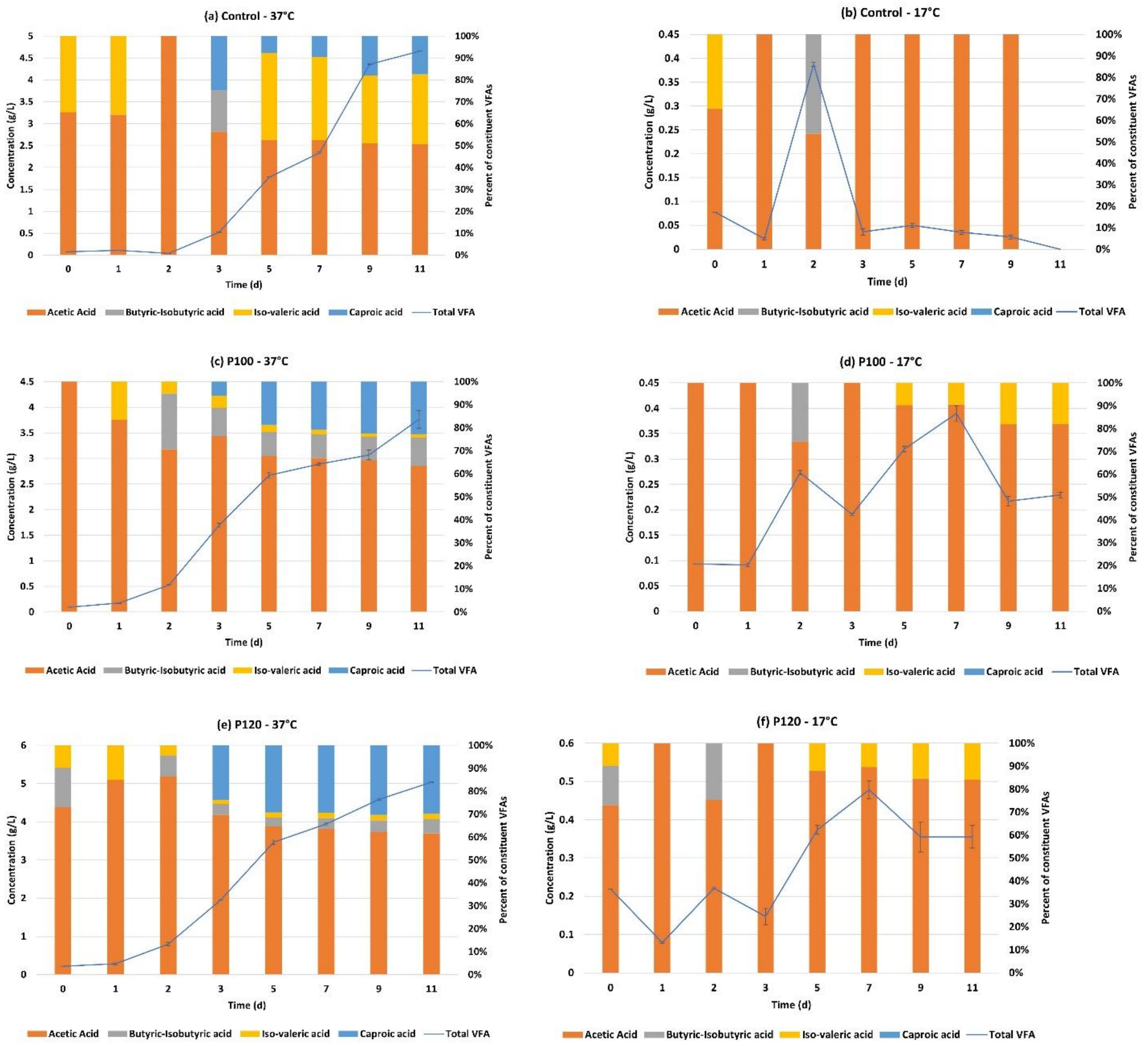


Figure 21. Percentage of constituent VFAs and total VFA generation trend. (a) Untreated food waste (control) at 37°C, (b) Untreated food waste (control) at 17°C (c) 100°C-pH9 Pretreatment and 37°C fermentation, (d) 100°C-pH9 Pretreatment and 17°C fermentation, (e) 120°C-pH9 Pretreatment and 37°C fermentation, and (f) 120°C-pH9 Pretreatment and 17°C fermentation

The values under mesophilic temperature were 2-3-folds higher than those of psychrophilic temperature along with 10-30% accumulation of caproic acid. However, the acetic acid values kept increasing till day 11 under 17°C. Thus, psychrophilic temperature allowed for a less complex VFA mixture with acetic acid as the major constituent. This reduces the complexity of downstream extraction. The delay in acetic acid production can be correlated to the initial lag phase for the inoculum, where they adapt to psychrophilic conditions [192].

Chain elongation was observed with increasing concentrations of caproic acid with increasing fermentation time at 37°C. A maximum concentration of 1.5 g/L caproic acid was observed on day 11 with pretreatment 2, forming about 23% of the total VFAs accumulated. The control and pretreatment 1 resulted in comparable values of approximately 1.1 g/L. The increasing values of caproic acid corresponded to the decreasing values of acetic and butyric acids, inferring the process of chain elongation. Chain elongation requires even-carbon VFAs (acetic and butyric acid) as well as accumulation of electron donors such as ethanol and lactic acid. The electron donor provides acetyl-CoA for reverse β-oxidation of the shorter chain VFAs like acetic and butyric acids. Each step of reverse β-oxidation increases the chain length by two carbon atoms. Ethanol is known to be a preferred electron donor to lactic acid due to its involvement in other metabolic pathways [286]. However, with food waste as the substrate, the accumulation of lactic acid is higher than ethanol. Operationally, this process takes place under a longer retention time, as is also observed in this study [205]. However, at lower pH values, the chain elongation is non-optimal, exacerbated by psychrophilic temperatures.

Under a psychrophilic temperature of 17°C, butyric acid was detected at minute concentration from day in pretreated sets. This also corresponds to the increased presence of acetic acid simultaneously. Here, the acetogenic bacteria can consume CO_2 and hydrogen gas to generate acetic acid via acetogenesis. Since this fermentation process was of mixed-acid type, the simultaneous generation of ethanol and lactic acid by lactic acid bacteria with genera *Lactobacillus* and *Lactococcus* [287], [288] would again provide necessary electron donors for increasing acetic acid. Compared to mesophilic temperature conditions, the acetic acid was produced more in a longer period. With further studies, different operational parameters can be tested in psychrophilic temperatures to selectively enhance particular VFAs.

Bacterial diversity changes after pretreatment under psychrophilic temperature

The initial changes in bacterial diversity were analyzed under psychrophilic temperature to compare the difference between untreated and pretreated substrate. The dominant taxon at the phylum level found under control conditions was *Firmicutes*. The lesser presence was from *Chloroflexi, Bacteriodota,* and *Proteobacteria*. Under pretreated conditions, *Proteobacteria* was found to be the dominant taxon along with *Firmicutes*. At the genus level, the abundance of *Sporosarcina* under control conditions was shifted to *Hafnia_obesumbacterium* on day 0. On day 2 and 3, further shift in abundance was observed towards *Enterococcus*, *Fonticella,Carnobacterium,* and *Yersinia*. Pretreatment 1 condition also showed more prevalence of *Bacillus,* and *Clostridium_sensu_stricto_11* (Fig.22). The overall bacterial diversity increased after pretreatment, changing the dynamics of colonization under psychrophilic temperature.

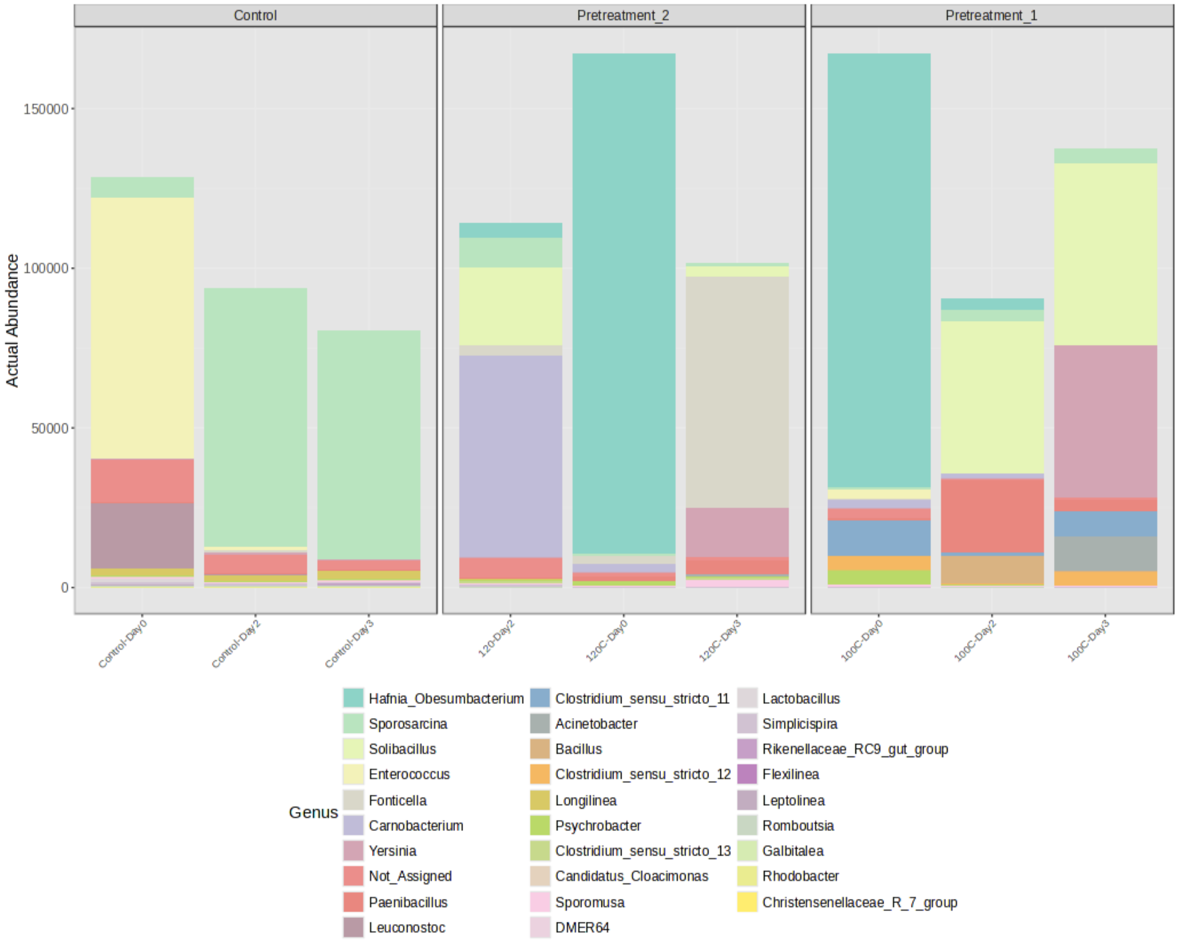
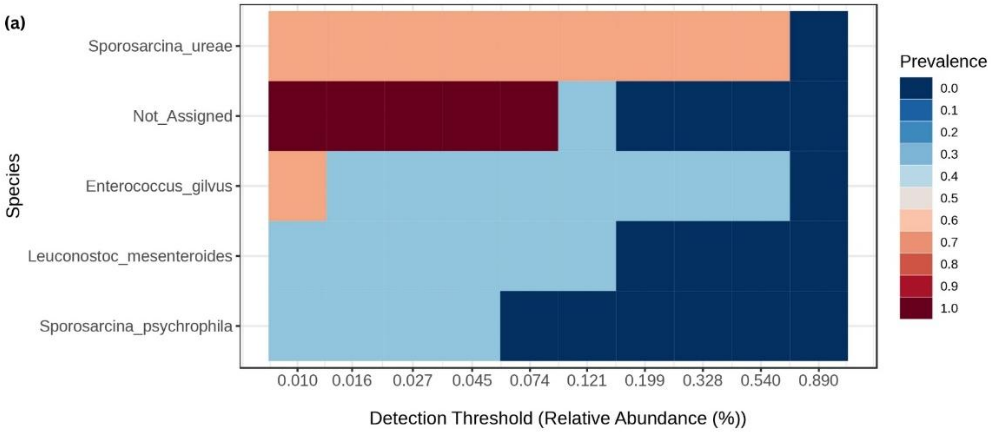


Figure 22. Bar graph showing actual abundance of different genera under 17ºC.

To assess the core bacterial species during fermentation, the heat map was generated (Fig.23), showing relative abundance (%) and prevalence on a scale of 0.0 to 1.0. The highest relative abundance under control was not assigned to a specific species when checked against SILVA database. However, it was followed by *Solibacillus silvestris*. These are closely related to fermentative bacteria of genus *Bacillus* and often found in clusters together. This fermentative bacteria shows high proteolytic enzymatic activity, often used for the enzyme production of alkaline protease [210]. Similarly, following an unassigned category, high relative abundance is observed for *Hafnia_obesumbacterium_alvei* alongside *Solibacillus silvestris,* followed by *Carnobacterium_inhibens*. *Hafnia_obesumbacterium* spp., compete with lactic acid producing bacteria to promote production of other fermentative metabolites. As observed with plant-based substrates, these species promote protein breakdown. Their abundance shifts with respect to lactic acid producers and are sensitive to acidic pH [289]. Under low temperatures, *Hafnia alvei* is known to produce volatile compounds by transforming methionine and cysteine amino acids via deamination and transamination [290]. Thus, psychrophilic temperature of 17°C promoted its increased abundance and the subsequent VFA production under pretreated conditions that reduced its competing lactic acid producers.



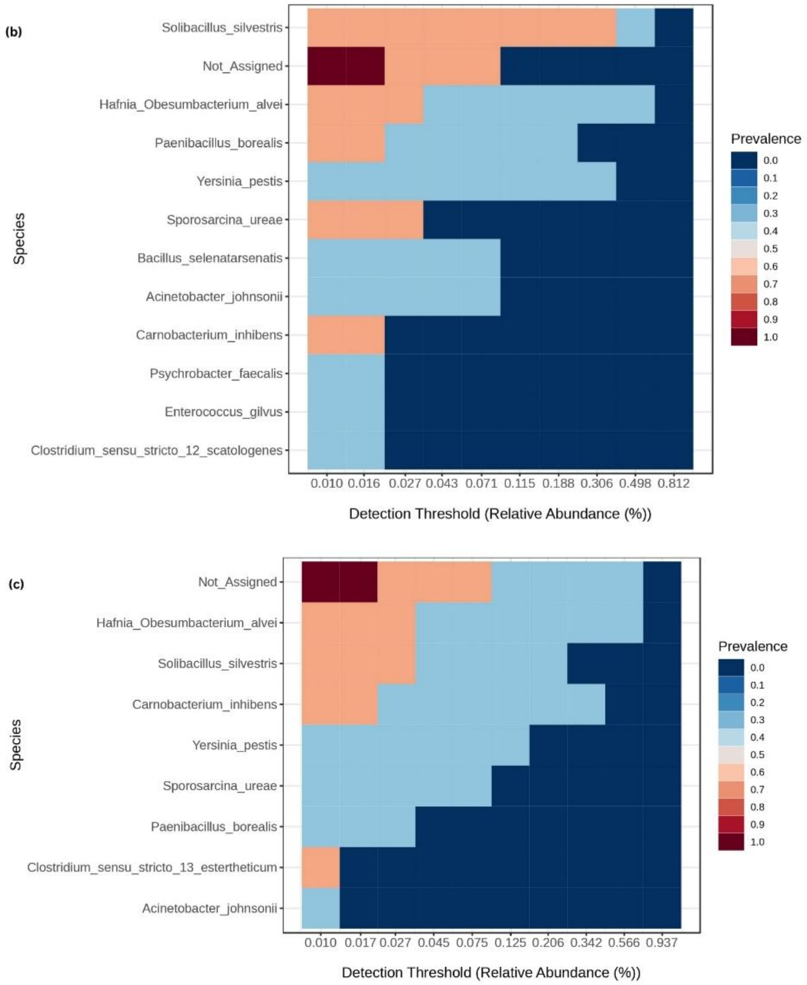


Figure 23. Heat map for relative abundance (%) of species under **(a)** control and **(b)** pretreatment 1 conditions, and **(c)** pretreatment 2 conditions at 17°C.

Furthermore, the fermentative bacteria from genus *Fonticella* showed increased abundance under pretreated sets. These bacteria enhance acetic acid production and show high resistance to harsh and unfavourable environmental conditions such as low temperature [291]. In a metagenomic study by Zhang *et al*., it was observed that during VFA production, fatty acid biosynthesis and pyruvate metabolism are upregulated by the *FASN* gene expressed by *Fonticella* spp [292]. This upregulation was closely linked to sufficient availability of the substrates that correlates to the enhanced hydrolysis with pretreatment in our study where acetic acid was the abundant VFA.

Power consumption during incubation at two temperatures (Preliminary studies)

The operation costs associated with temperature maintenance can be considerable when the outside ambient temperature is low, such as between 25-20°C. The energy consumption by the shaker incubator was calculated for maintaining the two studied operational temperatures with the outside ambient temperature of 25 and 20°C respectively. The two temperatures were selected as the ambient laboratory temperature is set within this range. The heating power for maintaining 37°C for 11 days in the shaker incubator as per the two ambient temperatures was 146 kWh and 145 kWh respectively. While the heating power for maintaining 17°C for 11 days in the shaker incubator was 64 kWh and 2 kWh respectively. Thus, the power consumed at 37°C as compared to 17°C was more than twice as much at the outside temperature of 25°C and considerably more at the outside temperature of 20°C. The total costs for power consumption at the two temperatures are shown in Table 13 with the unit cost used from Ontario region of Canada for the non-residential sector under the tiered category with charges based on overall usage (Ontario Energy Board). This emphasizes the reduction in associated costs for temperature maintenance which can vary regionally as per their respective power price. To build on the findings of this study, a thorough techno-economic assessment of the fermentation process can be conducted to provide a comprehensive analysis of the associated costs including the additional pretreatment.

Table 13: Costs for temperature maintenance at 37°C and 17°C in a shaker incubator

Operating temperature	**Ambient temperature outside the incubator**	**Heating Power (kWh)**	**Unit cost (CAD/kWh)**	**Total cost (CAD/kWh)**
37°C	25°C	146	12.5	1825
37°C	20°C	145	12.5	1812.5
17°C	25°C	64	12.5	800
17°C	20°C	2	12.5	25

6.1.6 Conclusions

Performing substrate pretreatment and operating the process at psychrophilic temperature enhanced the hydrolysis and acidogenesis compared to the untreated substrate. Once adaptation to the low temperature happened, enhanced production of acetic acid was observed by 2-fold. The combined conditions of substrate pretreatment and psychrophilic temperature allowed for a less complex VFA mixture with acetic acid as the predominant VFA. The analysis of changes in microbial diversity showed increased abundance of *Hafnia* and *Fonticella* species under 17°C and pretreated substrate sets. These are known for the production of acetic acid and compete with lactic acid producers. The reduced production of other VFAs and metabolites can also make downstream extraction easier by the selective enhancement of acetic acid production. Moreover, a decreased presence of propionic acid is favourable for fermentation as it is known to be inhibitory to the process.

6.2 Rhamnolipids-enhanced volatile fatty acid production under psychrophilic temperature using food waste

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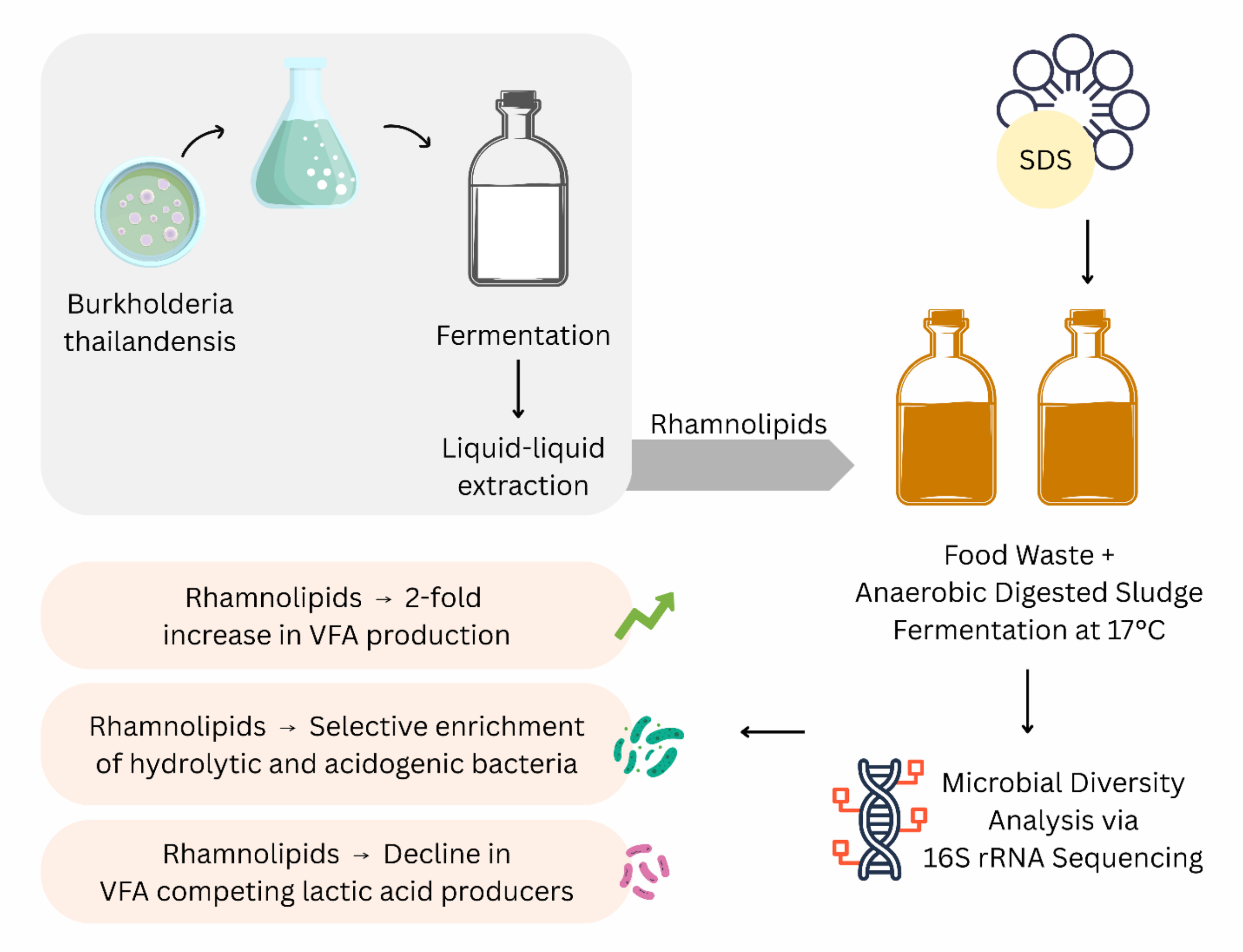
Contribution Statement: For the fermentation part, I conducted the background literature review; designed the experiments; performed all laboratory work, data collection, and primary analyses; created figures/tables; and wrote the manuscript. The primary work for rhamnolipid production was performed by the second author. This chapter is adapted from the published article, with experimental setup concisely summarized here and full methodological details provided in Chapter 4, and formatting aligned with the thesis.

6.2.1 Abstract

Psychrophilic fermentation offers a low-energy route for organic waste valorization, however reduced microbial activity at low temperatures poses a barrier to effective hydrolysis and volatile fatty acid (VFA) production. This study explores the use of biosurfactant rhamnolipids against synthetic surfactant sodium dodecyl sulfate (SDS) to enhance the fermentation efficiency of food waste at 17 °C. Two surfactant dosages (0.2 and 0.3 g/g TSS) were compared for both rhamnolipids and SDS with control (no surfactant) in batch anaerobic conditions. SDS, despite its strong surfactant properties, resulted in marginal improvements in VFA accumulation (~10–12%), likely due to its lower biocompatibility and cytotoxicity at higher concentrations, which may suppress functional fermentative microbes. In contrast, rhamnolipid addition led to a twofold increase in VFA production (up to 4.4 g/L), with dominant abundance of acetic, butyric, and isobutyric acids. The amphiphilic nature of rhamnolipids promoted a selective emulsification of lipid fractions and its improved bioavailability under cold conditions. Microbial community analysis revealed that rhamnolipids selectively enriched hydrolytic and acidogenic taxa such as *Pseudomonas fragi*, *Clostridium* spp., and *P. veronii*, consistent with its role in enhancing substrate emulsification and enzyme accessibility. The downregulation of lactic acid-producing species like *Carnobacterium inhibens* suggested a redirection of metabolic flux towards VFA production over lactic acid accumulation. The occurrence of *Leuconostoc gelidum*, *Romboutsia ilealis*, and *Gordonia rubripertincta* under rhamnolipid conditions from day 0 onwards suggested a targeted microbial succession aligning with the metabolic role of rhamnolipids in enhancing hydrolysis and acidogenesis. In contrast, these species were found only by day 11 in the control set. Overall, this study positions rhamnolipids as functionally superior to conventional surfactants like SDS in psychrophilic anaerobic fermentation, not only through enhanced physicochemical solubilization but also via selective microbial shift. These findings support the integration of biosurfactants in enhancing the efficiency of low-temperature waste-to-VFA conversions.

Keywords: rhamnolipids, sodium dodecyl sulfate, acidogenic fermentation, food waste, species enrichment

6.2.2 Graphical Abstract



6.2.3 Introduction

Psychrophilic fermentation is gaining traction as a strategy for waste valorization particularly where maintaining mesophilic temperatures imposes high energy costs. Despite this potential, very few studies have examined acidogenic fermentation under psychrophilic conditions, and even fewer have explored strategies to selectively enhance specific VFAs at low temperatures. In our previous study, we demonstrated the feasibility of operating acidogenic fermentation of food waste at 17 °C. A selective VFA production i.e., high butyrate in a mixed culture system, and targeted microbial community shifts under psychrophilic conditions were successfully achieved [6]. This highlighted the potential for low-energy bioconversion, but also underscored key limitations in overall VFA yield. This was due to a constrained hydrolysis and poor substrate solubilization at low temperatures. Addressing these bottlenecks is therefore crucial to developing a psychrophilic acidogenic process, which remains scarcely represented in the literature.

Presently, hydrolysis is a rate-limiting step in mixed culture acidogenic fermentation as without an efficient disintegration of the organic matter, there would not be a full utilization of the substrate to produce VFAs. Different substrate pretreatment methods are utilized to enhance the hydrolysis step, such as thermal, chemical, ultrasonic, etc. Specifically, for VFA biosynthesis, biosurfactants such as rhamnolipids have emerged as biocompatible agents that could enhance organic solubilization and VFA accumulation in substrates such as primary and waste-activated sludge [2]. Unlike other biosurfactants such as surfactin or sophorolipids, rhamnolipids have been shown to retain function in both aerobic and anaerobic environments and to facilitate hydrolysis of hydrophobic substrates. For example, rhamnolipids enhance the release of dissolved organic matter and increase volatile fatty acid production in anaerobic codigestion systems [294]. Their molecular structure, comprising rhamnose sugars linked to β-hydroxy fatty acids, enables amphiphilic interactions that improve substrate accessibility and emulsification without significantly inhibiting fermentative bacteria. A study by Johnravindar et al., showed an enhanced hydrolysis and acidogenesis in anaerobic digestion with increasing rhamnolipid dosages from 0.02 to 0.25 g/g total suspended solids (TSS), with a maximum VFA concentration of 9.53±0.53 g/L achieved at the highest rhamnolipid dosage. These authors reported a more than threefold increase in VFA production compared to the control [3]. Using waste-activated sludge as a substrate, Li et al. also observed a nearly three-fold increase in VFA production and an accumulation of 2.6±0.3

g/L VFA using a rhamnolipids dosage of 0.04 g/g TSS [2]. A combined effect of rhamnolipids (0.003 g/g SS), alkali conditions (pH 10), and microwave on the disintegration of dairy waste activated sludge showed a 55% increase in the soluble chemical oxygen demand (SCOD) [4]. Another similar study by He et al. observed a positive combined effect of rhamnolipids (0.2 g/g TSS) and alkaline pH 10, yielding a 4-fold increase in the total VFA [5]. There are various other studies employing rhamnolipids in largely the mesophilic digestion of sludge, concluding at least a 3-fold increase in VFA generation [6], [7]. However, the application of rhamnolipids for low temperature acidogenic fermentation of food waste has not been studied, to the best of our knowledge.

Generally, the degradation of polysaccharides and proteins is faster than fats due to the additional requirement of emulsification for them to be broken down. Moreover, lipases required for this emulsification are also not as abundant as glycosidases and proteases in the microbial consortia derived from anaerobic sludge. Rhamnolipids essentially solubilize hydrophobic compounds and increase their solubility for uptake by the microorganisms, thereby assisting with the degradation of all three macro-constituents (carbohydrates, fats, and proteins) of the substrate. The fat content in sludge ranges between 5-10%, while in food waste, it ranges between 10-30%, making food waste a richer substrate for fat solubilization by rhamnolipids, and offering more potential for enhanced microbial uptake and subsequent conversion into VFAs during fermentation. Biosurfactants were selected over chemical surfactant due to their superior biocompatibility, lower toxicity, and enhanced interaction with complex organic substrates.

The addition of rhamnolipids in psychrophilic fermentation of food waste and sludge could enhance the solubilization and microbial degradation of fats, along with polysaccharides and proteins, into VFAs, resulting in higher VFA yields compared to the fermentation without rhamnolipid addition. In our previous work, we observed that achieving industrially relevant VFA concentrations from food waste necessitates high substrate loadings, typically within the 9–20 g COD L^{-1} range. Yet, high-VS systems often suffer from poor hydrolysis, mass transfer limitations, and process instability [295] especially under psychrophilic or sub-mesophilic conditions. Here, we thus assess the effect of rhamnolipids on VFA production as opposed to chemical surfactant, sodium dodecyl sulphate (SDS) under psychrophilic conditions. This study also contributes to the development of circular biorefinery concepts by demonstrating how value-added compounds such as rhamnolipids can be reintegrated into fermentation processes to enhance hydrolysis and acidogenesis. Building upon our previously developed psychrophilic fermentation framework for targeted butyric acid production (Kumar et al., 2024), this study demonstrates the effectiveness of rhamnolipid-assisted hydrolysis in overcoming the hydrolytic limitations typically observed at low temperatures. Therefore, this study aimed to (i) evaluate the effect of fermentation-derived rhamnolipids addition on hydrolysis and VFA production during psychrophilic fermentation of food waste; (ii) compare the performance against a a chemical surfactant (SDS) and a non-supplemented control and; and (iii) analyze changes in soluble COD, pH, individual VFA profiles, and microbial community structure to elucidate the underlying mechanisms of enhancement. The findings provide new insights into the feasibility of biosurfactant-assisted VFA fermentation as a low-energy, cold-climate waste valorization approach.

6.2.4 Materials and Methods

Rhamnolipids were produced using skim milk to simulate solids-not-fat (SNF) waste stream from dairy processing using *Burkholderia thailandensis* E264, a non-pathogenic microorganism. The production and structural analysis of rhamnolipids are described under Chapter 4, Section 4.5.

Anaerobically digested sludge was collected from the Humber wastewater treatment plant in Ontario, Canada, to be used as an inoculum. Food waste used as the substrate was collected from the restaurants' generated organic waste at York Lanes, York University in Toronto, Canada. This was a post-consumption food and consisted of vegetables, fruits, meat, noodles, rice, and bread. The characterization for both the inoculum and substrate were performed as described under Chapter 4, Section 4.1 (Table 14).

Table 14. Characterization of substrate (food waste) and inoculum (anaerobically digested sludge)

Characteristics	Food Waste	Anaerobically Digested Sludge
pH	5.1 ± 0.09	7.16 ± 0.01
Total Solids (TS) (g/L)	89 ± 3.3	11.5 ± 0.5
Volatile solids (VS) (g/L)	83.8 ± 2.7	8.04 ± 0.3
Total Chemical Oxygen Demand (TCOD) (g/L)	82 ± 0.6	12.2 ± 0.03

Soluble Chemical Oxygen Demand (SCOD) (g/L)	70.3 ± 0.5	9.8 ± 0.06
Total Nitrogen (TN) (g/L)	4.15 ± 0.2	1.85 ± 0.05
Total Kjeldahl Nitrogen (TKN) (g/L)	4.6 ± 0.02	1.79 ± 0.03

The experimental setup for comparing the VFA production with rhamnolipid and chemical surfactant addition is described under Chapter 4, Section 4.6. The method for the analytical, microbial community diversity, and statistical analysis are described under Chapter 4, Section 4.9, 4.10, and 4.11 respectively. The microbial diversity shift was statistically correlated between the control set and the two dosages of rhamnolipid addition through Principal Coordinate Analysis (PCoA) using MicrobiomeAnalyst 2.0 (Quebec, Canada).

6.2.5 Results and Discussion

Production and characterization of rhamnolipids

Skim milk, characterized by a high TOC (11.48 g/L), lactose (22.82 g/L), protein (35.42 g/L), and sufficient TN (0.162 g/L), provided a rich and balanced source of carbon and nitrogen, supporting robust microbial growth and rhamnolipids production. A rhamnolipids production of 3.5 g/L was obtained using skim milk as the sole production medium. The FTIR spectra of the rhamnolipids produced on skim milk were compared to those of commercial rhamnolipids to confirm the presence of characteristic functional groups (Figure 24). Both spectra exhibited a broad absorption band around 3200–3400 cm^{-1}, indicating the presence of hydroxyl groups, typical of rhamnose sugar units. Peaks observed between 2950–2850 cm^{-1} correspond to aliphatic C–H stretching vibrations, confirming the lipid chain component in the rhamnolipid structure. A prominent band around 1740 cm^{-1} in both samples represents ester carbonyl (C=O) stretching, a key indicator of rhamnolipid ester bonds [184]. Additionally, the fingerprint region between 1250–1000 cm^{-1} showed complex peaks associated with C–O–C and C–O stretches, consistent with glycosidic linkages in rhamnolipids. While minor variations in intensity and peak sharpness were observed, likely due to feedstock composition, degree of purity, or extraction differences, the overall spectral alignment with commercial standards verified the successful biosynthesis of rhamnolipids using skim milk.

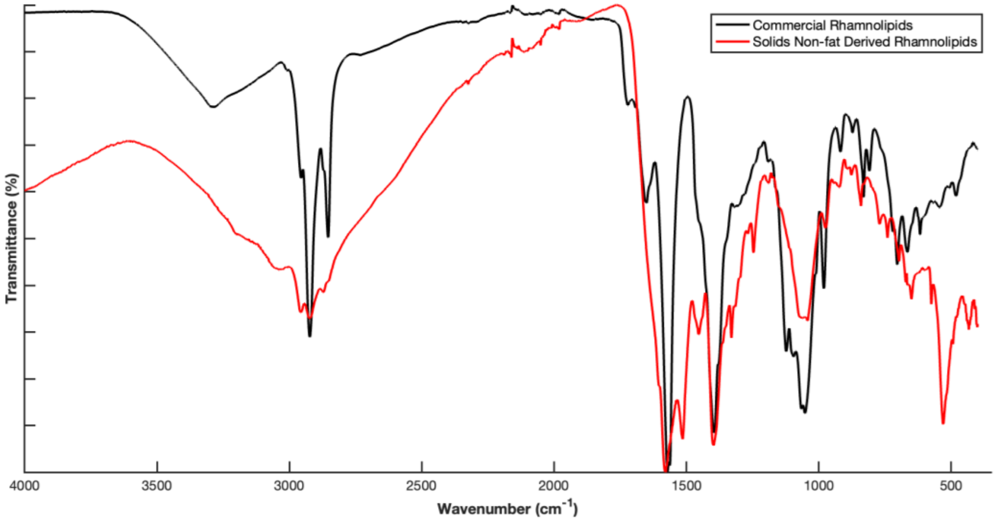


Figure 24. FTIR spectra of SNF-derived rhamnolipids and commercial rhamnolipids

The congener composition of bacteria-derived rhamnolipids was analyzed by direct infusion in MS. The results are shown in Figure 25. The rhamnolipid congeners identified in MS and their relative abundances were Rha-C14-C14 (34.12%), Rha-C14-C16 (32.54%), Rha-Rha-C14-C14 (12.12%), and Rha-Rha-C14-C16/C16-C14 (21.22%). *B. thailandensis* is known to produce long chain mono- and di-rhamnolipids when grown on suitable feedstocks, and as shown in our previous studies [296]. The distribution of congeners indicated that skim milk provided a balanced nutrient profile (carbon, nitrogen and other micronutrients) that could support the biosynthesis of a wide range of rhamnolipid congeners.

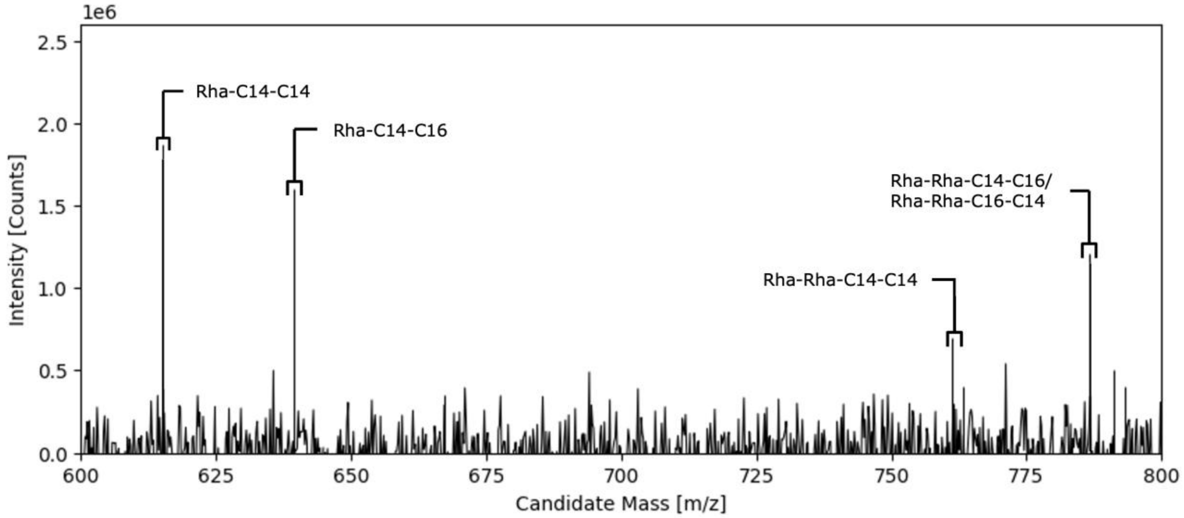


Figure 25. Mass spectrum showing the congener composition of rhamnolipids produced by B. thailandensis on skim milk, highlighting the dominant mono- and di-rhamnolipids.

Effect of rhamnolipid addition on food waste solubilization

Fermentation of food waste was run to assess the effect of two dosages of rhamnolipids on VFA production and its composition. Dosages of 0.2 g/g TSS and 0.3 g/g TSS were tested for rhamnolipid, which were selected based on prior studies involving anaerobic fermentation of substrates like waste activated sludge, where rhamnolipid dosages typically ranged from 0.02 to 0.4 g/g TSS [296], [297], [298]. Given that food waste generally contains a higher lipid content compared to waste activated sludge, and considering the amphiphilic nature of rhamnolipids that enhances hydrolysis of lipid-rich substrates, higher dosages were adopted to maximize solubilization and subsequent VFA yield under acidogenic conditions. The two dosages were chosen to represent incremental increases within the upper effective range, enabling comparison of rhamnolipid performance under psychrophilic conditions. This range was chosen based on previous studies that identified 0.2–0.5 g/g TSS as optimal for improving hydrolysis and VFA production, with higher concentrations yielding diminishing returns or even process instability (Yi et al., 2013). Elevated biosurfactant levels can lead to excessive foaming, stable emulsion formation, or disruption of microbial membranes, all of which can hinder reactor performance (Al-Tahhan et al., 2000). Moreover, rhamnolipids at high concentrations are known to alter cell-surface properties through lipopolysaccharide release and bilayer expansion, potentially perturbing mixed microbial consortia.

With the addition of rhamnolipids to VFA fermentation, the SCOD values (Figure 26a) increased with time as hydrolysis and acidogenesis progressed, reaching a peak around day 9 under all conditions. The subsequent decline in SCOD, despite continued lipid degradation, likely resulted from the conversion of soluble intermediates into VFAs and gases, biomass assimilation, and partial precipitation of VFA carboxylate salts with cations under alkaline conditions [299], [300]. Addition of rhamnolipids showed a maximum SCOD values between 26.4-27.1 g/L, while the control had the maximum SCOD value of 25.5 g/L. However, on performing ANOVA, the difference in SCOD values between the control and rhamnolipid conditions was not found to be significant with a *p*-value >0.05. While SCOD values were not significantly different between control and rhamnolipid treatments, it must be noted that SCOD is a cumulative metric that does not distinguish between the various types of soluble metabolites. It is therefore plausible that the control condition comprised different hydrolysis intermediates than the rhamnolipid-treated sets. This is supported by the distinct VFA trend and profiles observed in Figure 27 below, as well as the shifts in microbial community structure.

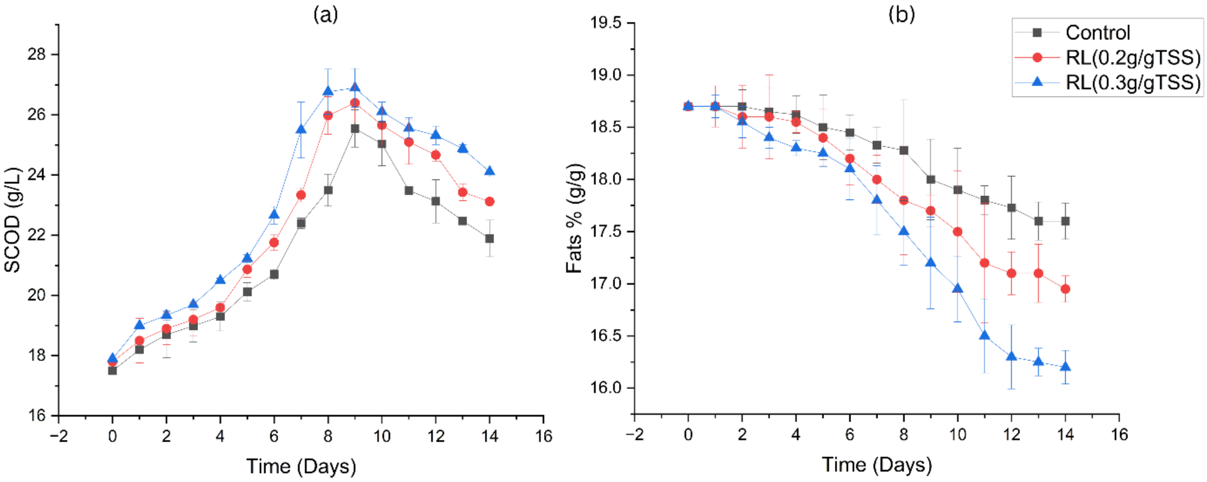


Figure 26. The kinetics of (a) SCOD production and (b) fat (%) remaining during the course of fermentation of food waste with and without rhamnolipids addition.

The total VFA production with different rhamnolipid dosages with respect to time is shown in Figure 27a. The higher dosage of RL-0.3 g/g TSS gave the highest total VFA concentrations of 4.4 g/L. This was closely followed by the dosage of 0.2 g/g TSS, which gave a total VFA concentration of 4 g/L. When compared to the control conditions with no rhamnolipid addition, the difference was found to be significant at a *p*-value <0.05. The control set had the highest VFA concentration of 2.2 g/L. Since the SCOD changes showed a similar trend for all the conditions, yet total VFA were found twice as much as in the control, it might point out to the generation of other co-metabolites in the control set. The daily measurement of total gas production by the water displacement method showed that the gas formation reduced to nearly 0 after 7 days. In the control set, however, there was relatively more gas formation observed from day 6 to day 9 than in the rhamnolipid sets (date not shown). As observed by Luo *et al* [301], in waste-activated sludge, the rhamnolipid addition under alkaline conditions enhanced the levels of soluble protein and carbohydrates that eventually led to an increased production of VFAs and ammoniacal nitrogen. This was attributed to the improved solubilization activity of rhamnolipids under alkaline conditions that increased the membrane hydrophobicity and cell permeability, resulting in increased levels of extracellular carbohydrates and proteins. Furthermore, these conditions also led to the dissociation of acidic groups in the extracellular polymeric substances (EPS) layer of the bacteria. Since a similar effect was not observed in our study with the control set, the metabolic shift was thus relatively less favourable towards VFA accumulation. Other metabolites in acidogenic fermentation can include lactic acid, ethanol, and ketones [302]. In a two-phase anaerobic digestion study by Xu et al. [303], the effect of rhamnolipid addition was distinctly observed for the acidogenesis stage. It was found that the VFA concentration increased 5 times to 3.5±0.09 gCOD/L from 0.5±0.05 gCOD/L with no rhamnolipids addition., showing an enhanced hydrolysis and acidogenesis. Although the SCOD values were comparable between the control and rhamnolipid-treated systems, the composition and transformation efficiency of the solubilized organics differed. In the rhamnolipid-amended sets, the enhanced substrate–microbe interaction under alkaline conditions led to a higher total VFA accumulation and a greater proportion of butyric and acetic acids. This indicated an improved metabolic conversion of soluble intermediates into acidogenic products. In contrast, the control group, despite similar SCOD, showed lower VFA yields, implying that a larger share of the soluble organics remained as non-fermentative intermediates (e.g., soluble carbohydrates, amino acids, or EPS fragments). This distinction underscores that rhamnolipids improved bioconversion efficiency rather than overall solubilization capacity.

A key mechanism of action of surfactants is to increase the solubility of lipids by forming an emulsion, as they reduce the interfacial tension at the water-oil interface, and in doing so, enhance the fat availability for microbial degradation [304]. This effect was supported by the presence of rhamnolipids which was observed by the declining total fat content (%) as the fermentation progressed (Figure 26b). While the control set showed a reducing fat content (%) with time, the effect was enhanced with rhamnolipids addition with increasing dosage. To the knowledge of the authors, this fat-reduction effect of rhamnolipids has not been observed with organic waste like food waste. Several studies have reported this same effect, however with other organic wastes such as digested sludge with synthesized organic waste, petroleum hydrocarbons, etc. and chemical surfactants such as SDS and Triton X-100 [304], [305]. Zhu et al., observed the effect of SDS in the digestion of a mixture of soluble starch, vegetable protein and soybean oil. They found a reduced microbial activity, extended incubation time in batch-type anaerobic digestion while degrading the soybean oil [304]. Mohanty and Mukherji showed the emulsification of non-aqueous phase liquids (components of petroleum waste) by Triton X-100 and subsequently increased the microbial degradation of the substrate [305].

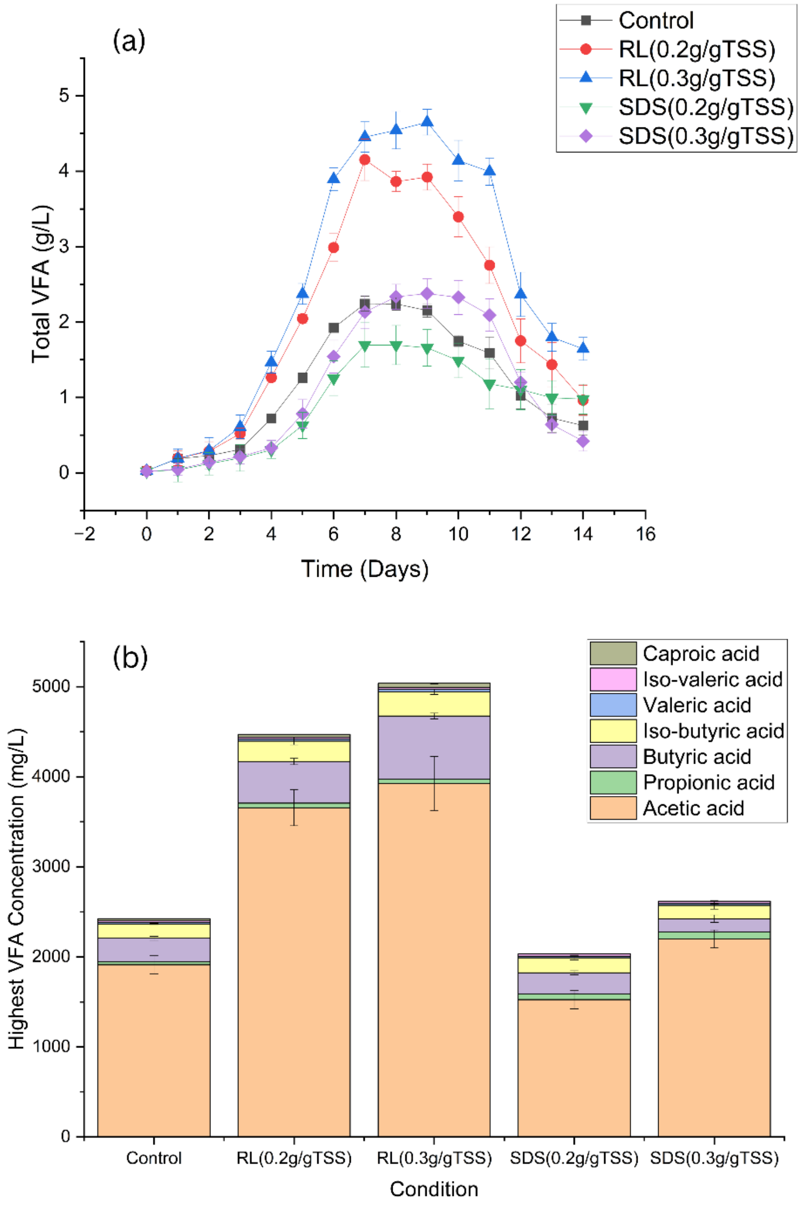


Figure 27. VFA production upon addition of rhamnolipid and chemical surfactant. (a) Concentration of total volatile fatty acids (VFA) and the (b) composition of the highest VFA concentration under control set and different dosages of rhamnolipids and sodium dodecyl sulfate

VFA production and steering of metabolic pathway

In terms of VFA distribution and the highest accumulated concentration of all VFAs, the dominant VFA across all conditions was acetic acid (Figure 27b). The highest concentrations observed were nearly 4 g/L in rhamnolipid dosage 0.3 g/gTSS. This was more than a 2-fold increase from the control set at 1.9 g/L. The higher dosage also showed prolonged accumulation time of acetic acid till 11 days, while 0.2 g/gTSS dosage showed decline right after day 8. Considering the 3 g VS added per 300 mL system, the corresponding yield was approximately 0.33 g VFA g^{-1} VS added for the 0.3 g g^{-1} TSS dosage and 0.16 g VFA g^{-1} VS added for the control. Such a trend was also observed by Li *et al* [306], where they observed prolonged accumulation time of acetic acid with a maximum concentration at 1.5 g/L using waste activated sludge as a substrate. The application of rhamnolipids promoted the activity of acidogenic bacteria by enhancing the available soluble carbon. Their study showed increment in propionic acid and valeric acid which supported the further process in biogas development. In contrast, in this study, butyric acid was the second prevalent VFA after acetic acid. The order of VFAs with respect to their highest accumulated concentration was acetic acid > butyric acid > caproic acid > propionic acid > valeric acid. The declining concentration of acetic acid coincided with the increasing concentration of butyric and caproic acid, thus showing possible β-chain elongation. Propionic acid and valeric acid showed increased (almost 2-fold) production under rhamnolipid sets as opposed to the control.

The control set of butyric acid showed increasing accumulation till day 10 and 11 at 260 mg/L, which then plateaued and declined afterwards. The rhamnolipid 0.2 g/g TSS dosage showed a maximum accumulation at 462 mg/L on day 11. This was nearly a two-fold enhancement over the control. However, with 0.3 g/g TSS dosage, the butyric acid trend was increasing until day 14 as well, with the highest accumulation at 703 mg/L, an increment of 2.7 times that the control set. More studies employing rhamnolipids under alkaline conditions such as by He *et al* [307], observed propionic acid and valeric acid to be more abundant than butyric or caproic acid. Most of the studies reported primarily used sludge as a substrate for biogas (methane) production, where methanogenesis was active. In contrast, the present study focused solely on the acidogenic stage, where methane formation was intentionally suppressed to favor VFA accumulation. From a metabolic perspective, the accumulation of acetic acid is attributed to the classical acetyl-CoA pathway via pyruvate decarboxylation and subsequent oxidation [308], [309], [310] The significant increase in butyric acid suggests the activation of the butyrate production pathway, wherein acetyl-CoA is converted to butyryl-CoA through condensation and reduction steps, followed by the action of butyrate kinase (buk) or butyryl-CoA:acetate CoA-transferase. The sustained increase in butyrate under higher rhamnolipid dosage in this study indicated that the carbon flux was diverted toward butyrate synthesis, potentially due to the selective enrichment of

Clostridium spp., which is known to harbor these metabolic routes. Caproic acid, observed in increasing amounts in this study, is a product of chain elongation via the reverse β-oxidation pathway, where acetate or butyrate serve as electron acceptors and are elongated using ethanol or other reduced intermediates. This further indicated that rhamnolipids not only enhanced solubilization but also created conditions favorable for syntrophic interactions or redox-balanced chain elongation in this work.

While, in this study, methanogenesis was arrested to promote the acidogenic fermentation, a key factor here would be the difference in the substrate type, directing the rhamnolipid effect and a possible shift in the metabolic pathways in a manner where rhamnolipid addition promoted chain elongation pathways to produce even-chain VFAs over odd-chain VFAs. Since the rhamnolipid addition affects the EPS layer of certain bacteria, which provides enhanced tolerance to a changing environment, this might have resulted in an overall reduction of the microbial diversity in the system. Firmicutes play a key role in degrading organics in the hydrolysis process under anaerobic conditions and they were found to increase in abundance with rhamnolipid treatment of waste activated sludge [116]. Proteobacteria have also been shown to increase in numbers and are known for VFA production and acetic acid accumulation. At the class level, *Clostridia* were observed to increase from 4% to 14%. The rhamnolipid treatment was thus conducive to the shift in microbial diversity for VFA production [311].

A study by Wang *et al* [149] studied the effect of rhamnolipid in treating mariculture solid waste. They also observed an enhanced accumulation of acetic acid with rhamnolipid treatment from 50% in control to 70% in a treated set. The excitation emission matrix (EEM) analysis showed a release of biodegradable substances. These substances act as electron shuttles in the fermentation process, improving electron transfer, conducive to VFA production. Similar remarks were provided in another study [312]. Another mechanism of rhamnolipids' positive effect is due to the protection of enzymes from metal ions via micelle formation [150]. Wang *et al* [149], also observed the difference in microbial diversity with rhamnolipid treatment and found similar increase in Firmicutes from 5.8% to 41.7%. However, Proteobacteria were reduced from 28.85 to 10.7%, which is known for the enrichment of acetic, propionic and valeric acid. The metabolic pathways related to VFA synthesis (specifically lipid and amino acid metabolism) were observed to have been enriched during fermentation with rhamnolipids. The genes encoding glutamate, aspartate, L-cystine transporters (for transport of extracellular protein) were found to be enhanced by 9.4 times than the control, while genes associated with carbohydrate transport also showed an increased expression.

Comparison of VFA production between rhamnolipids and SDS addition

The experimental results demonstrated that the addition of rhamnolipids significantly enhanced VFA production during psychrophilic fermentation of food waste, outperforming both the SDS-treated and control setups. The cumulative VFA concentration in the rhamnolipid-added sets was consistently higher across the fermentation period, suggesting an improved hydrolysis-acidogenesis synergy driven by the biosurfactant's emulsifying properties. Rhamnolipids, being microbial origin biosurfactants, possess both hydrophilic and hydrophobic domains, allowing them to reduce surface tension and emulsify complex macromolecules such as lipids and proteins. Enhanced solubilization led to more efficient enzymatic access and hydrolysis, thereby accelerating the release of monomers and their conversion to VFAs. A comparative study between rhamnolipids and SDS addition to waste activated sludge for hydrogen production by Zhou et al. [313], also showed similar results with respect to VFA accumulation. They found total VFA concentration under rhamnolipid addition to be 1.16 times higher than SDS addition, yielding nearly 6 gCOD/L VFA.

Conversely, SDS-treated set exhibited no significant improvement in VFA yield compared to the control. Despite its strong surfactant properties, SDS lacks biocompatibility and may selectively suppress acidogenic and hydrolytic microbial populations critical for VFA generation. These findings align with previous studies where synthetic surfactants, while improving solubilization, often led to microbial inhibition under anaerobic conditions [313]. Quan et al. [314] observed this inhibitory effect of SDS on microbial community while reduction in VFA production using food waste as opposed to the control. The acidogenic bacteria belonging to *Clostridium* reduced from 82.71% to 23.72% in abundance, leading to the low VFA concentration. Furthermore, in this study, SDS addition also delayed the total VFA peak (Figure 3a), showing its negative impact on acidogenesis, while rhamnolipids addition was favourable under psychrophilic temperature. The comparable performance of SDS and the control also suggested that under the tested conditions, the positive effects of SDS on solubilization were either minimal or outweighed by its negative impacts on microbial metabolism. SDS is a strong anionic surfactant known to disrupt cell membranes and denature proteins, which can negatively affect the viability and functionality of anaerobic fermentative microbes [315]. Unlike biosurfactants, which interact gently with microbial membranes and promote enzymatic accessibility, SDS lacks microbial selectivity and biocompatibility [316]. Similar observations, albeit under mesophilic conditions, were also observed by Zhou et al., who reported that while SDS enhanced the solubilization of waste activated sludge by disrupting extracellular polymeric substances, it also exerted inhibitory effects on fermentative bacteria such as *Megasphaera* and *Oscillibacter*. In contrast, rhamnolipids, also anionic surfactants, appear to maintain or even promote microbial functionality, as further supported by microbial community analysis (Section 3.3). The addition of rhamnolipids and SDS showed a difference in the VFA composition as well. While both treatments showed acetic acid dominance, rhamnolipids-treated sets showed an enhancement of butyric and isobutyric acid following acetic acid. On the other hand, SDS-treated sets showed an enhancement of propionic and valeric acids. Generally, butyric and isobutyric acid pathways are associated with more reduced conditions where NADH needs to be oxidized efficiently [317], [318]. With rhamnolipids addition contributing to lipid solubilization and selective microbial shift, the enhancement of butyric and isobutyric acid was observed. In contrast, SDS-treated sets showed increased levels of propionic and valeric acids, suggesting a shift toward alternative fermentation pathways such as succinate pathway [319]. In low-temperature systems, where microbial resilience is already limited, the inhibitory effects of SDS are likely amplified, reinforcing the advantage of biosurfactants as more bio-based and selective alternatives.

Abundance and trends in microbial diversity shift with rhamnolipid treatment

The Shannon alpha diversity index provides a composite measure of both species' richness and evenness, reflecting the ecological complexity of microbial communities. In this study, Shannon diversity analysis (Figure 28a) revealed notable shifts in community structure in response to rhamnolipid addition. The 0.3 g/g TSS rhamnolipid treatment (RL2) consistently exhibited higher Shannon diversity values compared to the control and other treatments across most time points, indicating a more diverse and functionally resilient microbial ecosystem. This increase in diversity suggests that higher rhamnolipid dosages foster a broader range of microbial niches. The control group displayed a comparatively lower diversity, pointing toward a narrower functional guild dominated by a few adapted fermenters. Interestingly, the 0.2 g/g TSS rhamnolipid treatment

(RL1) showed intermediate Shannon index values, supporting the idea of a dose-dependent microbial response. These trends correlate well with VFA production patterns, particularly the enhanced butyric and caproic acid accumulation in RL2, implying that increased microbial diversity may enable a more distributed metabolic network, supporting chain elongation and redox balancing.

Principal coordinates analysis (PCoA) revealed clear distinctions in microbial community structure between the control and rhamnolipid-treated groups (Figure 28b). Axis 1, accounting for 41% of the variation, separated the treatments along a gradient of rhamnolipid dosage. Both rhamnolipid groups (0.2 and 0.3 g/g) are displaced from the control group along Axis 1, indicating a substantial shift in community composition due to biosurfactant addition. The shift is more pronounced for the RL-0.3 g/g group, further emphasizing a dose-dependent effect of rhamnolipids on microbial structure. This indicates that rhamnolipids not only increased diversity but also selected for distinct taxa, leading to unique microbial consortia. Partial overlap among groups points to the existence of a shared core microbiota, while peripheral clustering shows the selection of niche-specific taxa under rhamnolipid influence.

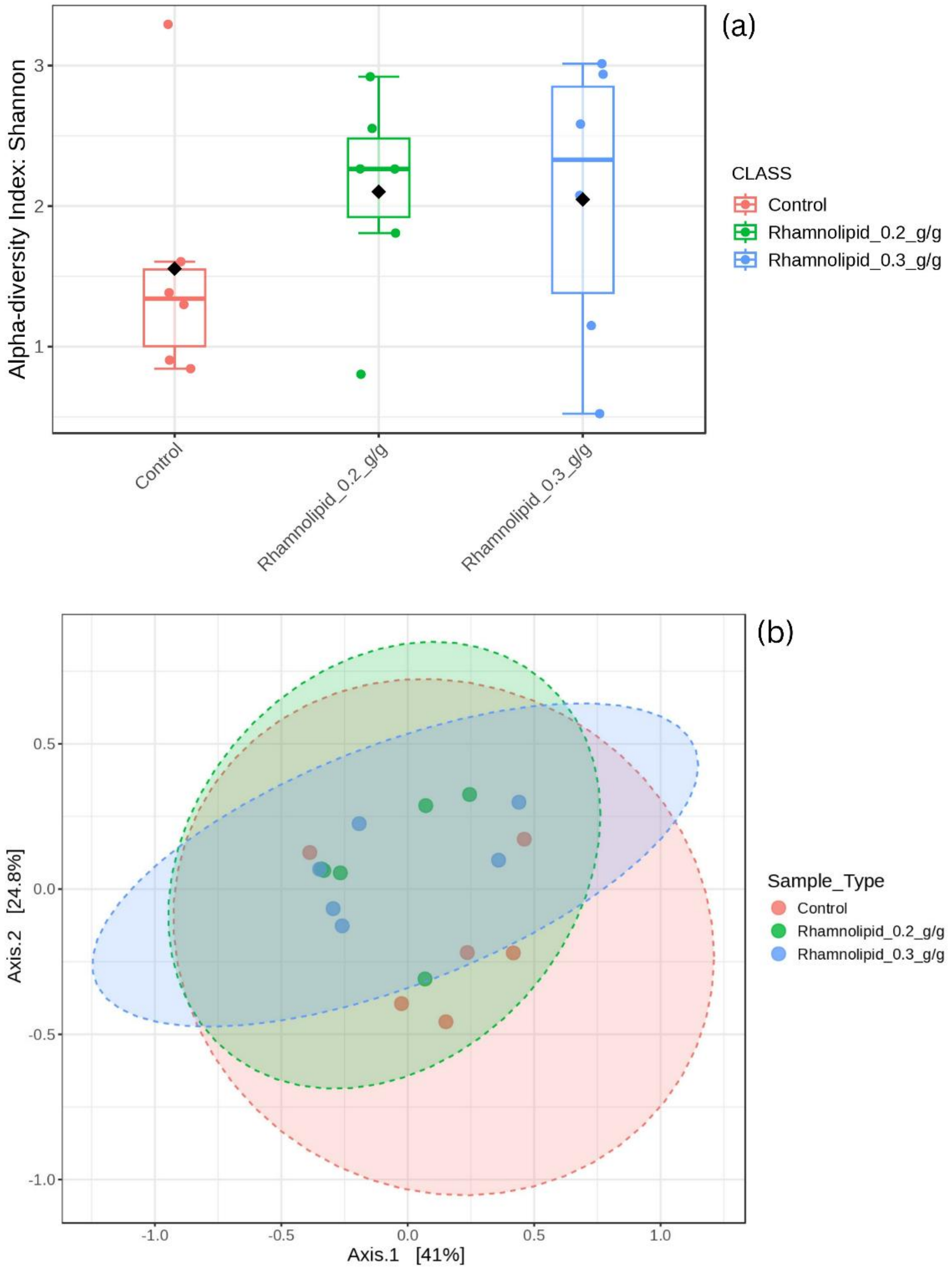


Figure 28. Microbial community profiles under rhamnolipid assisted acidogenic fermentation and control with no rhamnolipids addition. (a) Box plot for Shannon Alpha diversity index and (b) Principal coordinates analysis (PCoA) for beta diversity of microbial communities across different fermentation conditions.

The analysis for the abundance of microbial species (Figure 29) showed an overall decrease in microbial abundance under rhamnolipid-treated sets. This suggests that rhamnolipids exerted a selective pressure on the microbial community, inhibiting the growth of certain bacteria as compared to the control. The shift in substrate composition with fermentation over time favoured *Pseudomonas* growth, especially under rhamnolipids set at 0.3 g/g TSS dosage. This genus is known to tolerate surfactants and producing rhamnolipids [320]. Furthermore, inhibition of competing bacteria allowed *Pseudomonas veronii* and *Pseudomonas fragi* to thrive. Among them, *P. fragi* is known for its ability to degrade lipids and proteins, contributing to VFA production by further producing biosurfactants. It further supports the dominance of other *Pseudomonas* species to thrive, especially under psychrophilic conditions [321], [322]. Thus, rhamnolipids not only enhanced lipid solubilization but also positively reinforced biosurfactant-producing psychrotolerant bacteria's abundance, leading to VFA production.

The lactic acid bacterium *Carnobacterium inhibens* was observed to decline in rhamnolipid-treated sets. This observation suggests that the competitive advantage afforded to the *Pseudomonas* species may inhibit lactic acid production,a key factor in achieving a shift towards VFA production over traditional lactic acid fermentation. The fluctuations observed in other species,such as the resurgence of *Leuconostoc gelidum*, *Gordonia rubripertincta*, *Clostridium* species, *Romboutsia ilealis*, and *Arthrobacter alpinus* further exemplify the complexity of microbial interactions under varying conditions. *Leuconostoc gelidum* is another psychrotrophic lactic acid bacteria [323] initially present in rhamnolipids treatments may compete with *Pseudomonas* spp. for resources but decline under rhamnolipid pressure, while eventually increasing in control sets. *Gordonia rubripertincta* is a versatile species that generates bioactive compounds and extracellular enzymes [324], [325]. While not much is known about its role in fermentation processes, other species under this genus are gaining attraction for potential biotechnological roles [326]. The acidogenic *Clostridium* species showed higher abundance at later time points in the control set as fermentation progressed. While under rhamnolipid-treated sets, they were found at earlier time points. The presence of rhamnolipids initially provided a selective advantage, facilitating microbial access to substrates and potentially enhancing the breakdown of complex organic materials, where *Clostridium* species could access simpler substrates earlier. The combined effects of rhamnolipid treatment and the dynamic microbial community suggest a complex relationship between microbial abundance and VFA production. While *Clostridium* species are integral to VFA production, their altered temporal dynamics under rhamnolipid treatment indicate that optimizing conditions for these bacteria could enhance total VFA yields.

Building on these observations, the early appearance of *Clostridium* species under rhamnolipid treatment points to a potential shift in the fermentation trajectory, favoring acidogenesis over lactic acid fermentation. Rhamnolipids likely disrupt microbial hierarchies by altering substrate availability and competitive interactions, thus enabling Clostridium and other acidogenic species to outcompete slower-growing or less adaptable lactic acid bacteria. This temporal advancement in Clostridium abundance suggests that biosurfactants may not only enhance hydrolysis but also modulate community succession in favor of VFA-producing taxa. Such shifts underscore the importance of microbial ecology in fermentation systems, where tailored interventions, like biosurfactant addition—can be leveraged to steer metabolic outputs toward more desirable end-products like VFAs, rather than intermediates such as lactic acid.

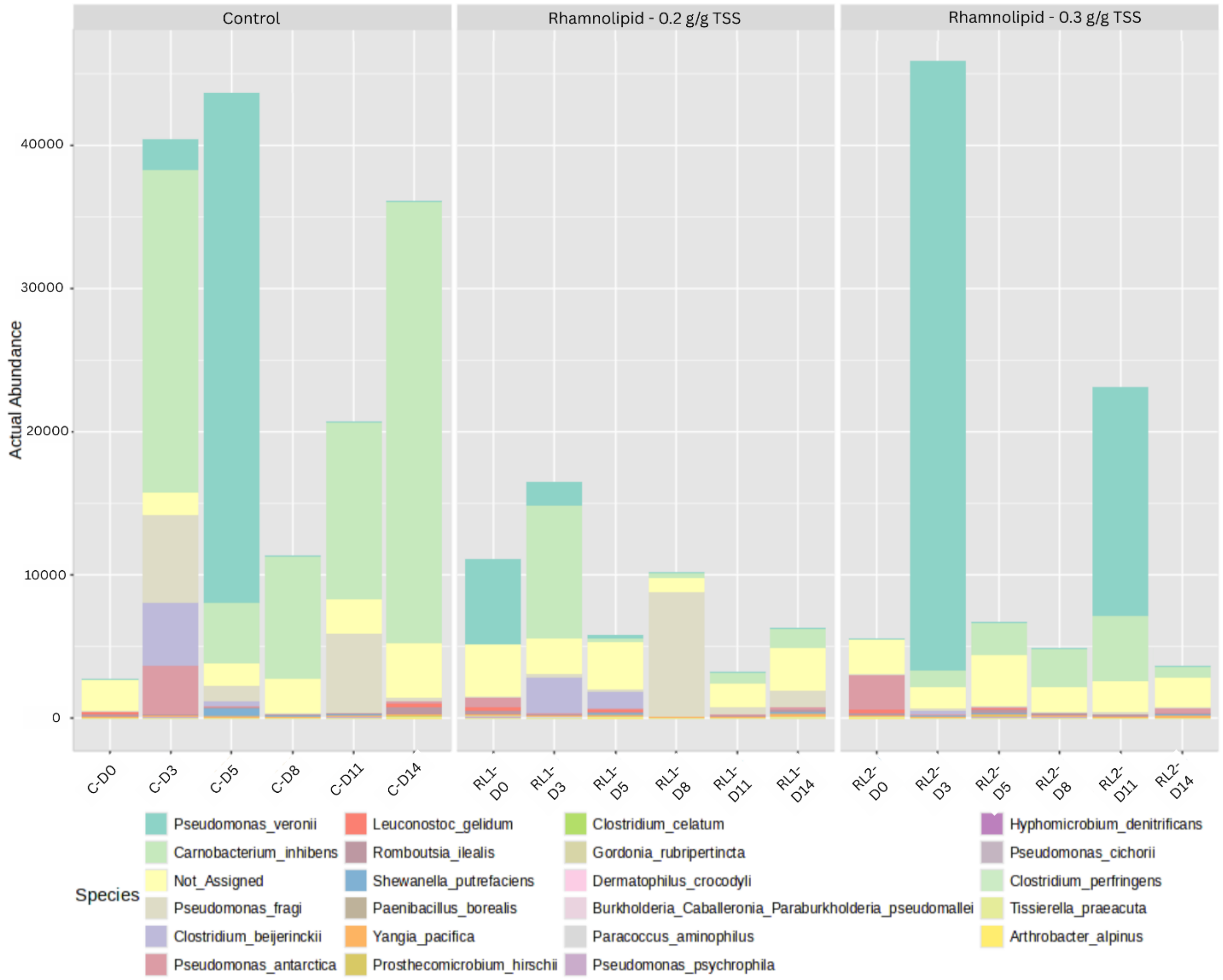


Figure 29. Abundance of species in control and rhamnolipids treated set at 0.2 g/g TSS dosage (RL1) and 0.3 g/g TSS dosage (RL2) on day 0, 3, 5, 8, 11, and 14 (D0, D3, D5, D8, D11, D14).

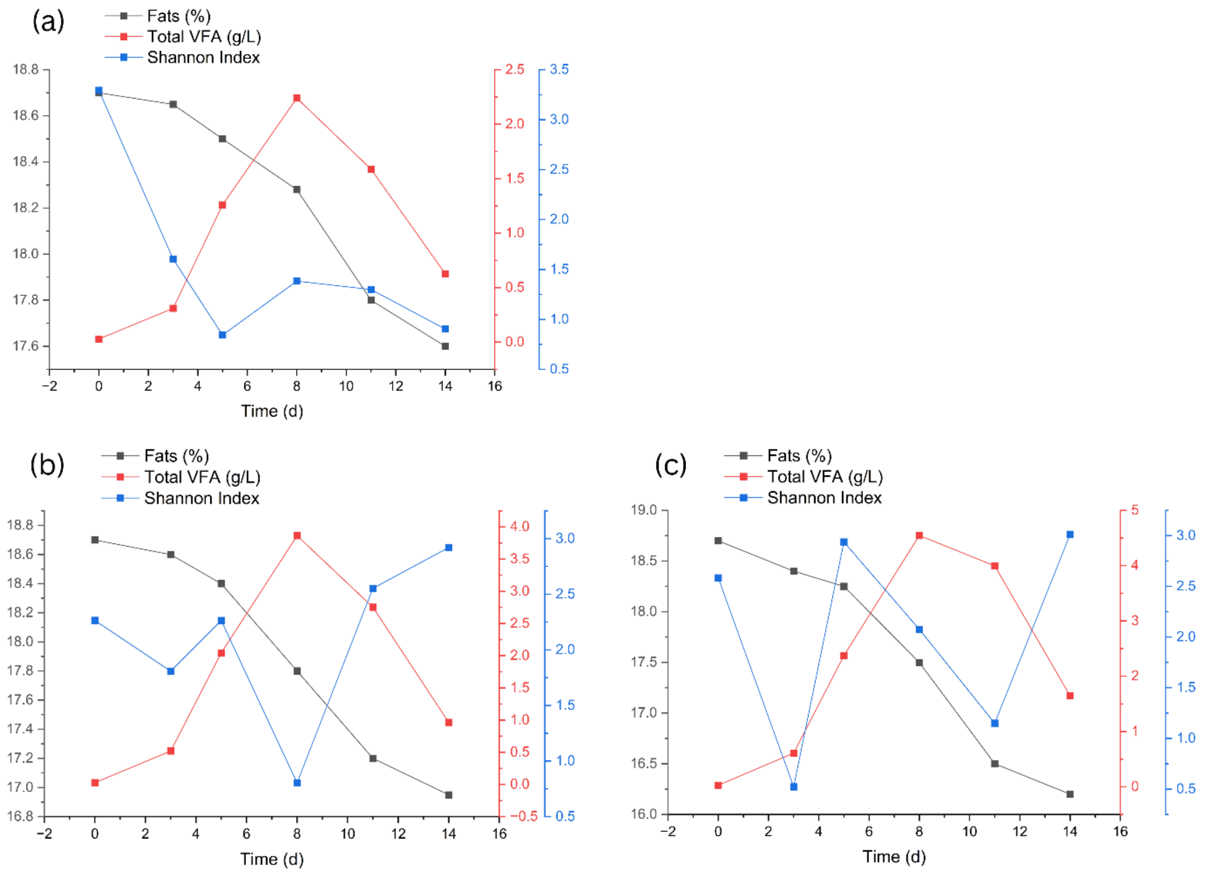


Figure 30. Trends in fat content (%), total volatile fatty acid concentration (g/L), and Shannon diversity index during fermentation of food waste under (a) control, (b) rhamnolipid 0.2 g/g TSS, and (c) rhamnolipid 0.3 g/g TSS conditions

Various studies assessing the microbial diversity shift or trends on the addition of rhamnolipids and SDS in anaerobic digestion and fermentation systems concluded the enrichment of hydrolytic and acidogenic bacteria belonging to the class *Actinobacteria, Clostridia, Bacilli, Alphaproteobacteria* and *Bacteroidia*, among others [304], [327], [328]. Likewise, the enrichment of these classes was also observed in this study with rhamnolipids addition. Under psychrophilic temperatures, this selective enrichment by rhamnolipid treatment favoured psychrotolerant bacteria towards VFA enhancement.

The integration of fat reduction, total VFA production, and Shannon diversity index across treatments (Figure 30) reveals a strong correlation between biosurfactant-induced solubilization, microbial community restructuring, and fermentation efficiency. In the control set (Figure 30a), fat degradation was minimal, and total VFA accumulation remained low (~2.2 g/L), corresponding to a gradual decline in microbial diversity. This suggests that the limited bioavailability of lipids constrained microbial recruitment and metabolic diversity, as also observed in untreated high-fat waste systems [329]. In contrast, the rhamnolipid-treated sets showed markedly improved performance. At 0.2 g/g TSS (Figure 30b), fat content dropped steadily with a corresponding rise in VFA yield (~4.0 g/L by day 8), coinciding with an increase in Shannon diversity. This implies that biosurfactant-enhanced solubilization facilitated broader substrate accessibility, enabling diverse microbial populations to engage in sequential hydrolysis and acidogenesis, similar to observations by Fu et al. (2022) where rhamnolipids enhanced VFA accumulation from sludge [330]. At the higher dosage of 0.3 g/g TSS (Figure 30c), the most significant fat reduction (~3% drop) and highest total VFA yield (~4.6 g/L) were achieved, accompanied by a rebound in microbial diversity after an initial drop. This late-stage recovery may reflect the selective enrichment of functional taxa, such as *Clostridium* and *Pseudomonas fragi*, known for their roles in lipid degradation and butyrate production [329], [331]. Moreover, the delayed peak in VFA under RL2 suggests a more sustained carbon release and prolonged acidogenic activity. This is particularly important under psychrophilic conditions, where hydrolysis and acidogenesis are typically rate-limiting. As biosurfactants, rhamnolipids lower interfacial tension and emulsify hydrophobic fat globules, thereby increasing the surface area available for enzymatic action. This facilitates the activity of lipases, which hydrolyze triacylglycerols into diacylglycerols, monoacylglycerols, and ultimately free fatty acids and glycerol. These hydrolysis products become more readily bioavailable due to increased membrane permeability, which rhamnolipids are also known to enhance. Glycerol is phosphorylated to dihydroxyacetone phosphate, which enters the glycolytic pathway, whereas free fatty acids can undergo β-oxidation to yield acetyl-CoA. Acetyl-CoA acts as the central metabolic intermediate for VFA biosynthesis, particularly for acetic and butyric acid through the actions of acetate kinase, butyrate kinase, and associated transferases. Thus, rhamnolipid-mediated lipid solubilization contributes directly to an increase in acetyl-CoA flux, driving higher VFA yields. In parallel, rhamnolipids induce a shift in microbial community structure by suppressing lactic acid bacteria and promoting the enrichment of acidogenic bacteria [332]. This selective pressure redirects carbon away from lactate toward VFA production pathways. The combined effects of biochemical solubilization, enzymatic accessibility, improved metabolite uptake, and community realignment explain the observed increase in VFA yield and selectivity under rhamnolipid-treated conditions. These findings highlight the potential for rhamnolipid-assisted fermentation to improve substrate hydrolysis and acidogenesis at low temperatures, suggesting opportunities for scalable integration into cold-climate waste valorization systems. Future work can incorporate microbial community profiling and substrate-specific degradation analyses for both biosurfactant and chemical surfactant systems to provide deeper mechanistic understanding.

6.2.6 Conclusions

This study demonstrated that rhamnolipid addition not only enhanced VFA production during psychrophilic fermentation of food waste but also led to substantial shifts in the microbial community structure. Compared to both the control and SDS-treated sets, rhamnolipid-treated sets achieved a higher VFA concentration, particularly of acetic, butyric, and isobutyric acids. Key species such as *Pseudomonas fragi* and *P. veronii*, known for their surfactant tolerance and lipid degradation capabilities, were selectively enriched under rhamnolipids treatment. In contrast, lactic acid producers like *Carnobacterium inhibens* declined, indicating a shift away from lactate-dominant pathways. Additionally, early appearance of species like *Clostridium* spp., *Leuconostoc gelidum*, and *Gordonia rubripertincta* under rhamnolipid conditions suggested an enhanced onset fermentation. These findings underscore the dual role of rhamnolipids in both facilitating substrate solubilization and engineering a more psychrotolerant consortium. In contrast, SDS showed a limited benefit and potential microbial inhibition. Overall, rhamnolipids presented a promising, bio-based additive for optimizing the fermentation efficiency and microbial stability in food waste fermentation system.

CHAPTER 7

Supporting Microbial Activity for Psychrophilic Fermentation and Targeted VFA Production

7.1 - Microbial Steering in Psychrophilic Fermentation: Bioaugmentation as a Tool for Butyric Acid Enrichment from Food Waste

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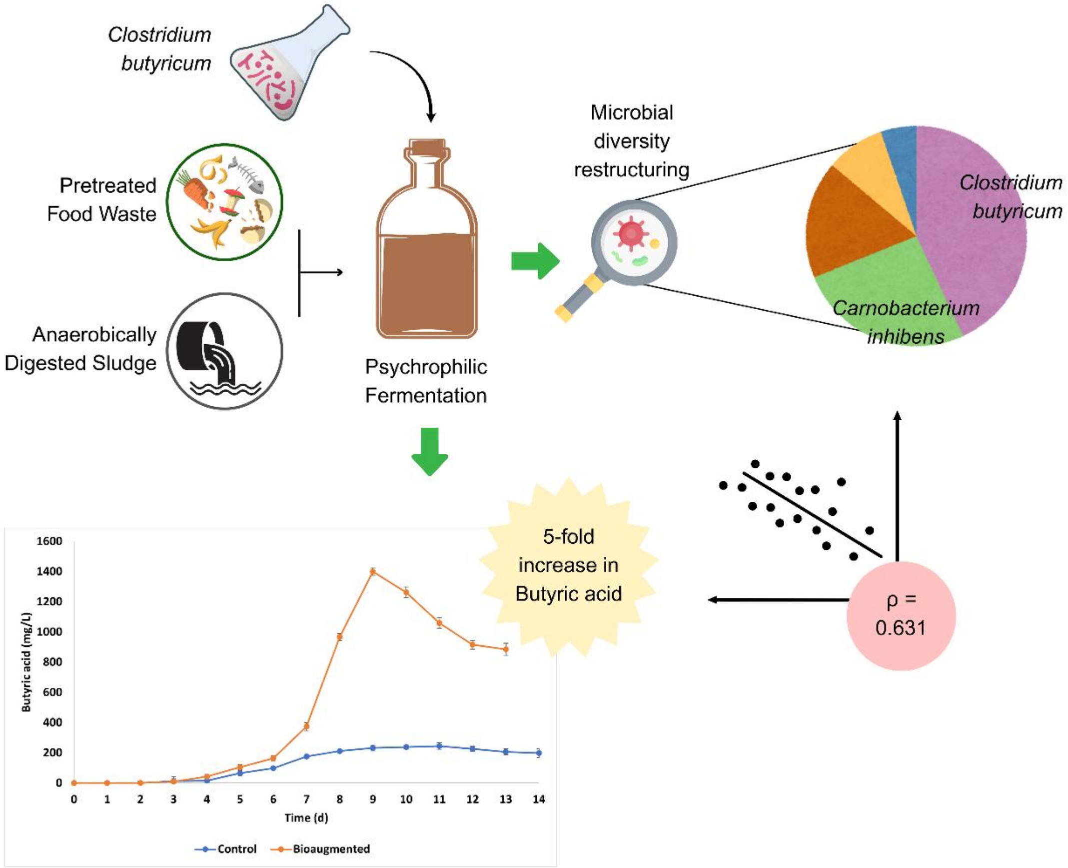
Contribution Statement: I conducted the background literature review; designed the experiments; performed all laboratory work, data collection, and primary analyses; created figures/tables; and wrote the manuscript. This chapter is adapted from the submitted article, with experimental setup concisely summarized here and full methodological details provided in Chapter 4, and formatting aligned with the thesis.

7.1.1 Abstract

This study investigated the impact of bioaugmentation with *Clostridium butyricum* on volatile fatty acid (VFA) production and microbial community dynamics during anaerobic fermentation of food waste under psychrophilic conditions (17 °C). Batch experiments were conducted using mixed cultures derived from anaerobically digested sludge, with and without bioaugmentation. The bioaugmented system achieved a maximum VFA concentration of 4,585 mg/L, more than twice that of the control (2089 mg/L), with butyric acid levels enhanced by 5.3-folds over control. Notably, butyric acid selectivity increased to 36.4% in the bioaugmented set compared to 20.2% in the control. Acetic acid remained the dominant acid in both systems, likely due to the competitive presence of acetogenic bacteria. 16S rRNA sequencing revealed significant microbial restructuring in the bioaugmented system, marked by a sustained increase in *Clostridium butyricum* and *Carnobacterium inhibens*. A statistically significant Spearman correlation ($\rho = 0.631$, $p = 0.028$) between *C. butyricum* abundance and butyric acid concentration confirmed its functional contribution to enhanced butyrate production. Redundancy analysis showed that microbial community shifts were closely linked to butyric acid production, with high-butyric-acid samples clustering separately along the primary ordination axis. This pattern reflected the influence of bioaugmentation under low-temperature conditions. In contrast, the control system displayed higher proportions of *Pseudomonas* species and unassigned taxa in later stages, reflecting a less favorable community structure and reduced metabolic efficiency. Collectively, these findings highlight the efficacy of targeted bioaugmentation for enhancing butyric acid production under mixed culture psychrophilic conditions and provide insights into community-level interactions that drive metabolic outcomes in low-temperature fermentation.

Keywords: bioaugmentation, butyric acid, selective VFA, *Clostridium butyricum*, acidogenic fermentation, food waste

7.1.2 Graphical Abstract



7.1.3 Introduction

The conversion of organic waste streams into value-added products through anaerobic fermentation has gained increasing attention in recent years as part of the circular bioeconomy approach. Among the valuable intermediates generated through this process are volatile fatty acids (VFAs), a group of short-chain organic acids that include C2-C6 carbon chain. These compounds serve as precursors for biofuels, bioplastics, and other biochemicals. However, optimizing the production of specific VFAs, particularly butyric acid, remains a challenge in mixed-culture systems, which are inherently complex due to microbial competition, metabolic cross-feeding, and sensitivity to operational variables such as temperature, substrate type, and pH [333]. Butyric acid is particularly desirable due to its applications in the chemical, pharmaceutical, and food industries [2]. Traditionally, butyric acid is produced through petrochemical routes or pure culture fermentation [334]. Yet, the use of waste-derived substrates under mixed microbial consortia offers a more sustainable alternative. However, in such systems, the yield and selectivity toward butyric acid are often limited by the absence or low activity of functional microbial groups capable of directing carbon flow efficiently to butyric acid pathway [335]. In this context, bioaugmentation, the intentional introduction of functional microbial strains, emerges as a promising strategy to steer metabolic pathways toward a targeted product (VFA) formation.

Bioaugmentation has been successfully applied in various wastewater and fermentation processes to enhance pollutant degradation [336], [337], methane production [338], [339], and more recently, VFA generation (Atasoy & Cetecioglu, 2021; Murali et al., 2021). In anaerobic fermentation, this approach can address the inherent instability of the microbial consortia by enriching the community with specialized strains that either accelerate rate-limiting steps, such as hydrolysis, or reinforce metabolic pathways of interest, such as acidogenesis. The presence of key microbial populations not only improves the substrate conversion efficiency but also reduces the accumulation of undesired by-products, enhancing the process stability and output consistency. Despite its potential, the application of bioaugmentation for butyric acid enhancement has mostly been explored under mesophilic conditions using substrates such as sludge, cheese industry wastewater, rice straw, and sugarcane vinasse [342], [343], [344]. Reports have demonstrated improved yields under mesophilic temperatures when *Clostridium* species, especially *Clostridium butyricum* were used due to their robust butyric acid-producing capabilities [345], [346], [347].

These species are known to ferment a broad range of carbohydrates into butyrate and display resilience to fluctuating conditions in complex fermentation systems without being overtaken by faster-growing species [113], [348]. However, in real waste streams such as food waste and sewage sludge, the inherent variability in substrate composition and native microbial diversity presents additional significant challenges in maintaining the dominance of butyrate producers. This issue is further amplified under psychrophilic conditions (≤20°C), where microbial metabolic activity is inherently reduced. Few studies have explored the bioaugmentation efficacy at such low temperatures and that too for the anaerobic digestion process to enhance biogas [346]. In our previous study with food waste [6], psychrophilic temperature sustained butyric acid longer than mesophilic conditions and thus offered a means of a targeted approach. Microbial diversity analysis showed that the most dominant genera of bacterial species belonged to *Paenibacillus, Solibacillus, Sporosarcina, Bacillus,* and *Lactobacillus*. However, their key roles and characteristics pertinent to fermentation are psychrotolerance, proteolytic enzyme production, nitrogen fixation, and antimicrobial production [210], [349], [350]. This supported the butyric acid producers via hydrolytic activities. Among the known butyric acid producers found in the samples were several species under the genus *Clostridium*. These are capable of utilizing complex substrates for butyric acid production and are tolerant to synergistic fermentation [6], making them potential candidates for bioaugmentation.

In this study, we addressed this notable gap in the literature by investigating the use of *Clostridium butyricum* as a bioaugmentation agent to enhance butyric acid production in a psychrophilic mixed-culture fermentation system treating food waste. This strain was selected based on our previous work [6] and other reported studies by [351], and also its compatibility with the existing microbial consortium [352]. Moreover, it can tolerate low temperature conditions and is often found coexisting with *C. tyrobutyricum* in natural and food-associated environments, suggesting ecological compatibility as reported previously in some reports [353], [354]. By focusing on microbial dynamics, VFA profiles, and community shifts, the study contributes to developing more controlled and efficient fermentation processes for selective bioproduct (VFA) generation from organic waste.

7.1.4 Materials and methods

Anaerobically digested sludge was sourced from the Humber Wastewater Treatment Facility located in Ontario, Canada, to serve as the inoculum for this study. The substrate, organic food waste, was collected from post-consumer leftovers at various restaurants in York Lanes, situated within York University, Toronto. Both the substrate and the inoculum were characterised with details presented in Table 15, as described in Chapter 4, Section 4.1.

Table 15. Characterization of inoculum (food waste) and inoculum (anaerobically digested sludge)

Characteristics	**Study on temperature effect on VFA**	
	Food Waste	Anaerobically Digested Sludge
pH	5.10 ± 0.09	7.16 ± 0.01
Total Solids (TS) (g/L)	89.0 ± 3.3	11.5 ± 0.5
Volatile solids (VS) (g/L)	83.8 ± 2.7	8.04 ± 0.30
Total Chemical Oxygen Demand (TCOD) (g/L)	82.0 ± 0.6	12.2 ± 0.03
Soluble Chemical Oxygen Demand (SCOD) (g/L)	70.3 ± 0.5	9.80 ± 0.06
Total Nitrogen (TN) (g/L)	4.15 ± 0.20	1.85 ± 0.05
Total Kjeldahl Nitrogen (TKN) (g/L)	4.60 ± 0.02	1.79 ± 0.03

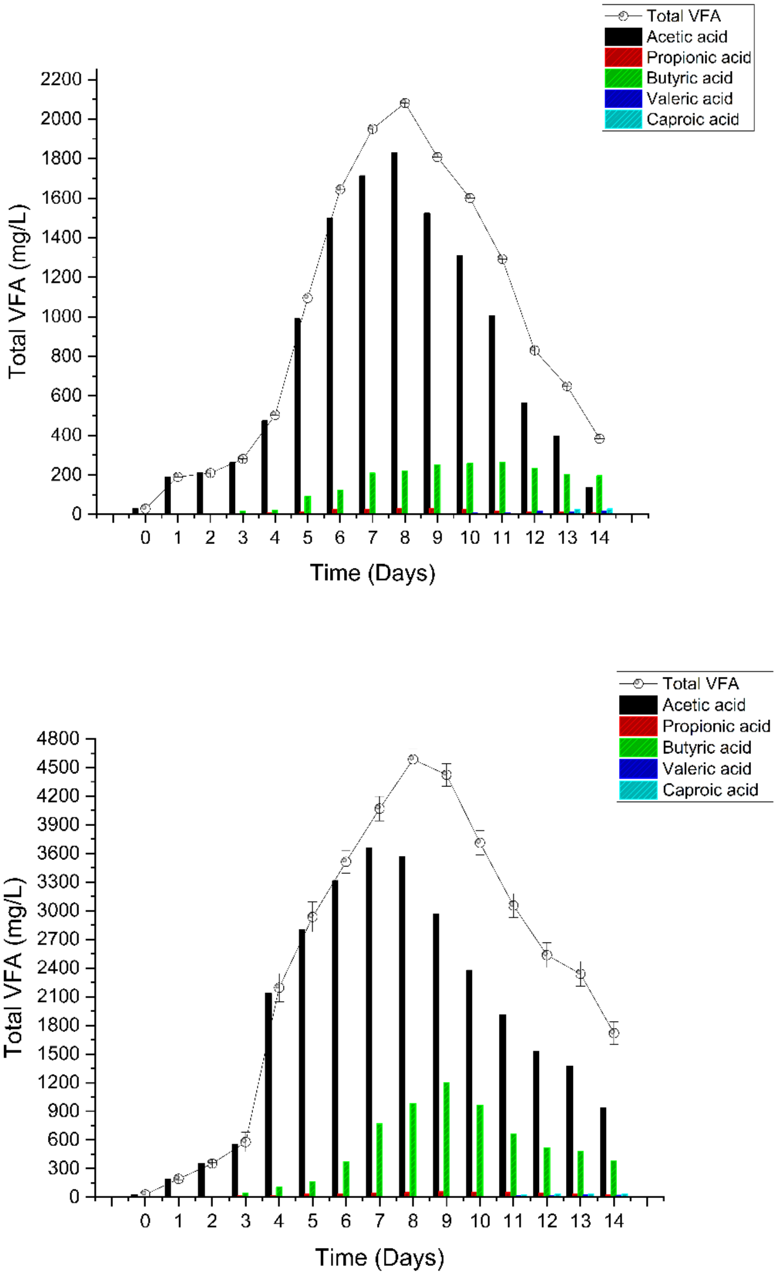
Preparation of *Clostridium butyricum* and the experimental setup for fermentation with bioaugmentation is described under Chapter 4 - Section 4.7 and Section 4.8 respectively. The analytical, microbial diversity, and statistical analysis were performed as described under Chapter 4, Section 4.9, 4.10. and 4.11 respectively.

To investigate the relationship between microbial composition and butyric acid production, microbial abundance data were first aggregated at the species level. A Spearman's rank correlation analysis was conducted between the relative abundance of *Clostridium butyricum* and butyric acid concentration (g/L) using the cor.test() function in R, with method set to "spearman". To assess broader microbial community influence on fermentation outcomes, redundancy analysis (RDA) was performed using the rda() function from the vegan package. Prior to RDA, microbial abundance data were Hellinger-transformed using decostand() to account for compositional effects. The ordination plot was constructed with samples colored by butyric acid concentration (centered), enabling visual interpretation of the association between community structure and butyrate levels. All statistical analyses and visualizations were performed in R (v4.x) using the vegan, ggplot2, and tidyverse packages.

7.1.5 Results and Discussion

Effect of bioaugmentation on VFA production

The result of total and individual VFA production to evaluate the effects of bioaugmentation are represented in Figure 31. The overall VFA concentration was enhanced in the bioaugmented set with 4585 mg/L, which was 2 times higher than that obtained in the control set (2,089 mg/L). Considering the 3 g VS added per 250 mL system, the corresponding VFA yield was 0.38 g VFA g^{-1} VS added for the bioaugmented set and 0.17 g VFA g^{-1} VS added for the control. This improvement in both concentration and yield reflects the positive influence of bioaugmentation on substrate conversion efficiency and acidogenic activity. This total increase in VFA also correlated with the increase in SCOD values in the bioaugmented set (Figure 32). On performing the two-factor ANOVA to test the significance of the difference in VFA production in control and bioaugmented sets, the p-value was found to be <0.05, and thus the results were statistically significant. This enhancement was distinctly observed after day 3 when the bioaugmentation was performed. Building on our preliminary studies [6], and the observed trends of VFA composition, day 3 was selected for the system to produce acetic acid sufficiently close to its peak to be used as the substrate for chain elongation. The individual VFAs also showed an increase in their concentration after bioaugmentation of the system.



(a)

(b)

Figure 31: Total and individual VFA concentration in the (a) control and the (b) bioaugmented set

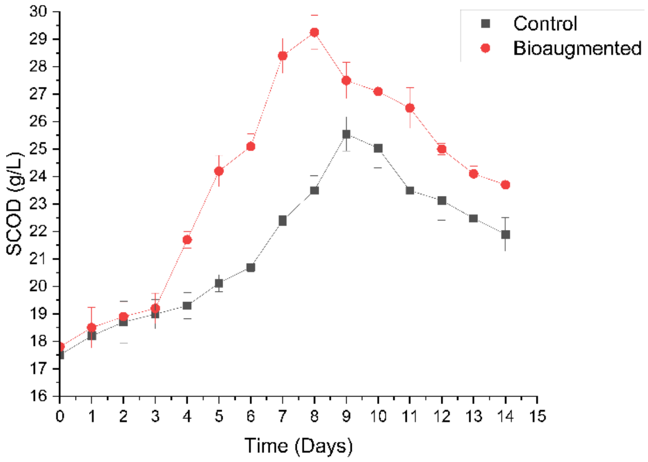


Figure 32: Soluble chemical oxygen demand changes in the control and bioaugmented set

The role of *Clostridium butyricum* has been studied to enhance VFA production in mixed-culture systems using food waste, among various other substrates as well. Ortigueira et al., showed that along with H_2 production, butyric acid production also improved by 3-times after bioaugmentation, reaching a peak of 2.5 g/L concentration [151]. Another similar study by Diez et al., showed butyric acid production reaching 2 g/L in systems bioaugmented by a combination of *Clostridium butyricum, Clostridium beijerinckii, Lactobacillus plantarum,* and *Lactobacillus pentosus* [355]. These studies, however, observed these results using sterile food waste and under mesophilic conditions. In studies employing substrates under non-sterile conditions such as dairy industry wastewater, showed about 1,587 mg/L of butyric acid produced after bioaugmentation with *Clostridium butyricum* [153]. However, the present study showed 1,401 mg/L of butyric acid concentration after bioaugmentation with *Clostridium butyricum,* under psychrophilic temperature of 17 ºC. This selective approach was based on our previous study comparing the effect of psychrophilic temperature on the composition of VFAs produced, showing sustained accumulation of butyric acid over other VFAs [6]. To further evaluate the efficiency of carbon conversion toward butyrate in the bioaugmented system, the theoretical maximum yield was estimated from the initial COD. Assuming complete conversion of organic matter exclusively to butyrate, the theoretical butyric acid concentration was calculated using a COD equivalence factor of 1.82 gCOD/g butyrate. Based on the initial COD of 18 g/L, the corresponding theoretical maximum concentration of butyric acid was approximately 9.9 g/L (equivalent to 0.82 g/g VS added for 3 g

VS). In comparison, the bioaugmented system produced 1.4 g/L of butyric acid, representing approximately 14 % of the theoretical ceiling, whereas the non-augmented control attained only ~6 %. Under mesophilic conditions, *C. butyricum* typically exhibits robust metabolic activity and rapid substrate utilization, leading to high butyric acid yields. In contrast, under psychrophilic conditions, while overall microbial activity is reduced, *C. butyricum* appears to adapt by engaging in cooperative interactions with psychrotolerant species, allowing for functional acidogenesis to proceed effectively, albeit at a slower rate. The shift from dominance to syntrophy may explain how comparable yields are achieved even at low temperatures. Building on this selectivity, the bioaugmentation strategy enhanced the butyric acid concentration by 5.3-times as compared to the control. Moreover, the selectivity of butyric acid under the bioaugmented set was found to be 36.4%, higher than 20.2% in the control set. This confirms the role of bioaugmentation in supporting butyric acid production. This is particularly significant given that low temperatures generally slow down metabolic rates, leading to reduced fermentation efficiencies. The ability of *C. butyricum* to maintain high levels of butyric acid production under psychrophilic conditions highlights its metabolic adaptability.

Among other VFAs obtained in this fermentation process, acetic acid was found to be the dominant VFA. This abundance along with lactic acid is generally observed when using food waste as a substrate owing to its native microflora [355]. Correlation analysis of *Clostridium butyricum* and individual VFAs performed by Atasoy et al., showed the enhancement of acetic, butyric and valeric acid in their study by a syntrophic relationship [153]. Such syntrophy is likely mediated through metabolite cross-feeding, where hydrolytic and fermentative bacteria produce short-chain substrates (e.g., lactate, ethanol, or acetate) that *C. butyricum* can further convert into butyric acid. This cooperative interaction facilitates a metabolic shift, especially when hydrogen partial pressures are regulated, enabling reductive pathways such as butyrate synthesis to dominate [356]. While the dominant metabolite production by a specific bacterium is studied and reported in mono-cultures, the bioaugmentation approach in mixed-cultures does enhance the targeted metabolite but also changes the composition differently. Because of a new syntrophy of the microorganisms in the bioaugmented system, metabolic pathways are affected by different routes [357]. Santiago et al. showed that the native microorganisms of food waste, such as *Eneterobacteriaceae, Enterobacterales*, and *Enterobacter* are negatively correlated with hydrogen and butyric acid production but positively correlated with acetic acid production [358]. Additionally, these microorganisms showed a negative correlation with *Clostridium butyricum* as well, thus explaining the relative abundance of acetic acid over butyric acid. This antagonistic relationship may be explained by competition for essential nutrients (e.g., glucose and peptides), as well as the release of antimicrobial secondary metabolites. Furthermore, suppression of *C. butyricum* by native flora could limit its metabolic dominance unless supported by favorable environmental conditions (e.g., pH, temperature).

While these reported observations correspond to the studies under mesophilic temperatures, reports under psychrophilic fermentation targeting VFAs are scant as per the author's present review of the literature. The psychrophilic anaerobic digestion studies have explored the microbial community diversity in these biosystems, showing the dominance of *Syntrophomonas, Syntrophus, Bacteroides,* and *Geobacter* in addition to *Clostridium* genera [192]. The shift in diversity has been reported to favour acidogenic bacteria over hydrolytic bacteria, however, this can vary between different substrate types. Furthermore, lower temperatures show increased acetic acid yields over other metabolites [359]. This is often attributed to thermodynamic constraints under psychrophilic conditions that favor acetate as a terminal product, especially when hydrogen levels are low [360]. At lower temperatures, metabolic reactions that produce more reduced compounds such as butyrate or propionate become less energetically favorable, as their associated Gibbs free energy changes are closer to equilibrium. In contrast, acetate production remains thermodynamically favorable due to its lower energy demand and simpler electron transfer steps, particularly under hydrogen-limited conditions [361]. Therefore, achieving high butyrate selectivity in such a setting, enabled by bioaugmentation is indicative of effective metabolic steering.

Microbial shift enhancing butyric acid production, and the corresponding bottleneck

Bioaugmentation with *Clostridium butyricum* alters the microbial community dynamics, influencing competition, cross-feeding, and syntrophic interactions. It favours the conversion of sugars to butyric acid and reduces the dominance of lactate producers that tend to dominate substrates like food and dairy waste [362]. *C. butyricum* utilises NADH to produce butyric acid via butyryl-CoA pathway but also produces acetic acid via acetyl phosphate route. Although butyric acid production is favoured via bioaugmentation, the simultaneous increase in the acetic acid shows the presence of acetogenic bacteria as competitors (NADH consumers), converting the corresponding hydrogen gas produced to acetic acid. As observed by Sin et al., acetogenic bacteria such as *Streptococcus equinus* and *Megasphaera elsdenii* outcompete *Clostridium butyricum* [363]. This creates a bottleneck, with acetic acid abundance, despite enhanced butyric acid production.

The microbial community dynamics observed in this study demonstrate the substantial impact of bioaugmentation on the temporal evolution of species composition during anaerobic fermentation under psychrophilic conditions (Figure 31). Early time points (day 0 and day 4) showed relatively balanced contributions to both treatment arms, but as the fermentation progressed, the microbial communities diverged substantially. The bioaugmented condition became increasingly dominated by members of the *Clostridia* and *Bacilli* classes, both known for their roles in anaerobic degradation and metabolic versatility, which suggests that bioaugmentation effectively enriched specific functional groups likely introduced or selectively favored by the treatment. In contrast, the control condition demonstrated a broader taxonomic spread with increasing representation of *Gammaproteobacteria*, a group often associated with fast growth in nutrient-enriched conditions. By day 14, the divergence between the two conditions was most pronounced. While the bioaugmented communities remained relatively centered around a few key functional classes, the control samples showed a more diffuse community structure with contributions from a larger number of less-abundant taxa including *Planctomycetes, Verrucomicrobia,* and *Acidimicrobiia*. This divergence implies that bioaugmentation selectively enriched targeted taxa.

At the species level, at the initial time point (day 0), both the control and bioaugmented sets exhibited a relatively low microbial abundance, with a significant proportion of sequences unassigned. This was expected given the unadapted status of the system and the complex, heterogeneous nature of the inoculated mixed culture. Notably, *C. butyricum* was present at very low abundance in both sets, confirming its role as a minor constituent in the native microbial community prior to augmentation. As bioaugmentation was performed on day 3, by day 4, microbial growth had markedly increased in both sets, but a clear divergence in community composition emerged. In the bioaugmented set, there was a strong enrichment of *Carnobacterium inhibens*, alongside moderate levels of *Pseudomonas veronii* and *Clostridium butyricum*. This pattern suggested early synergistic interactions between the introduced *Clostridium* and psychrotolerant cohabitants such as *Carnobacterium*, an organism known for its ability to thrive under low-temperature and anaerobic conditions and for its proteolytic capabilities. Proteolytic activity from *Carnobacterium* and similar taxa can liberate peptides and amino acids from complex organic matter, thus increasing the availability of fermentable substrates for *C. butyricum*, a phenomenon aligned with cooperative substrate hydrolysis described in low-temperature digesters [364]. In contrast, the control set displayed a more heterogeneous microbial composition dominated by *Pseudomonas fragi* and unassigned taxa, suggesting unstructured microbial proliferation with little directional selection toward butyrate-producing species.

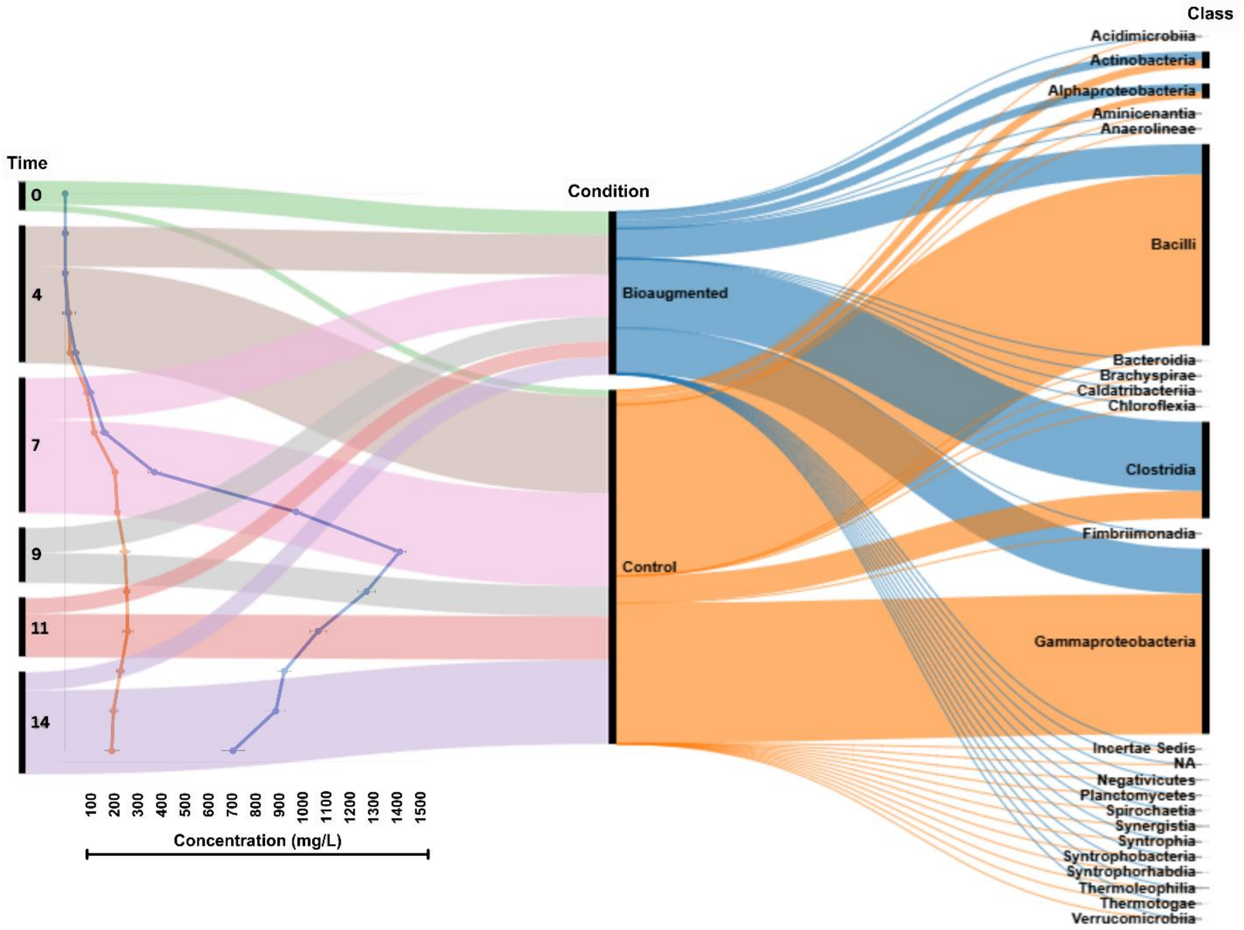


Figure 31. Alluvial plot showing temporal microbial diversity (class) shift and concurrent butyric acid production across two conditions: Bioaugmented (blue) and Control (orange). The left axis represents time points (0, 4, 7, 9, 11, and 14 days), which flow into the middle panel, indicating the treatment group each sample belongs to. From there, flows continue to the rightmost panel, which lists identified bacterial classes. Alongside the left axis, the line graph shows the butyric acid concentration trend. The width of each stream is proportional to the relative abundance of each bacterial class in a given condition and time point.

The effect of bioaugmentation became most evident by day 7. In the control sample, a pronounced increase in unassigned taxa was observed, likely indicating instability or overgrowth of uncharacterized microbes with fermentative roles. In contrast, the bioaugmented sample showed a marked increase in the abundance of *Clostridium butyricum*, indicating successful colonization and activity of the introduced strain. This coincided with a relative decline in competing *Pseudomonas* species, suggesting competitive exclusion or outperformance by *Clostridium* under the selective conditions of the fermentation process. The increase in *Clostridium butyricum* during this phase likely contributed to the observed enhancement in butyric acid production, aligning with previous findings where targeted bioaugmentation led to significant increases in VFA yields [170]. Such shifts are often stabilized through feedback mechanisms involving pH and redox potential regulation, ensuring a favorable niche for *Clostridium* proliferation.

From day 9 to day 14, a dynamic restructuring of the microbial community occurred. While *Clostridium butyricum* maintained its presence in the bioaugmented set, there was a concurrent rise in *Carnobacterium inhibens* and a re-emergence of *Pseudomonas* species, particularly *P. fragi* and *P. veronii*. The resurgence of these bacteria suggested the onset of microbial succession and potential resource competition in later fermentation stages. However, unlike the control set where no single taxon dominated, the bioaugmented system retained a functionally structured community with a higher relative abundance of known fermentative taxa, reflecting more stable and directed microbial interactions. This highlights the role of bioaugmentation not only in promoting specific metabolic outputs, such as butyric acid enhancement, but also in reshaping and stabilizing the microbial ecology of mixed-culture systems. Interestingly, the persistent dominance of *Carnobacterium inhibens* in the bioaugmented condition across several time points (notably B4, B11, and B14) suggested that this species may play a supportive role in enhancing butyrate production. It is plausible that *Carnobacterium,* through proteolytic and psychrotolerant capabilities, facilitated substrate breakdown and nutrient availability for *Clostridium butyricum* [365]. This cooperative interaction where hydrolytic and psychrotolerant bacteria in co-cultures can stimulate solventogenic *Clostridia* through amino acid-based cross-feeding and pH buffering [366]. This hypothesis is supported by earlier findings highlighting the roles of psychrotolerant, hydrolytic bacteria such as *Paenibacillus* and *Solibacillus* in assisting fermentation under low temperatures [210], [349].

On the other hand, the microbial trajectory in the control condition reflects a system prone to instability and dominated by less-desirable, non-butyrate-producing taxa. The persistence of *Pseudomonas fragi* and the high proportion of unassigned species throughout the fermentation period may contribute to suboptimal VFA profiles and process inconsistency. Further examination of the unassigned taxa, although lacking species-level resolution, revealed meaningful trends when classified at higher taxonomic levels. These groups were primarily composed of members from the orders *Actinobacteria* PeM15, *Mycobacteriales, Enterobacterales, Hyphomicrobiales,* and *Rhodobacterales*. Their presence in the bioaugmented system might be the result of new ecological niches being created after adding *Clostridium butyricum.* As fermentation conditions changed, especially with the production of new metabolites, these lesser-known organisms may have found opportunities to grow temporarily. This idea is supported by Sim et al. [367], who observed a similar rise in low-abundance or unclassified organisms during early stages of bioaugmented digestion processes. For example, *Enterobacterales* are often involved in acetate and lactate production, and may act as competitors to butyrate producers like C. *butyricum* [368]. Meanwhile, others like *Hyphomicrobiales* and *Rhodobacterales* are not typically involved in fermentation and may represent residual DNA or non-fermentative background species [369]. Thus, the microbial community was adapting to the presence of the new strain and shifting resource availability. Over time, the system in the bioaugmented set became more stable, with consistent dominance of *Clostridium butyricum* and *Carnobacterium inhibens*. In contrast, the control system showed a spike in unassigned taxa only at day 14, likely due to late-stage fermentation stress or declining system performance. This may have allowed weaker or inefficient fermenters to grow, leading to a less structured and less efficient microbial community. This contrast reinforces the benefit of bioaugmentation in steering the microbial community toward targeted fermentative outcomes, especially when working with highly variable substrates such as food waste and sludge under psychrophilic temperatures.

Correlation between *Clostridium butyricum* enrichment with butyric acid enhancement

To determine whether bioaugmentation with *Clostridium butyricum* significantly influenced butyric acid production, a Spearman correlation analysis was conducted between the relative abundance of *C. butyricum* and the measured butyric acid concentrations across all samples. A moderate but statistically significant positive correlation was observed ($\rho = 0.631$, $p = 0.028$), supporting the hypothesis that *C. butyricum* plays a key role in enhancing butyric acid formation during psychrophilic fermentation of food waste. To further explore the influence of microbial community structure on fermentation outcomes, RDA was employed as a constrained ordination technique. Based on species-level microbial profiles and butyric acid data, the RDA approximation plot revealed clear clustering of samples along the primary ordination axis, with color gradients representing centered butyric acid concentrations (Figure 32). Samples with elevated butyric acid were distinctly positioned, indicating that variation in microbial composition, most notably the presence and abundance of *C. butyricum* is a major explanatory factor for differences in butyrate yield. These multivariate and univariate findings, taken together, provide strong evidence that the observed increase in butyric acid concentrations in bioaugmented samples is not incidental but biologically driven by shifts in community structure resulting from the inoculation strategy. The statistical significance of this correlation, combined with the ecological insights from RDA, underscores the efficacy of bioaugmentation as a targeted strategy for steering anaerobic fermentation toward desired metabolic products, even under psychrophilic constraints.

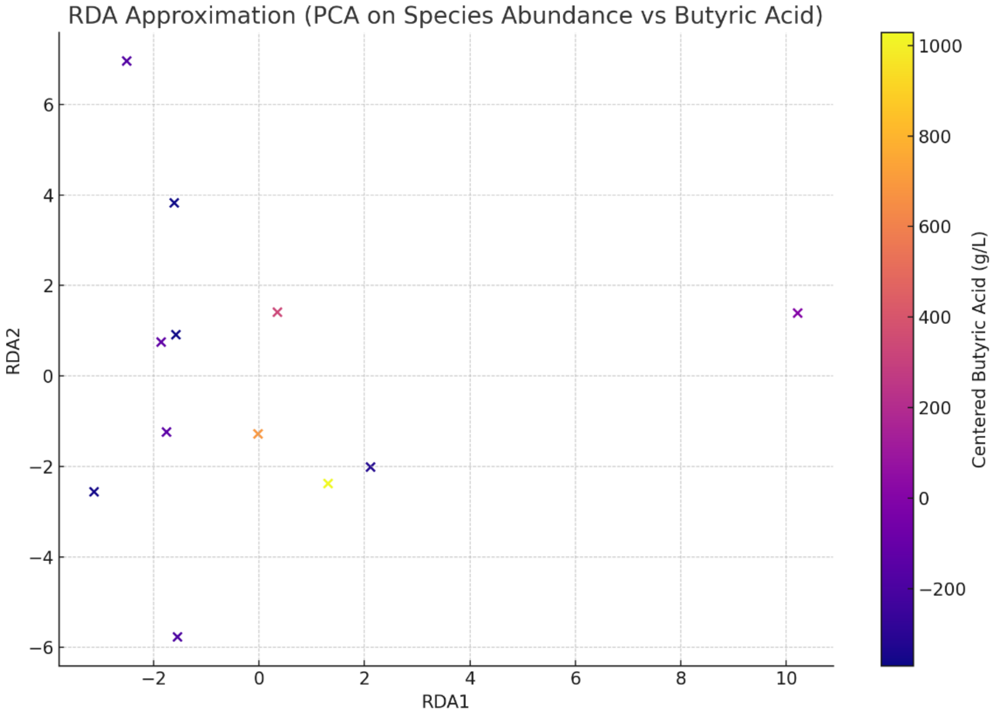


Figure 32. Redundancy Analysis (RDA) approximation showing the relationship between microbial species composition and butyric acid concentrations across fermentation samples. Each point represents a sample, projected in the ordination space derived from PCA of species-level

microbial abundance. The color gradient indicates the centered concentration of butyric acid (g/L). Samples with similar microbial community structures are positioned closely together, and a distinct clustering of samples with elevated butyric acid levels supports the influence of community shifts, particularly the role of *Clostridium butyricum* on fermentation outcomes.

7.1.6 Conclusion

Bioaugmentation with *Clostridium butyricum* significantly enhanced the butyric acid production and shaped the microbial community dynamics during psychrophilic anaerobic fermentation of food waste. The augmented system achieved a peak VFA concentration of 4.6 g/L, with butyric acid reaching 1.4 g/L. Butyrate selectivity improved to 36.4% compared to 20% under the non-augmented fermentation. Key microbial players, including *C. butyricum* and *Carnobacterium inhibens*, were enriched, while competing and less efficient taxa were suppressed. Although early increases in unassigned taxa were noted in bioaugmented condition, they reflected transient shifts rather than instability. Overall, this study demonstrates that bioaugmentation is an effective strategy to steer mixed microbial communities toward targeted metabolite production under low-temperature conditions. This offers a viable approach for optimizing fermentation processes involving complex and variable substrates like food waste.

CHAPTER 8

Conclusions and Recommendations

8.1 Conclusions

The following conclusions were drawn from this research:

8.1.1 The initial phase of the study examined the role of psychrophilic temperature (17 °C) in shaping the acidogenic fermentation profile and microbial ecology. Compared to mesophilic (37 °C) and intermediate (27 °C) conditions, psychrophilic fermentation resulted in a slower rate of hydrolysis and acidogenesis but with a notable shift toward the selective accumulation of butyric and acetic acids. Acetic acid remained the dominant metabolite owing to its thermodynamic favorability at low temperatures; however, the relative increase in butyric acid indicated an adaptive microbial redirection. This shift suggests that psychrotolerant fermentative communities possess inherent flexibility to modulate metabolic outputs even under low-energy conditions. Targeting butyric acid as the preferred product, despite acetate dominance, provided an important mechanistic insight into how process steering can enhance carbon reduction efficiency and redox balance in psychrophilic systems. The enrichment of psychrotolerant hydrolytic and acidogenic bacteria under these conditions demonstrated that microbial adaptation can sustain functional acidogenesis without thermal input. Nevertheless, the slower kinetics and incomplete hydrolysis observed underscore a trade-off between process stability and productivity, emphasizing the need for biostimulation and bioaugmentation strategies that accelerate substrate solubilization while maintaining low operational energy demand.

8.1.2 A literature-based meta-analysis reinforced the sensitivity of VFA production to substrate characteristics (e.g., COD, VS, macronutrient composition) and operational parameters (e.g., temperature, pH, inoculum type). It also highlighted the high heterogeneity and lack of standardization across reported studies, especially regarding food waste variability. This emphasized the need for tailored strategies specific to substrate-inoculum combinations. A key finding was the strong positive correlation of initial VS concentration of the substrate in the system with the VFA production. The effect of initial VS concentration was then experimentally studied, revealing that VFA production positively correlated with increasing VS concentration up to a saturation point. Beyond this, inhibitory effects possibly due to acid accumulation or nutrient limitations were observed. Notably, butyric acid concentration peaked at the highest tested VS loading (3.5 gVS/250 mL), suggesting the importance of optimizing organic loading for targeted metabolite recovery. While these findings provide valuable generalizations, the meta-analysis was constrained by inconsistent reporting of inoculum-to-substrate ratios, differing analytical methods, and limited representation from cold-climate studies. Future meta-analyses should integrate kinetic modeling or machine-learning approaches to better quantify nonlinear interactions between parameters.

8.1.3 To overcome the hydrolysis bottleneck associated with psychrophilic fermentation, two strategies were employed: thermal alkaline pretreatment and the addition of biosurfactant rhamnolipids. Both approaches significantly enhanced solubilization of organic matter and subsequent VFA production from food waste at 17 °C. Thermal alkaline pretreatment (120 °C, pH 9) improved hydrolysis efficiency by breaking down complex polymers into soluble forms, leading to a marked increase in soluble COD and higher VFA yields relative to untreated controls. This pretreatment also influenced the VFA profile, promoting the accumulation of butyric and acetic acids. Microbial analysis showed a shift in community composition favoring fermentative and hydrolytic taxa, enabling improved metabolic performance even at low temperatures. Furthermore, rhamnolipid biosurfactants, applied at 0.3 g/g TSS, demonstrated a superior performance compared to the synthetic surfactant SDS. Rhamnolipids led to a twofold enhancement, reaching up to 4.4 g/L of VFA. Rhamnolipid addition also induced a selective microbial shift. Enrichment of hydrolytic and acidogenic species such as *Pseudomonas fragi, Clostridium* spp., and *P. veronii* was observed, aligning with redirection toward VFA production by concurrent suppression of lactic acid producers like *Carnobacterium inhibens*. Collectively, these findings demonstrate that both thermal-alkaline pretreatment and rhamnolipid addition significantly enhance fermentation efficiency at low temperatures by improving substrate accessibility and actively modulating microbial communities to favor acidogenesis. These interventions provide robust strategies for enabling high-efficiency VFA production in energy-constrained psychrophilic systems. Despite these promising results, scaling such strategies requires economic and environmental assessment of reagent use and long-term stability. Biosurfactant cost, recovery potential, and microbial adaptation during extended operation remain practical constraints.

8.1.4 In the final phase, based on previously performed microbial community analyses, targeted bioaugmentation using *Clostridium butyricum* was employed to direct the fermentation process toward increased butyric acid production. The bioaugmented system exhibited a substantial increase in both total VFA concentration and butyrate selectivity compared to the non-augmented control. Peak butyric acid levels increased by over five-fold, while total VFA concentration more than doubled, indicating improved metabolic efficiency. 16S rRNA gene sequencing and metagenomic analysis revealed that *C. butyricum* successfully colonized and persisted under psychrophilic conditions, promoting butyrate production via the butyryl-CoA pathway. The microbial community structure shifted significantly, with concurrent enrichment of beneficial taxa such as *Carnobacterium inhibens*, while competing fermenters and lactate producers (e.g., *Leuconostoc, Paenibacillus, Aeromonas*) were suppressed or restructured. The functional analysis suggested enhanced sugar metabolism, amino acid degradation, and syntrophic interactions that collectively favored acidogenesis. The introduced strain acted as a metabolic catalyst, redirecting carbon flux toward butyryl-CoA formation and stabilizing the redox balance required for sustained butyrate production under low-temperature conditions. Moreover, unassigned and novel taxa, predominantly from the Actinobacteria class (e.g., PeM15), also showed dynamic changes, indicating potential involvement in cross-feeding or secondary fermentation roles. These observations suggest that even under selective environmental pressures, microbial diversity can adapt through synergistic interactions, and that bioaugmentation can strategically harness such potential to achieve targeted metabolic outputs. However, bioaugmentation outcomes remain influenced by inoculum compatibility, competitive microbial interactions, and nutrient availability. Continuous or semi-continuous validation, metabolic flux modeling, and genomic tracking of augmented strains are recommended to confirm long-term stability and reproducibility.

8.2 Recommendations

1. To offset the cost of commercial rhamnolipids, future studies should investigate the possibility of producing biosurfactants in situ by integrating biosurfactant-producing microbes into the VFA fermentation system. This could be achieved through co-cultivation of specific bacteria, such as *Pseudomonas* spp., under controlled conditions that support both aerobic or microaerophilic phases for biosurfactant synthesis, followed by anaerobic conditions for fermentation.
2. This study showed an earlier microbial succession and a transient increase in specific taxa in the bioaugmented system as opposed to the control. Future work should test different inoculation times or repeated additions of *C. butyricum* to synchronize with acidogenesis and maximize community steering to butyric acid producers.
3. While 16S rRNA provides taxonomic insights, follow-up studies using metagenomics and metatranscriptomics would provide functional information on the activity of key metabolic pathways (e.g., butyryl-CoA, acetyl-phosphate routes) and identify regulatory bottlenecks under psychrophilic conditions.
4. Future studies should address downstream recovery technologies (e.g., membrane separation, electrodialysis) suited for psychrophilic fermentation broths. Special focus should be placed on selective recovery of butyric and acetic acids.
5. Transitioning to continuous or semi-continuous pilot-scale systems is essential to evaluate the long-term performance of rhamnolipid-enhanced and bioaugmented psychrophilic fermentation. Future studies should also include a techno-economic and life-cycle assessment (TEA–LCA) to assess the scalability, cost-effectiveness, and environmental sustainability of these processes compared with conventional anaerobic digestion. Such analysis will help identify economic bottlenecks, energy trade-offs, and environmental advantages associated with operating at low temperatures, providing a pathway toward industrial implementation of low-energy biorefinery systems.

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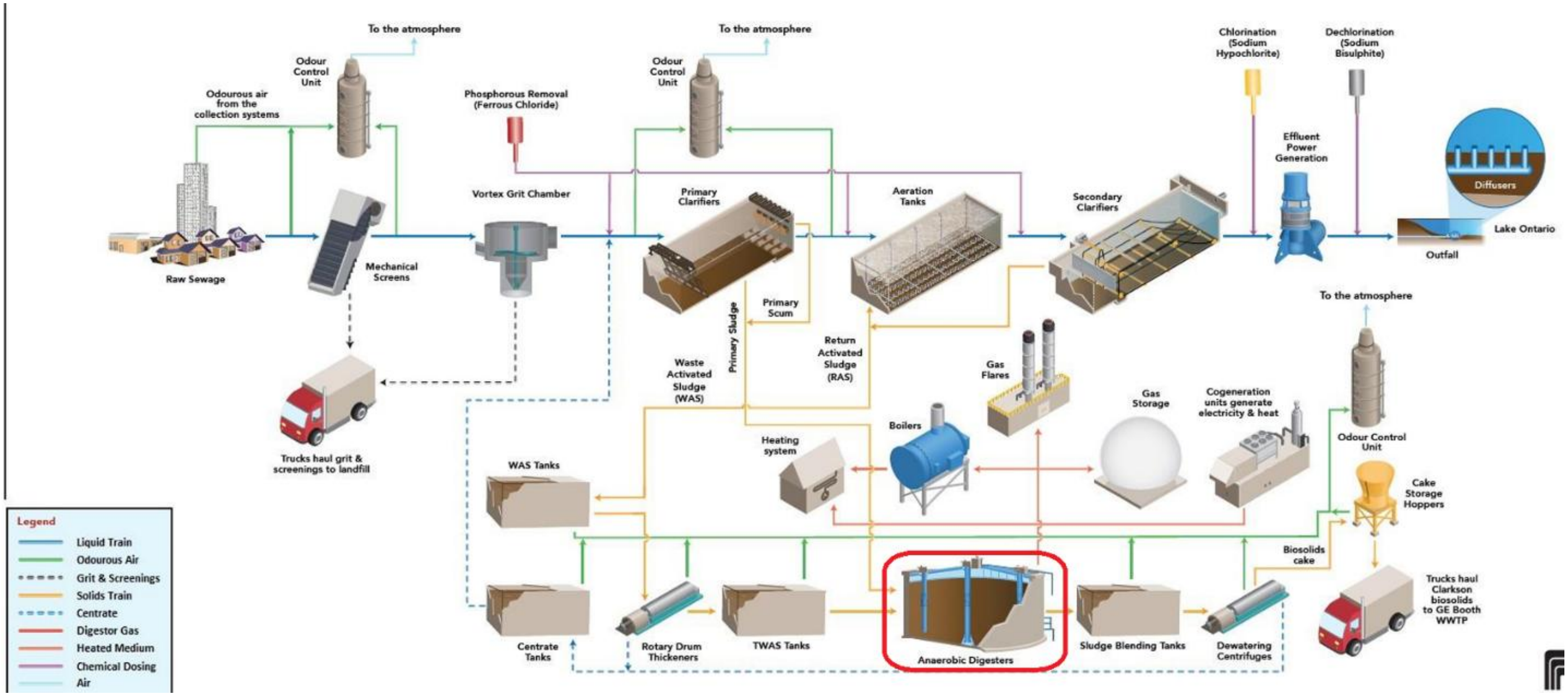
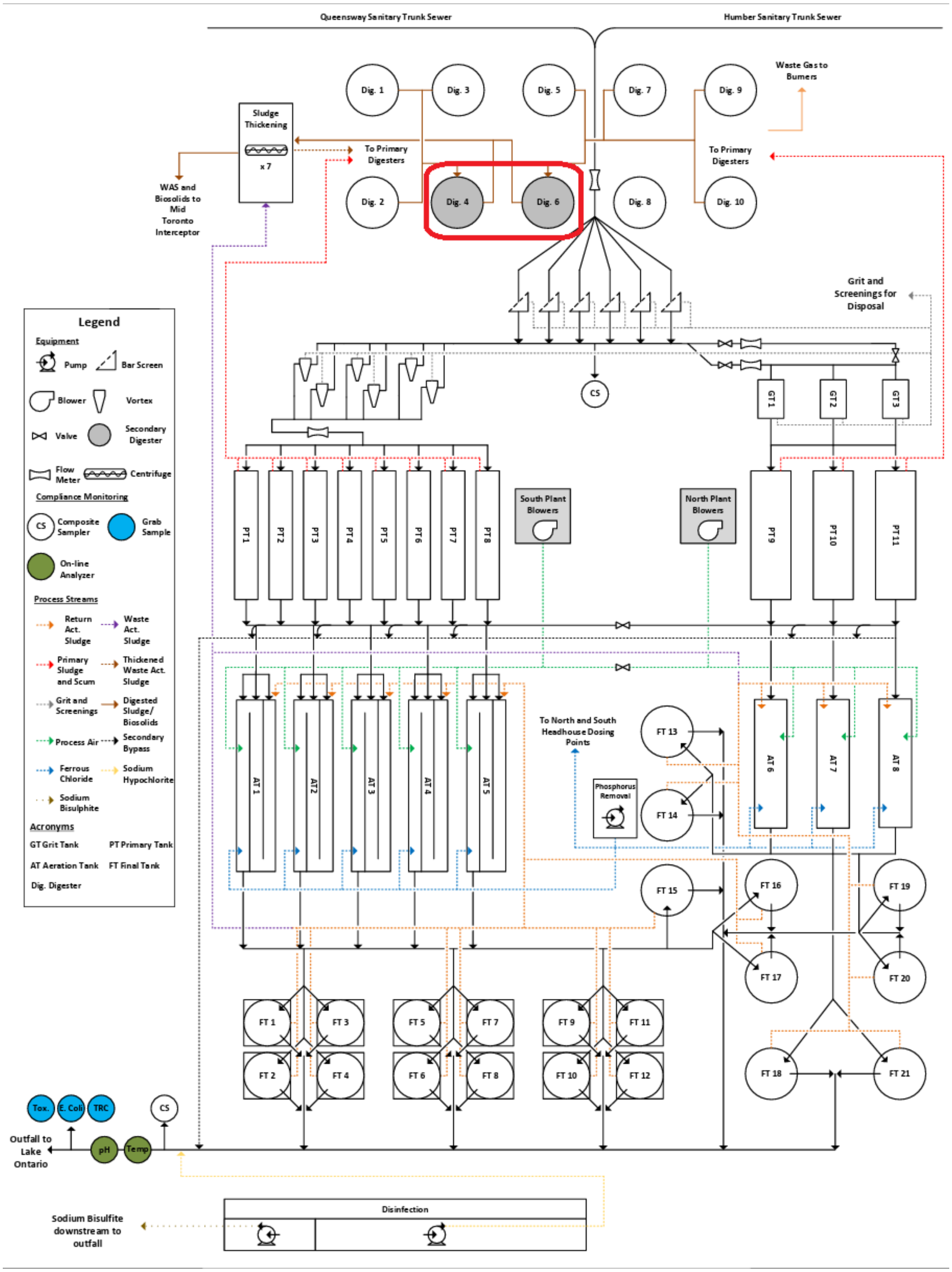
APPENDICES

Appendix 1: Schematic diagrams for the wastewater treatment plants for sludge collection

1. Clarkson Wastewater Treatment Plant: Sludge collection from anaerobic digestor (outlined in red)



2. Humber Wastewater Treatment Plant: Sludge collection from anaerobic digestor (outlined in red)



Appendix 2: Forest plots from meta-analysis study (Section 5.2)

Forest plots summarizing the meta-analysis assessing correlations between volatile fatty acid (VFA) concentrations and key operational parameters. The plots illustrate the strength and direction of associations, with effect sizes and confidence intervals derived from pooled study data.

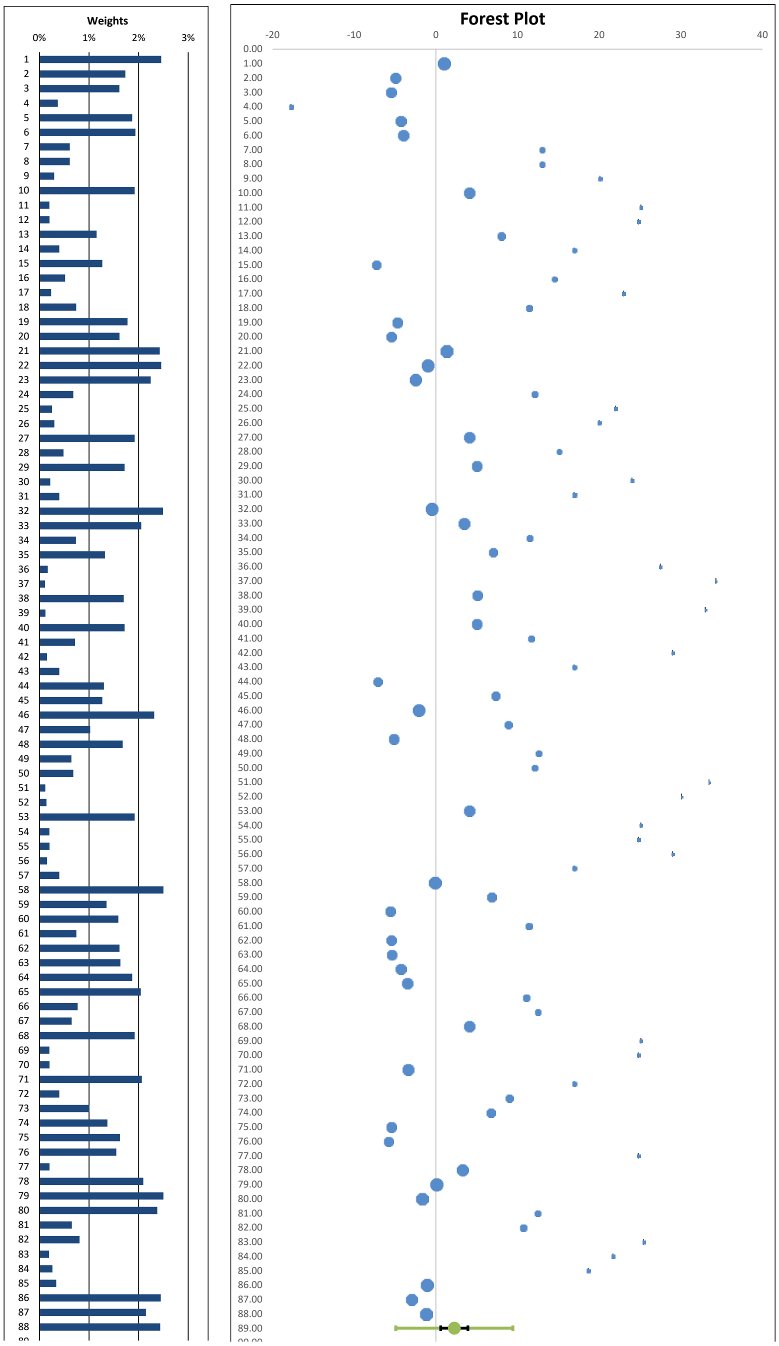


Figure: Forest plot for pH and VFA correlation

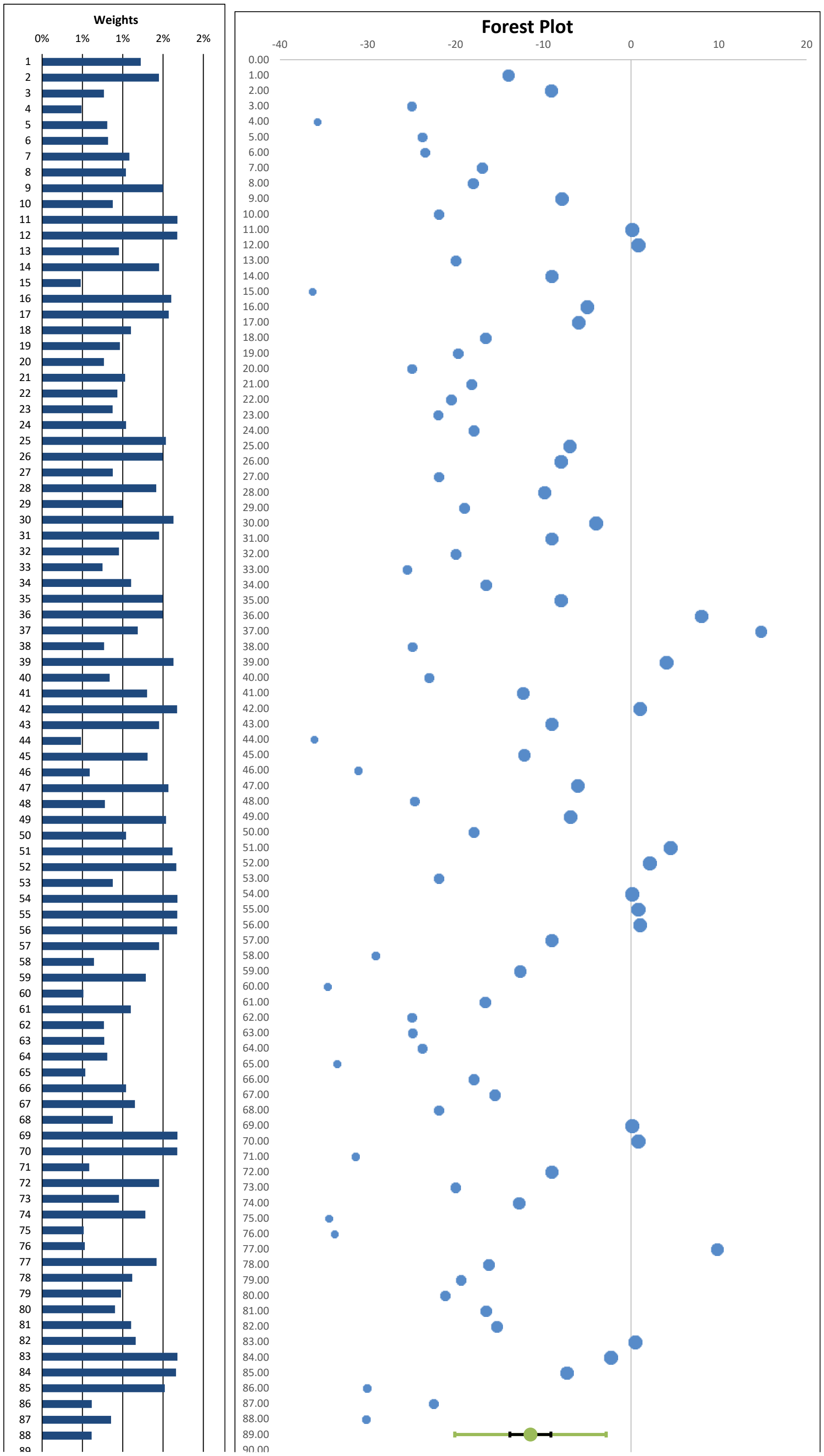


Figure: Forest plot for temperature and VFA correlation

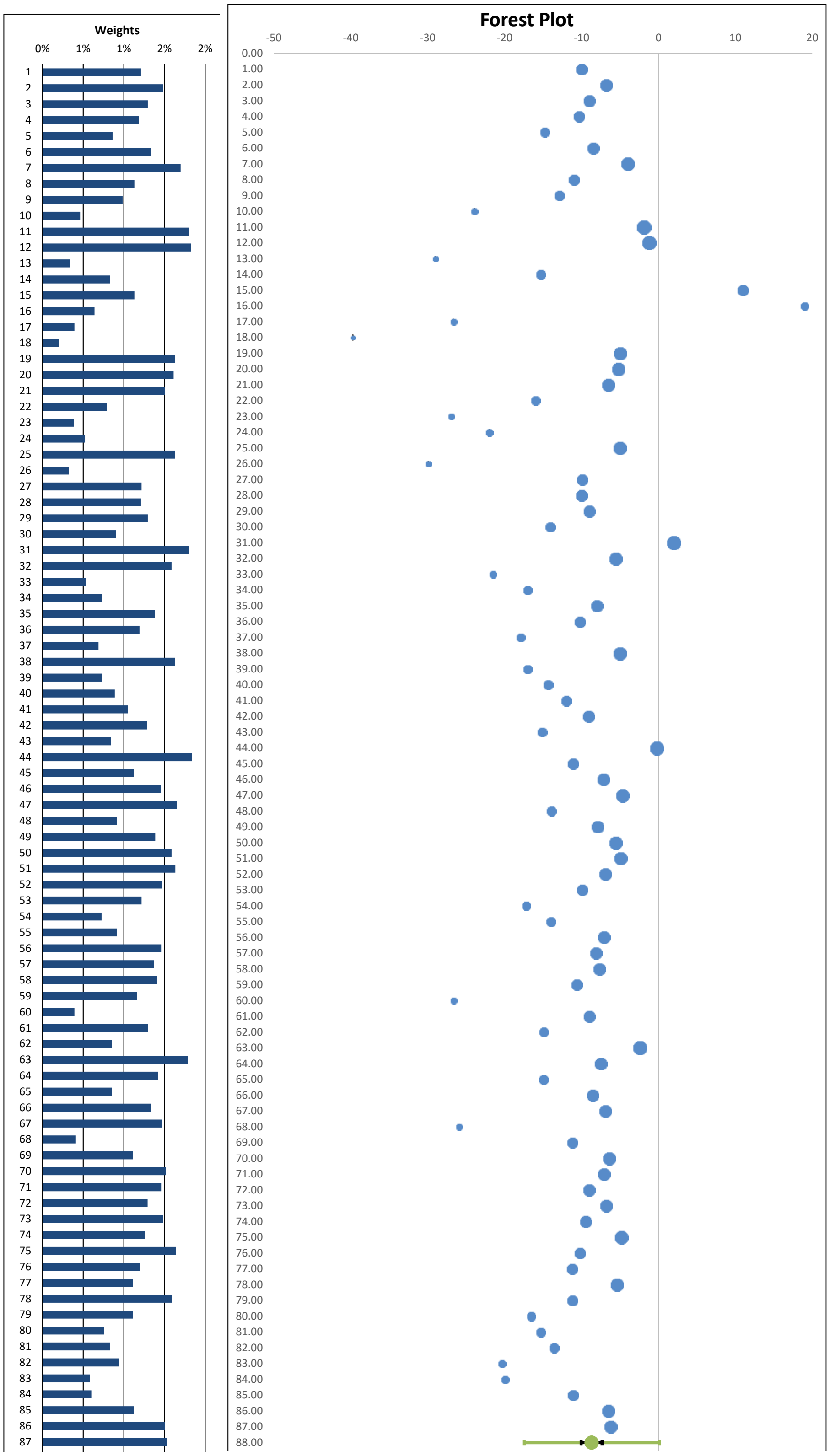


Figure: Forest plot for initial substrate concentration and VFA correlation

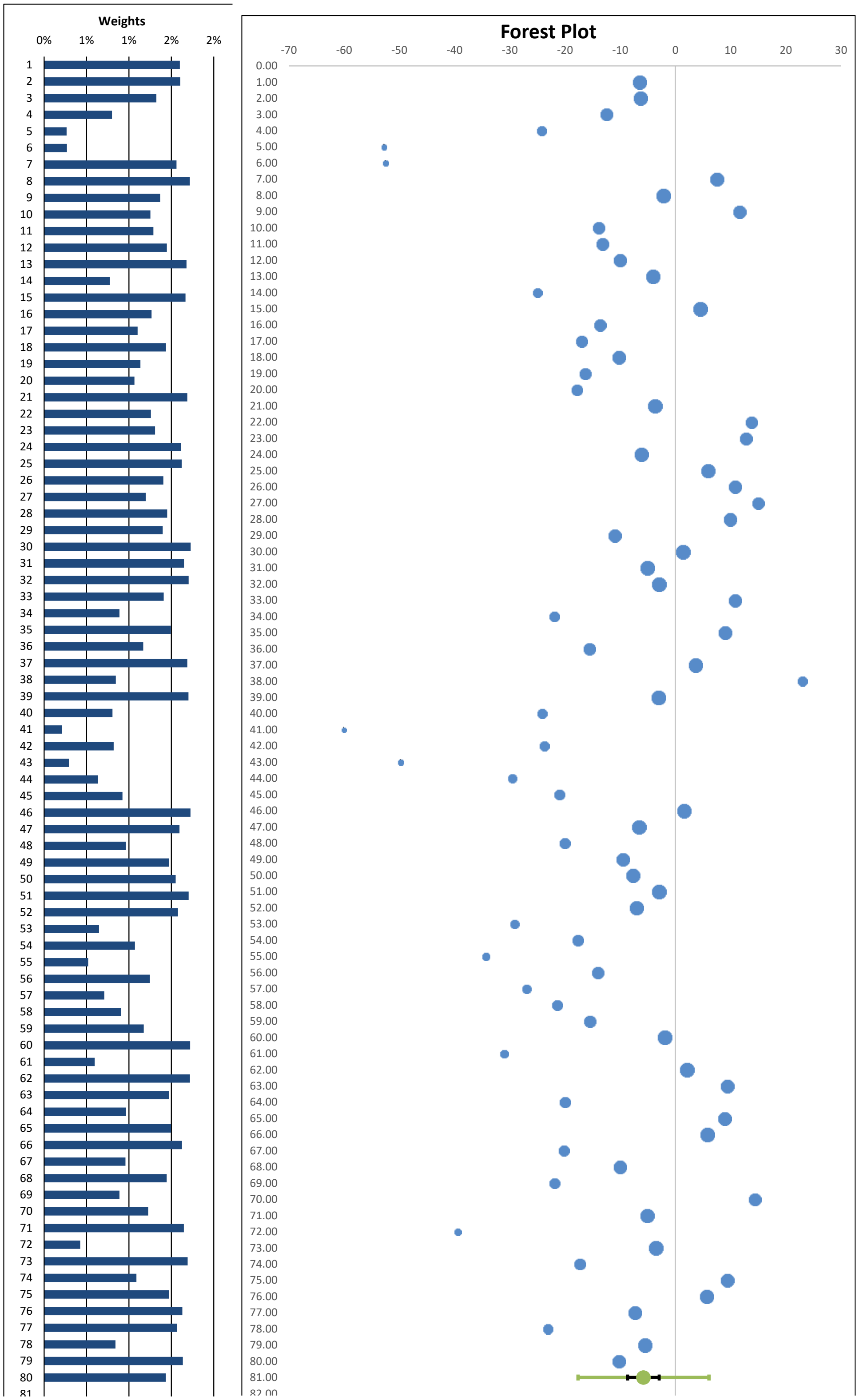


Figure: Forest plot for food waste VS and VFA correlation

Appendix 3: Impact of alkaline pH regulation on psychrophilic fermentation (Section 5.3)

Comparison of VFA production under controlled (pH 9) and uncontrolled pH conditions. Results indicate that maintaining pH 9 throughout fermentation substantially enhanced VFA accumulation compared to the uncontrolled set. Based on this outcome, pH 9 was selected for subsequent experiments evaluating the effect of varying initial volatile solids (VS) concentrations.

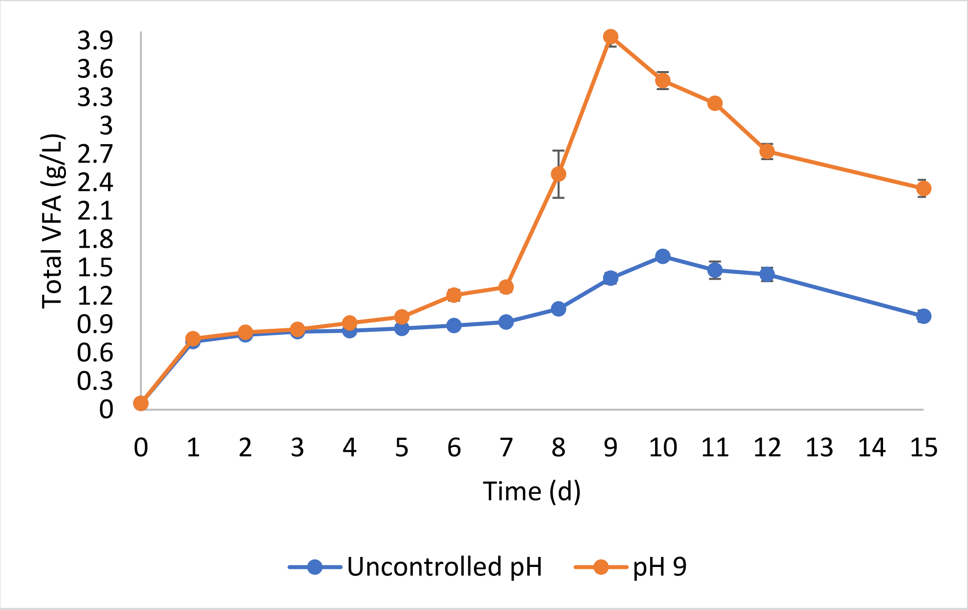


Figure: Total VFA concentration trend under uncontrolled and maintained (alkaline) pH conditions

Appendix 4: Proposed mechanism (literature-based) of rhamnolipid action in food waste fermentation (Section 6.2)

Literature-derived schematic illustrating the proposed mechanism by which rhamnolipids enhance volatile fatty acid (VFA) production. According to previous studies, rhamnolipids facilitate fat solubilization through emulsification, improving enzymatic accessibility for lipases to hydrolyze triacylglycerols (TAGs) into diacylglycerols (DAGs), monoacylglycerols (MAGs), and free fatty acids (FFAs). Glycerol released during hydrolysis can be phosphorylated to dihydroxyacetone phosphate (DHAP), entering the glycolytic pathway and contributing to acetyl-CoA formation for subsequent VFA biosynthesis. Concurrently, rhamnolipids may increase membrane permeability and promote microbial uptake of FFAs, while influencing community composition through lactic acid bacteria (LAB) suppression. This conceptual pathway is presented here for interpretive context and was not experimentally validated in the present study.

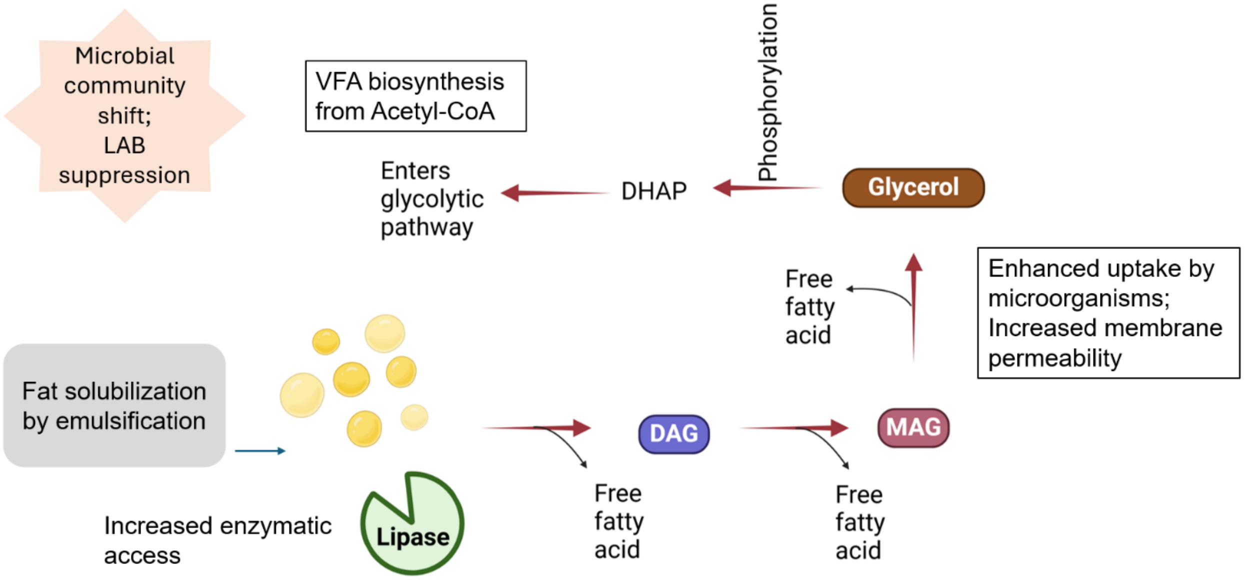


Figure: Literature-based schematic of the proposed role of rhamnolipids in enhancing VFA production from food waste via emulsification, improved hydrolysis, glycerol conversion, and enhanced microbial uptake.

Appendix 5: Culturing of anaerobic *Clostridium butyricum* (Section 7.1)

The schematic outlines the laboratory workflow for isolating and culturing Clostridium butyricum from commercially available prebiotic capsules prior to bioaugmentation experiments. Capsule contents were suspended in Reinforced Clostridial Medium (RCM) broth and streaked onto RCM agar plates. Plates were incubated anaerobically at 37 °C until distinct colonies appeared. Colonies were restreaked to confirm purity, and pure isolates were subsequently inoculated into liquid RCM broth. Anaerobic incubation was performed in sealed serum bottles purged with nitrogen gas until the culture reached the desired optical density ($OD_{600} \approx 1.0$). The resulting suspension was used for experimental inoculation.

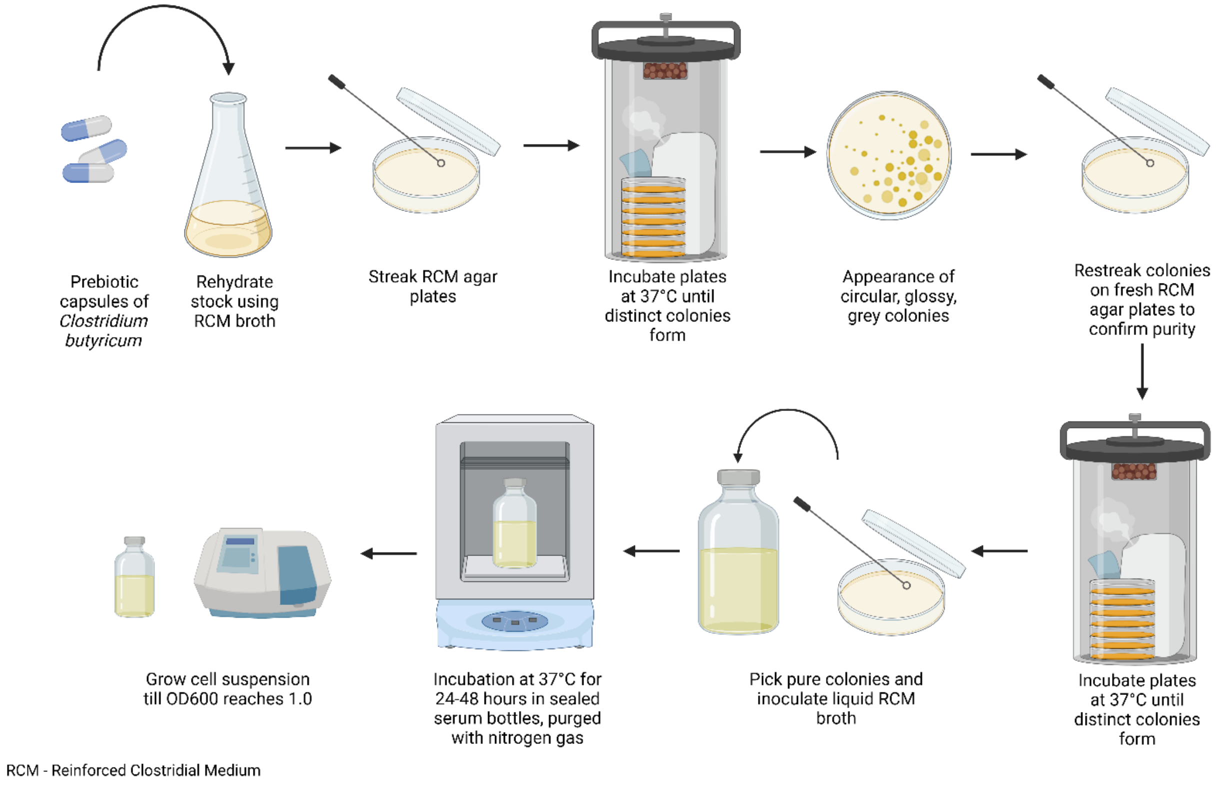


Figure: Workflow for isolating and culturing Clostridium butyricum from prebiotic capsules

Appendix 6 :Proposed metabolic pathway for conversion of lysine to butyric acid during acidogenic fermentation

Lysine degradation to butyric acid occurs via the cadaverine pathway under anaerobic conditions. Initially, lysine is decarboxylated by lysine decarboxylase to form cadaverine, which then undergoes oxidative deamination by amine oxidase, producing 5-aminovalerate. Subsequent reactions involving CoA transferase and β-oxidation steps yield butyryl-CoA, which is converted to butyrate through the action of butyrate kinase or phosphotransbutyrylase.

Simplified pathway:
Lysine → Cadaverine → 5-Aminovalerate → Glutarate semialdehyde → Butyryl-CoA → Butyric acid

Appendix 7: Note on Freezing pretreatment

In certain unrelated laboratory activities, food waste was frozen temporarily for storage convenience only and later used for separate experiments that are not part of this thesis. The freezing step in those instances was not a pretreatment method but simply a preservation measure to prevent microbial degradation prior to use.

Freezing and thawing are known to cause cellular disruption and partial solubilization of organic matter, which can influence hydrolysis rates. Therefore, all data and results presented in this thesis were obtained using freshly collected and immediately homogenized food waste, without any freeze–thaw storage, to maintain substrate integrity and experimental consistency.

Appendix 8: Metabolic pathways of lactic acid and VFA crossover

The lactate pathway plays a central role in mixed-culture acidogenic fermentation by serving as both a metabolic intermediate and a substrate for secondary VFA synthesis. In the present study, genera such as *Lactobacillus* and *Leuconostoc* are known lactate producers through the Embden–Meyerhof–Parnas (EMP) pathway, converting carbohydrates to pyruvate and subsequently to lactate via lactate dehydrogenase. Lactate can then be further oxidized to acetate, propionate, or butyrate depending on redox balance and microbial interactions.

Under anaerobic conditions, lactate oxidation to acetate or butyrate occurs through syntrophic associations involving *Clostridium*, *Pseudomonas*, or *Carnobacterium* spp., where lactate acts as an electron donor. Specifically, lactate is first converted to acetyl-CoA, which then enters the butyryl-CoA pathway for butyrate synthesis. This conversion is thermodynamically feasible when hydrogen-scavenging microbes maintain low H_2 partial pressure. The enrichment of *Clostridium* spp. in the bioaugmented system likely facilitated this conversion, explaining the concurrent decline in lactic acid-producing taxa and increase in butyrate concentration.

The coexistence of lactate-producing and lactate-utilizing bacteria therefore establishes a cross-feeding network, enhancing overall acidogenesis and supporting targeted butyrate formation. This interspecies lactate conversion pathway underscores the metabolic flexibility of mixed consortia under psychrophilic conditions and highlights lactate as a key intermediary in shaping the final VFA profile.

Appendix 9: Functional Genes and Enzymatic Roles in VFA production

This appendix provides a detailed molecular overview complementing the metagenomic discussion presented in Chapter 1. While the introduction outlined how pH modulates gene expression in anaerobic systems, this section expands on the specific genes, enzymes, and microbial taxa associated with different VFA biosynthetic routes.

The production of VFAs from food waste under anaerobic and psychrophilic conditions involves several interconnected metabolic pathways. These are primarily governed by carbohydrate degradation, amino acid fermentation, and redox-regulated reactions, which converge at the central intermediate acetyl-CoA. The distribution of metabolic flux from acetyl-CoA toward acetate, butyrate, or propionate is determined by the expression of key enzymes encoded by genes such as *ackA/pta, thl, buk, ldh,* and *hbd* [87].

The following table summarises the major functional genes reported in acidogenic fermentation, their corresponding enzymes, roles in metabolic conversion, and representative microbial genera typically associated with each pathway. This mapping provides a molecular-level perspective on how microbial communities coordinate carbon and electron flow toward specific VFAs, particularly under psychrophilic and low-pH conditions that favor acidogenesis.

Functional Gene	Encoded Enzyme / Role	Step in Fermentation	Representative Microbes	Dominant VFA / Product
ldh	Lactate dehydrogenase – interconverts pyruvate and lactate	Lactate pathway (acidogenesis)	*Lactobacillus*, *Leuconostoc*	Lactate → Butyrate (cross-feeding)
ackA, **pta**	Acetate kinase / phosphotransacetylase – converts acetyl-CoA → acetate + ATP	Acetogenesis	*Clostridium*, *Bacteroides*	Acetate
hydA	[FeFe]-hydrogenase – electron transfer and redox balance	Acidogenesis / H_2 evolution	*Clostridium*, *Desulfovibrio*	Indirectly supports butyrate
thl, **hbd**, **crt**, **bcd**, **etfA/B**, **ptb**, **buk**	Thiolase and dehydrogenases – chain elongation via butyryl-CoA	Butyrate synthesis	*Clostridium butyricum*, *C. tyrobutyricum*	Butyrate
mcrA	Methyl-coenzyme M reductase – key methanogenesis enzyme	Methanogenesis	*Methanosaeta*, *Methanobacterium*	Methane (consumes VFAs)

Functional Gene	Encoded Enzyme / Role	Step in Fermentation	Representative Microbes	Dominant VFA / Product
pfl	Pyruvate-formate lyase – redox balancing under alkaline stress	Mixed acid fermentation	*Enterococcus*, *Eubacterium*	Acetate / Formate
adhE	Alcohol dehydrogenase – acetyl-CoA → ethanol	Redox balance under stress	*Clostridium*, *Pseudomonas*	Alcohols (competes with VFAs)
narG, **phoA**, **groEL**, **dnaK**	Nitrate reductase / alkaline phosphatase / stress proteins	Alkaline adaptation	*Pseudomonas*, *Bacillus*	Stress adaptation
kamA, **kal**, **kce**	Lysine-degrading enzymes	Amino acid fermentation	*Clostridium*, *Peptoclostridium*	Butyrate + NH_3

Collectively, these genes and enzymatic routes demonstrate the metabolic plasticity of mixed microbial consortia involved in VFA production. For instance, *ldh* facilitates lactate formation under acid stress, which can later serve as a substrate for butyrate synthesis through the *thl–hbd–bcd* cascade. Similarly, *ackA* and *pta* regulate acetyl-CoA flux to acetate, contributing to ATP generation and system stability. The upregulation of *hydA* and *etf*A/B supports redox balance by linking electron transfer to hydrogen metabolism—critical for sustaining acidogenesis at low temperatures [88].

The inclusion of these molecular pathways provides a framework for interpreting functional gene expression in future metagenomic or metatranscriptomic studies, offering insight into how psychrophilic conditions selectively activate genes that favor VFA accumulation over methanogenesis.