UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE PESQUISA EM BIOENERGIA

PROGRAMA INTEGRADO (UNESP, USP E UNICAMP) DE PÓS-GRADUAÇÃO EM BIOENERGIA

Valorization of expired dairy products by anaerobic digestion

DANIELI FERNANDA CANAVER MARIN

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Thesis submitted to Bioenergy Research Institute, Sao Paulo State University UNESP, Rio Claro – SP, Brazil, as part of the requirements for obtaining a Doctor of Science degree.

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TÍTULO DA TESE: Valorization of Expired Dairy Products by Anaerobic Digestion

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To my dear husband Antonio.

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"The only source of knowledge is experience."

Albert Einstein.

ABSTRACT

The dairy industry is an important sector of the food industry, producing 928 million tons of milk in 2021. However, this sector is responsible for generating a high amount of waste, such as expired dairy products (EDP). Currently, the main destination of EDP are their disposal in landfills, causing environmental problems due to their high organic matter content. In this sense, this study proposed to evaluate strategies for using and valorizing EDP, transforming them into resources for bioproducts generation from anaerobic digestion (AD). As the first strategy, an autochthonous mixed culture was obtained from EDP using anaerobic batch reactors with *All Purpose Tween* (APT) culture medium as substrate. The *Lactobacillus* genus was identified with the highest relative abundance in the obtained culture with 78.4%. Subsequently, the autochthonous mixed culture was evaluated in anaerobic reactors using different substrates (xylose, arabinose, fructose, maltose, glucose, rhamnose, lactose, sucrose, EDP, and banana processing wastewater (BPW)). The mixed autochthonous culture grew on all tested substrates, with high carbohydrate removal (57.6-97.0%), producing lactic acid (LA) as the predominant soluble metabolite (2.40-7.85 g L^{-1}). The highest LA yield was obtained from BPW with 0.98 g g^{-1}. As the second strategy, it was evaluated the co-digestion of EDP with synthetic sewage (SS) in single-stage anaerobic reactors. Five assays were performed with the following percentages of EDP in the substrate mixture (1) 0%, (2) 5.0%, (3) 7.5%, (4) 10.0%, and (5) 15, 0% (v/v). Higher COD removal (89.2%) and higher CH_4 production (6583.6 NmL L^{-1}) were verified in Assay 4 (10.0% EDP). Higher CH_4 yield were obtained in Assay 3 (7.5% EDP) with 332.6 NmL gVS_{add}^{-1}. Acetoclastic (*Methanosaeta*) and hydrogenotrophic (*Methanolinea, Methanoregula,* and *Methanobacterium*) archaea were identified in assays 2, 3, and 4 (5.0% to 10.0% EDP), which probably contributed to the high efficiency of organic matter removal and high methane production. Assay 5 (15.0% EDP) was inhibited due to the accumulation of volatile acids in the anaerobic reactors. As the last strategy, the co-digestion of EDP with SS was evaluated from two-stage anaerobic systems. In the first stage (acidogenic reactors), three assays were assembled with the following substrate composition (v/v): (1) 10% EDP + 90% SS, (2) 15% EDP + 85% SS, and (3) 20% EDP + 80% SS. The liquid effluents generated at the end of the acidogenic stage of assays 1, 2, and 3 were used as substrates in the second stage (methanogenic reactors), corresponding to tests 4, 5, and 6, respectively. In the first stage, higher carbohydrate removal (92.3%) and higher hydrogen production (1309.3 NmL L_r^{-1}) were obtained in Assay 3 (20% EDP + 80% SS). In the second stage, higher COD removal (94.7%) and methane production (6813.4 NmL L_r^{-1}) were verified in Assay 5 (15% EDP + 85% acidified SS). Assay 6 (20% EDP + 80% acidified SS) was inhibited due to the accumulation of volatile fatty acids (11427.9 mg L^{-1}), mainly acetic acid (8455.6 mg L^{-1}). Thus, the experimental approaches of the studies carried out offer promising opportunities for EDP valorization through its application in biological processes. In addition, the use of expired dairy waste can contribute to its recovery by providing bioproducts and biofuels obtained from renewable sources.

Keywords: Expired dairy products; Anaerobic digestion; Methane; Hydrogen; Lactic acid.

RESUMO

A indústria de laticínios é um importante setor da indústria de alimentos com produção de 928 milhões de toneladas de leite em 2021. Entretanto, esse setor é responsável pela geração de uma elevada quantidade de resíduos, como os produtos lácteos vencidos (PLV). Atualmente, a principal destinação dos PLV é a sua disposição em aterros sanitários, causando problemas ambientais devido à sua elevada carga orgânica. Neste sentido, esse estudo propôs avaliar estratégias de utilização e valorização de PLV transformando-os em recursos para a geração de bioprodutos a partir do processo de digestão anaeróbia (DA). Como primeira estratégia uma cultura autóctone mista foi obtida a partir dos PLV usando reatores anaeróbios em batelada alimentados com meio de cultura *All Purpose Tween* (APT) como substrato. O gênero *Lactobacillus* foi identificado com maior abundância relativa na cultura obtida com 78.4%. Posteriormente, a cultura mista autóctone foi avaliada em reatores anaeróbios utilizando diferentes substratos (xilose, arabinose, frutose, maltose, glicose, ramnose, lactose, sacarose, PLV, e água residuária do processamento da banana (ARPB)). A cultura autóctone mista cresceu em todos os substratos testados, com elevadas remoções de carboidratos (57.6-97.0%) e produção predominante de ácido lático (AL) como metabólito solúvel (2,40-7,85 g L^{-1}). O maior rendimento de produção de AL foi obtido a partir da ARPB com 0,98 g g^{-1}. Como segunda estratégia, foi avaliada a co-digestão dos PLV com esgoto sintético (ES) em reatores anaeróbios em único estágio. Cinco ensaios foram realizados com as seguintes porcentagens de PLV na mistura de substratos (1) 0%, (2) 5,0%, (3) 7,5%, (4) 10,0%, e (5) 15,0% (v/v). A remoção mais elevada de DQO (89,2%) e maior produção de CH_4 (6583,6 NmL L^{-1}) foram verificadas no ensaio 4 (10,0% PLV). O rendimento mais elevado de CH_4 foi obtido no ensaio 3 (7,5% PLV) com 332.6 NmL gVS_{add}^{-1}. Archaeas acetoclásticas (*Methanosaeta*) e hidrogenotróficas (*Methanolinea, Methanoregula e Methanobacterium*) foram identificadas nos ensaios 2, 3 e 4 (5,0% a 10,0% PLV), o que provavelmente contribuiu para a eficiência elevada de remoção de matéria orgânica e alta produção de metano. O Ensaio 5 (15.0% PLV) foi inibido devido ao acúmulo de ácidos voláteis nos reatores anaeróbios. Como última estratégia, a co-digestão de PLV com ES foi avaliada a partir de sistemas anaeróbios em dois estágios. No primeiro estágio (reatores acidogênicos), foram montados três ensaios com a seguinte composição de substrato (v/v): (1) 10% PLV + 90% ES, (2) 15% PLV + 85% ES, e (3) 20% PLV + 80% ES. Os efluentes líquidos gerados ao final da etapa acidogênica dos ensaios 1, 2 e 3 foram utilizados como substratos no segundo estágio (reatores metanogênicos), correspondendo aos ensaios 4, 5 e 6, respectivamente. No primeiro estágio, a remoção mais elevada de carboidrato (92,3%) e maior produção de hidrogênio (1309.3 NmL L_r^{-1}) foram obtidas no ensaio 3 (20% PLV + 80% ES). No segundo estágio, a remoção mais elevada de DQO (94.7%) e produção de metano (6813.4 NmL L_r^{-1}) foram verificadas no ensaio 5 (15% PLV + 85% ES acidificado). O ensaio 6 (20% PLV + 80% ES acidificado) foi inibido devido ao acúmulo de ácidos graxos voláteis (11427,9 mg L^{-1}), principalmente ácido acético (8455,6 mg L^{-1}). Assim, as abordagens experimentais dos estudos realizados oferecem oportunidades promissoras para a valorização do PLV através da sua aplicação em processos biológicos. Além disso, a utilização de resíduos lácteos vencidos pode contribuir na sua valorização com o fornecimento de bioprodutos e biocombustíveis obtidos a partir de fontes renováveis.

Palavras-chave: Produtos lácteos vencidos; Digestão anaeróbia; Metano; Hidrogênio; Ácido lático.

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LIST OF ABBREVIATIONS AND ACRONYMS

AcoD - Anaerobic co-digestion

AD – Anaerobic digestion

APT - All Purpose Tween

ASBR - Anaerobic Sequential Batch Reactor

BPM - Biochemical potential of methane

BPW - Banana processing wastewater

CFU - Colony-forming unit

CG - Crude glycerol

COD – Chemistry Oxygen Demand

CSTR - Continuously stirred tank reactors

CW – Cheese whey

DF – Dark fermentation

DP – Dairy products

EDP – Expired dairy products

EGSB - Expanded granular sludge bed

EoL-DPs - End-of-Life Dairy Products

FOG - Fats, oils, and greases

FW – Food waste

GC - Gas chromatograph

HPB - Hydrogen-producing bacteria

HPLC - High-Performance Liquid Chromatography

HRT - Hydraulic retention time

LA – Lactic acid

LAB - Lactic acid bacteria

LCFA - Long-chain fatty acids

MRS - De Men, Rogrosa and Sharpe medium

NmL – Volume (mL) normalized by modified Gompertz equation

OLR - Organic loading rate

OUT - Operational taxonomic unit

PBS - Phosphate buffer saline solution

SAD – Single-stage anaerobic digestion

SS - Synthetic sewage

tC - total carbohydrates

TCD - Thermal conductivity detector

tCOD - total Chemistry Oxygen Demand

TS – Total solids

TSAD – Two-stage anaerobic digestion

UASB - Upflow anaerobic sludge blanket

VFA – Volatile fatty acids

VS – Volatile solids

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1 GENERAL INTRODUCTION

The dairy sector is one of the most important in the food industry, representing a relevant role in the world economy, with global production of 928 million tons in 2021 (FAO, 2021). Furthermore, the sector is projected to expand by 2025, particularly in the fresh dairy sector. (OECD; FAO, 2016).

However, dairy products are highly perishable, with a short shelf life (FANTOZZI et al., 2015). After expiration, these products are considered unfit for consumption, resulting in significant waste (SAKARIKA et al., 2020). The current management of this waste uses a small part for animal feed, and most of it is destined for landfill (ADGHIM et al., 2020; KOPSAHELIS et al., 2018).

Although dairy waste does not contain toxic substances, its disposal in landfills is harmful to the environment since it has a high organic matter content, easily biodegradable by microorganisms responsible for the production of greenhouse gases (DAT et al., 2020). Several studies demonstrate the adverse effects caused by the disposal of food waste in landfills and reinforce the urgency of converting these wastes into resources using sustainable techniques and processes (BRUNKLAUS B, REX E, CARLSSON E, 2018; OLIVEIRA; LAGO; DAL' MAGRO, 2021; VERZOLA, 2018).

Anaerobic digestion (AD) has been considered a suitable and efficient method for waste treatment. This biological process has great potential for waste valorization, turning it into raw material for biogas generation, which can be applied as a renewable energy source. AD also produces other value-added bioproducts, such as organic acids and alcohols, and reduces organic matter in waste, preventing environmental degradation (HUNTER; BLANCO; BORRION, 2021). Additionally, strategies such as anaerobic co-digestion and two-stage systems can also be used to improve AD efficiency, supporting the synergy and stability of the biological process (XU et al., 2018).

Expired dairy products (EDP) are composed of carbohydrates (57.5%), lipids (28.4%), and protein (14.0%) (SLOPIECKA et al., 2022), which makes them an excellent substrate for anaerobic digestion, as they can be easily degraded. Furthermore, EDP are the source of fermentative microorganisms, such as lactic acid bacteria, which can also be applied to obtaining products of biotechnological interest, such as lactic acid and ethanol (ESKIN; SHAHIDI, 2013; NOBLECOURT et al., 2018).

Many studies present the biotechnological potential of using other residues from the dairy industry (e.g., dairy wastewater and cheese whey) have been developed. However, few researches demonstrate the application of EDP with the same purpose. In this sense, the main objective of this work is to propose different strategies for using EDP by anaerobic digestion to valorize this waste, reducing its inappropriate disposal.

2 HYPOTHESES AND OBJECTIVES OF THIS THESIS

2.1 General Hypothesis and objective

The assays were conducted based on the general hypothesis that the expired dairy products (EDP), due to their high organic load, can be used as a potential substrate in anaerobic digestion, to obtain bioproducts, such as biogas (hydrogen and methane), organic acids and alcohols.

Thus, the general objective was to evaluate expired dairy products as a substrate for generate bioproducts using batch reactors from different strategies, such as obtaining autochthonous fermentative culture, anaerobic digestion in a single-stage, and anaerobic digestion in two-stage.

2.2 Specific Hypotheses and Objectives

2.2.1 Specific Hypothesis and Objective 1

Hypothesis 1: Expired dairy products are a natural source of fermentative microorganisms capable of degrading different carbon sources.
Objective 1: Obtain autochthonous fermentative culture from expired dairy products and evaluate it using different carbon sources.

2.2.2 Specific Hypothesis and Objective 2

Hypothesis 2: Expired dairy products due to their high organic matter can be co-digested with sewage (low organic matter), and both can be used as a substrate in single-stage anaerobic co-digestion.
Objective 2: In a single-stage system, evaluate methane generation in anaerobic batch reactors, fed with expired dairy products and sewage.

2.2.3 Specific Hypothesis and Objective 3

Hypothesis 3: Expired dairy products can be co-digested with sewage and used as a substrate in anaerobic co-digestion from two-stage biosystems; acidogenesis and methanogenesis.
Objective 3: Evaluate the anaerobic co-digestion of expired dairy products with sewage, using batch reactors in two-stage systems, aiming to generate biogas (hydrogen and methane) and other value-added products.

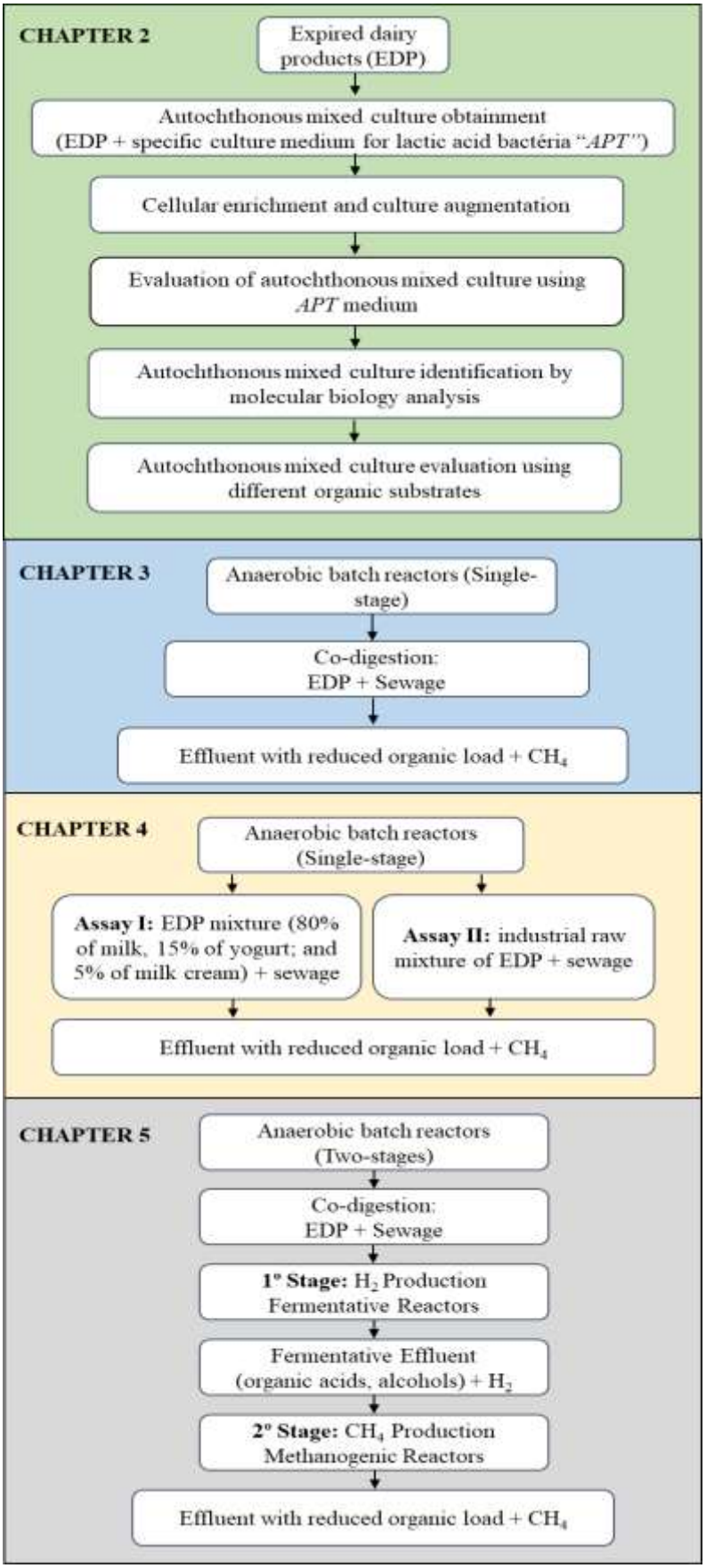
3 SCOPE OF THIS THESIS

This study was idealized based on the possibility to use expired dairy products (EDP) as a substrate in anaerobic digestion, due to their huge generation by the dairy industry and their current inappropriate destination. Therefore, this thesis was structured in chapters (I - V), where **Chapter I** presents the literature review, focusing on the dairy industry and its waste generation. In addition, anaerobic digestion was approached as an alternative for the EDP recovery, where the positive aspects of its use were reviewed, also its challenges and strategies to obtain a more efficient biological process. The other chapters (II – V) present the strategies used by the present study to evaluate the efficiency of biological processes in EDP valorization. The general experimental flowchart referring to these strategies is presented in **Figure 1**. In **Chapter II**, an autochthonous fermentative culture was obtained from EDP. This culture was identified and its biotechnological potential was evaluated by applying this culture in an anaerobic reactor using different substrates (xylose, arabinose, fructose, maltose, glucose, rhamnose, lactose, sucrose, EDP, and banana processing wastewater (BPW). Subsequently, EDP was used as a substrate from anaerobic co-digestion with synthetic sewage (SS) aiming for methane production. Therefore, in **Chapter III**, the co-digestion of EDP with SS was studied using anaerobic batch reactors in a single-stage. The main goal was to evaluate the effect of EDP increasing in biological process performance focusing on organic matter removal and achieving the highest methane yield with the tested parameters. The microbial community involved in anaerobic co-digestion was identified to better understand the reactor's performance.

Additionally, in **Chapter IV** a new study using single-stage anaerobic digestion was carried out in order to compare two distinct EDP types (Dairy Waste A and Dairy Waste B). This study was conducted as a way to assess whether EDP composition change would significantly affect methane production results. Dairy waste A was composed of a mixture with known concentrations of EDP (80% of milk + 15% of yogurt + 5% of milk cream), similar to the number of return products of the dairy industry. Dairy waste B was an industrial raw mixture (the same one used in Chapter III). Both dairy wastes were co-digested with SS using anaerobic batch reactors. This study was published in the journal “Engineering in Agriculture.” Also, as complementation of Chapter III, **Chapter V** evaluates the co-digestion of EDP with SS using a two-stage

system. This study was carried out as an attempt to overcome the inhibition of the biological process with the increase in the amount of EDP in the anaerobic reactors, as observed in Chapter III. Therefore, with the separation of the acidogenic and methanogenic stages, it was considered to treat a higher concentration of EDP achieving better reactors stability, in addition to obtaining better energy yield from hydrogen and methane production. This chapter also comprises a study of the microbiota of the two-stage, carried out through molecular biology analysis.

Figure 1 - General Experimental Flowchart



CHAPTER I

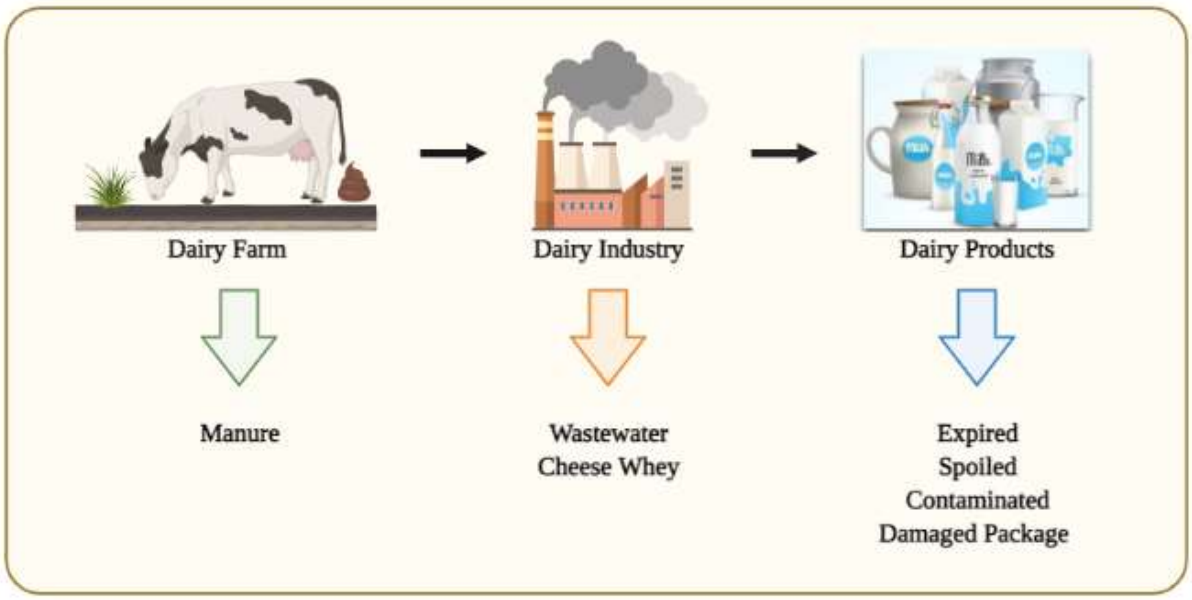
1 LITERATURE REVIEW

1.1 THE DAIRY INDUSTRY AND WASTE GENERATION

Population and economic growth, associated with improved quality of life, have led to a growing global demand for food. Dairy production is one of the most relevant components of the food industry. It generates a wide range of products (e.g., milk, milk powder, cheese, cream, yogurt, curd, butter, ice cream) consumed worldwide (KAUR, 2021). World milk production reached 928 million tons in 2021 (FAO, 2021). Brazil stood out in this sector as the sixth largest milk producer in the world, with the production of almost 36 million tons in the same year, which is highly positive to supply the nutritional demand of the population (FAO, 2022).

However, according to the Food and Agriculture Organization of the United Nations, it is estimated that approximately 1.3 billion tons of food are wasted every year, corresponding to a third of the total food produced for human consumption (FAO, 2013). At this point, the dairy industry is also highlighted as one of the major waste generators in its entire supply chain, from raw materials to products (AHMAD et al., 2019) (**Figure I.1**).

Figure I.1- Waste generated in the dairy industry supply chain.



The most abundant wastes in the dairy industry are wastewater and cheese whey. For every liter of processed milk, approximately 6-10 liters of wastewater are generated. Moreover, the production of one kilogram of cheese results in the

generation of approximately 9 liters of cheese whey (HAUSJELL et al., 2019; KAMBLE; BHARGAVA; PATIL, 2020). However, around 18% of milk production is wasted for several reasons (SAR et al., 2022). Dairy products have a short shelf-life and high perishability, often resulting in unsuitable products for sale or consumption, mainly because they have exceeded their expiration date. Additionally, many of these products return to the factory due to deterioration or damaged packaging (ALONSO et al., 2010; FANTOZZI et al., 2015).

Approximately 4-11 million tons of dairy waste are released annually into the environment. While not inherently toxic, this waste has a high organic matter, with COD concentrations between 13.0-63.2 g/L for dairy wastewater, 68.6-107.3 g/L for cheese whey cheese, and 112.8-297.2 g/L for expired dairy products. They are composed mainly of carbohydrates, protein, and fat (**Table I.1**). Consequently, their inappropriate disposal can harm the environment, causing eutrophication in water bodies, biogenic emissions, and air pollution, contributing to climate change (AHMAD et al., 2019; KOPSAHELIS et al., 2018).

Table I.1 – Dairy Waste Composition

Dairy Waste	COD (g/L)	Carbohydrate (g/L)	Protein (g/L)	Fat (g/L)	References
Cheese Whey	68.6	45.9	2.7	9.4	SADDOUD et al., 2007
Cheese Whey	77.5	35.3	8.7	-	TREU et al., 2019
Cheese Whey	107.3	70.0	34.4	-	PÉREZ-MORALES et al., 2021
Dairy Wastewater	13.0	-	-	4.2	AMINI et al., 2013
Dairy Wastewater	14.7-52.8	5.4-22.1	1.3-13.1	-	TAN et al., 2021
Dairy Wastewater	63.2	26.0	6.0	-	YE et al., 2023
Expired Dairy Products	112.8	26.8	-	-	KOPSAHELIS et al., 2018
Expired Dairy Products	191.0	47.2	-	6.64	SAKARIKA et al., 2020
Expired Dairy Products	185.3-297.2	52.3-78.5	-	12.4	MARIN et al., 2022

The processing of any pollution source must be done in agreement with the norms and regulations adopted by each country to prevent environmental degradation.

Currently, several treatment methods have been applied to treat dairy industry effluents, such as wetland, physicochemical treatment, and biological processes (activated sludge, aerated lagoon, and anaerobic) (CARVALHO; PRAZERES; RIVAS, 2013; HEALY; RODGERS; MULQUEEN, 2007; KAUR, 2021).

The primary destination for expired dairy products (EDP), considered solid waste, is either their utilization in animal feed or, more commonly, their disposal in landfills (ADGHIM et al., 2020; SAKARIKA et al., 2020). Both practices contradict Brazilian laws and regulations.

According to Normative Instruction No. 81 of 19th December 2018 of the Ministry of Agriculture, Livestock and Food Supply, Art. 16, it is not allowed to use expired food, as well as the use of trade returns, for the production of co-products intended for animal feed (BRASIL, 2018, p.17). Furthermore, Brazil's National Solid Waste Policy instituted by Law nº 12.305 of August 2nd, 2010, established the responsibility for developing solid waste reduction and reuse strategies to minimize its environmental impacts.

In addition to Brazil, landfilling of food waste is considered a global problem. This is because it contradicts laws and directives worldwide, such as Japan's Food Recycling Law of 2007, European Directive 2008/98/EC, and Turkey's regulation no. 27533 2012/03, Italian Law no. 166/16, India's Municipal Waste Management and Handling Rules 2000, among others. They all aim to reduce waste disposal by encouraging its prevention, reuse, recycling, and treatment.

Related to this issue, the recent development of the circular economy concept reinforces the importance of converting waste into resources and minimizing losses through the recovery and regeneration of products by their life cycle, using sustainable techniques and processes (MURRAY; SKENE; HAYNES, 2017; OLIVEIRA; LAGO; DAL' MAGRO, 2021).

In this context, anaerobic digestion (AD) is an effective alternative for valorizing dairy waste by converting them into valuable raw materials for producing biofuels and bioproducts. The biogas produced during AD can generate electricity, heat, and fuel for transportation, thus reducing greenhouse gas emissions. AD also offers environmental benefits reducing the negative impacts of landfilling. Additionally, AD encourages the transition to a circular economy by promoting resource efficiency, reducing waste and greenhouse gas emissions, and contributing to developing a more sustainable society (KOPSAHELIS et al., 2018).

Nevertheless, several studies have demonstrated the potential of using wastewater from the dairy industry and cheese whey to obtain biogas (FERREIRA et al., 2021; IVANCHENKO; YELATONTSEV; SAVENKOV, 2021; RINCÓN-PÉREZ et al., 2021; SIVAPRAKASAM; BALAJI, 2020). However, there is a lack of literature about using expired dairy products for the same purpose, strengthening the present study's importance.

1.2 Anaerobic digestion

Anaerobic digestion is widely recognized as an exciting alternative for treating and recovering organic waste (Adames et al., 2021). AD is a process carried out by a group of microorganisms in the absence of oxygen, generally divided into four phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (SIKORA et al., 2017).

The first step of the process, hydrolysis, is characterized by the degradation of complex organic matter (proteins, fats, and carbohydrates) into soluble molecules such as amino acids, fatty acids, and short-chain sugars (MERLIN CHRISTY; GOPINATH; DIVYA, 2014). In acidogenesis, the metabolites obtained from the hydrolysis phase are absorbed by bacteria and used in the metabolic cycle of these microorganisms, with consequent generation of volatile organic acids (acetic, propionic, lactic, and formic acids, among others), alcohols, and gases, such as H_2 e CO_2 (SRISOWMEYA; CHAKRAVARTHY; NANDHINI DEVI, 2020). According to Hassan & Nelson (2012) the common fermentative bacteria are *Lactobacillus*, *Eubacterium*, *Clostridium*, *Escherichia coli*, *Fusobacterium*, *Bacteroides*, *Leuconostoc*, and *Klebsiella*.

In acetogenesis, the soluble metabolites produced in acidogenesis are catabolized into acetic acid by a restricted group of homoacetogenic microorganisms (ZHANG; SHEN; NI, 2015). Homoacetogenic species are known in the genera *Acetobacterium*, *Acetoanaerobium*, *Acetogenium*, *Butyribacterium*, *Clostridium*, *Eubacterium*, and *Pelobacter* (BORJA, 2011).

In the final stage, methanogenesis, two groups of methanogenic archaea convert the intermediate compounds producing methane. Hydrogenotrophic archaea, reduce CO_2 to CH_4 using H_2 as a primary electron donor. Some genera also can use formate as the major electron donor (e.g., hydrogenotrophic genera are *Methanobacterium*, *Methanocellus, Methanomicrobium,* and *Methanolinea*). The second group is acetoclastic archaea, which produce CH_4 from acetate consumption. There are only two known genera in this group: *Methanosarcina* and *Methanosaeta*.

Methanosarcina also can utilize methanol and H_2 for methanogenesis (LIU; WHITMAN, 2008).

1.3 Degradation of expired dairy products main components

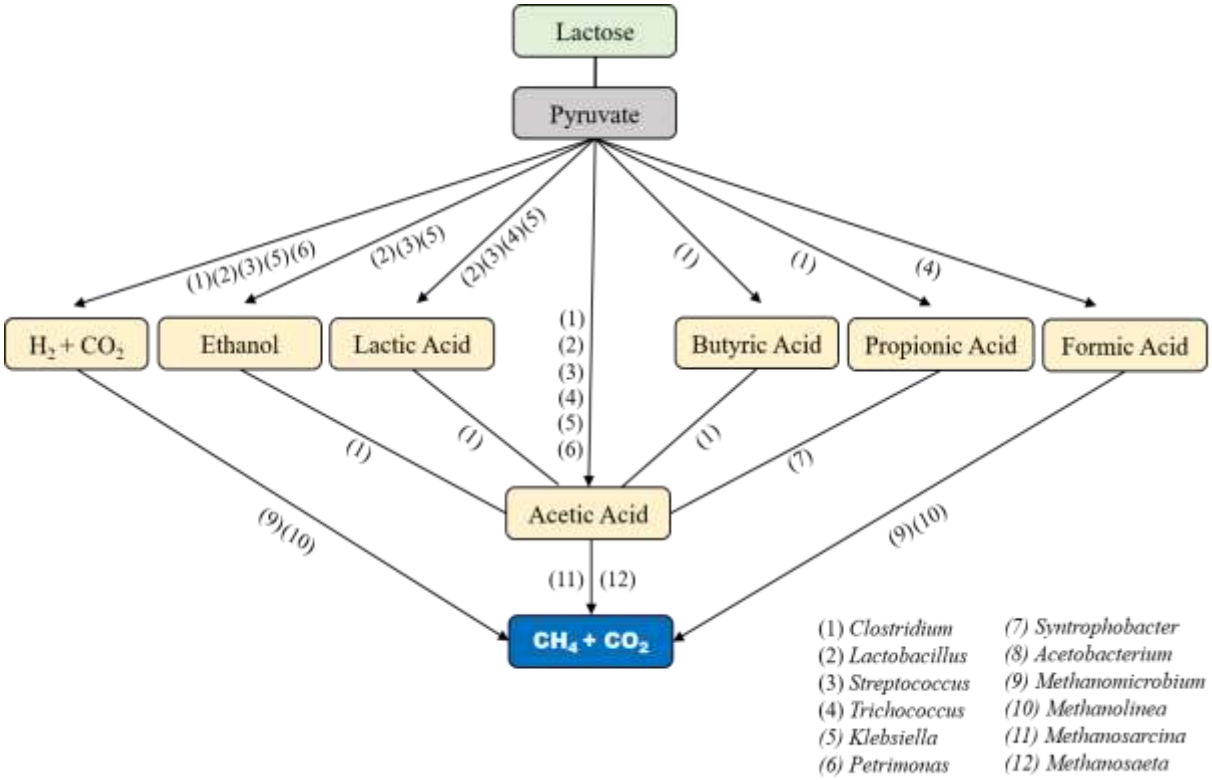
The major components of expired dairy products comprise carbohydrates (57.5%), lipids (28.4%), and protein (14.0%) (SLOPIECKA et al., 2022). The main carbohydrate in EDP is lactose, with an amount between 45-50 g/L (STAVROPOULOS et al., 2016). Casein is the most significant protein, accounting for approximately 70% of the total protein volume (BELLA; RAO, 2021). Lipids are basically composed of triglycerides with more than 97% (SAGE; DAUFIN; GESAN-GUIZIOU, 2008). The biodegradation pathways of these compounds are discussed below based on some literature studies.

1.4 Lactose degradation

Lactose, a disaccharide in milk, comprises glucose and galactose and can be broken down by lactase enzymes (BELLA; RAO, 2021). The conversion of lactose to methane involves multiple intermediate transformations between several microorganisms. A possible pathway for lactate degradation is presented in **Figure I.2**. Anaerobic bacteria mostly use the Embden Meyerhof-Parnas pathway (glycolysis) for lactose metabolism, which produces pyruvate and reduces NAD (NADH), transformed into lactate, acetate, ethanol, propionate, butyrate, H_2, and CO_2 (HASSAN; NELSON, 2012).

Clostridium genus ferments lactose to butyrate, acetate, propionic, ethanol, H_2, and CO_2. The metabolite yield produced generally depends on the metabolic end products. For example, when fermenting glucose, focusing on H_2 production, it is favored by the generation of acetic and butyric acid. The highest theoretical hydrogen yield is achieved when glucose is fermented to acetic acid with 4 mol of H_2/mol of glucose. However, if glucose is converted to butyric acid, the yield is reduced to 2 mol of H_2/mol of glucose. In contrast, propionic acid production consumes 1 mol of hydrogen per mol of propionic acid formed (STAVROPOULOS et al., 2016; WANG; YIN, 2021).

Figure I.2 - Possible pathway for lactate degradation to CH_4



Adapted from Hassan and Nelson (2012)

Lactic acid bacteria (LAB), such as the genera *Lactobacillus* and *Streptococcus*, can be classified into two groups based on the metabolites they produce during fermentation: homofermentative and heterofermentative. Homofermentative LAB mainly breaks down glucose into lactic acid, while heterofermentative LAB metabolizes glucose into lactic acid, acetic acid, ethanol, and CO_2 (KOMESU et al., 2017).

Lactic acid production in anaerobic reactors is often pointed out as responsible for decreased or inhibition of H_2 production in anaerobic systems. It can occur mainly due to substrate competition between LAB and hydrogen-producing bacteria (HPB) (CASTELLÓ et al., 2018). However, recent publications highlight hydrogen production from lactate, demonstrating the hydrogen-producing route with positive interactions between LAB and HPB (DETMAN et al., 2020; GARCÍA-DEPRAECT et al., 2021).

Hydrogen production from lactate occurs through two pathways: the acrylate pathway and the pyruvate-ferredoxin oxidoreductase pathway. In the acrylate pathway, the *Clostridium neopropionicum* specie can produce H_2 from lactate as a sole carbon source. Nevertheless, the pyruvate-ferredoxin oxidoreductase pathway is more widely used for H_2 production, where *Clostridium* species, including *C. tyrobutyricum*, *C.*

diolis, and *C. acetobutylicum*, use lactate and acetate as carbon and energy sources (GARCÍA-DEPRAECT et al., 2021; WANG; YIN, 2021).

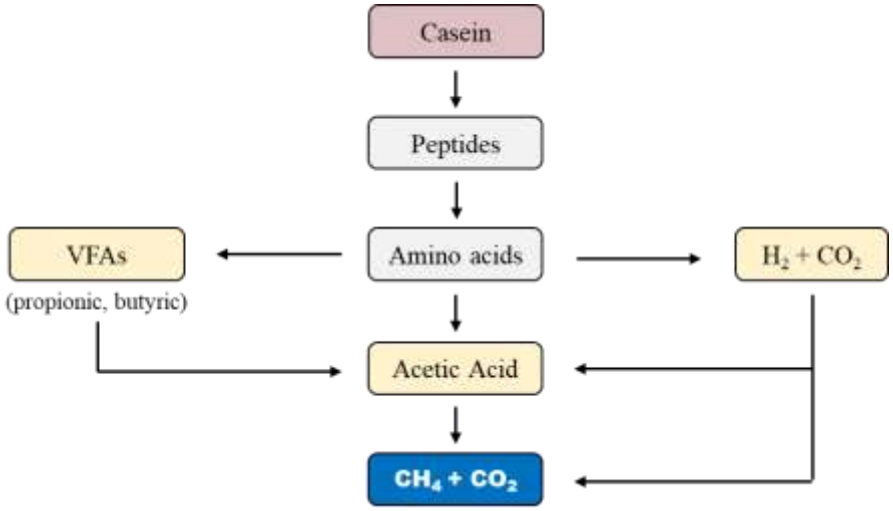
Trichococcus genus degraded glucose producing lactic acid, formic acid, and acetic acid (WANG et al., 2021). *Klebsiella* ferments lactose to acetate, ethanol, lactate and H_2 (HASSAN; NELSON, 2012). *Petrimonas* metabolize carbohydrates producing acetate and H_2 (GRABOWSKI et al., 2005).

Lactose is a readily degradable component fermenting at a fast rate, causing an imbalance between acidogenesis, acetogenesis, and methanogenesis. So, there is a chance of accumulation of VFAs which can lead to a drop in pH and an increase in the concentration of non-dissociated volatile fatty acids. This condition inhibits the methanogenesis process, resulting in the biological process failure (BELLA; RAO, 2021; KOPSAHELIS et al., 2018).

1.5 Casein Degradation

Extracellular proteases hydrolyze casein into peptides, which are subsequently degraded by peptidases to amino acids. They are further broken down into simpler compounds, such as organic acids (propionic and butyric), ammonia, CO_2, and a small amount of H_2 and H_2S (SIKORA et al., 2017). The conversion of casein into methane is presented in **Figure I.3**.

Figure I.3 - Anaerobic degradation of casein.



Adapted from Hassan and Nelson (2012)

Amino acid oxidation can occur via two routes: the Stickland reaction, where pairs of amino acids can be degraded, or through hydrogen-consuming bacteria for

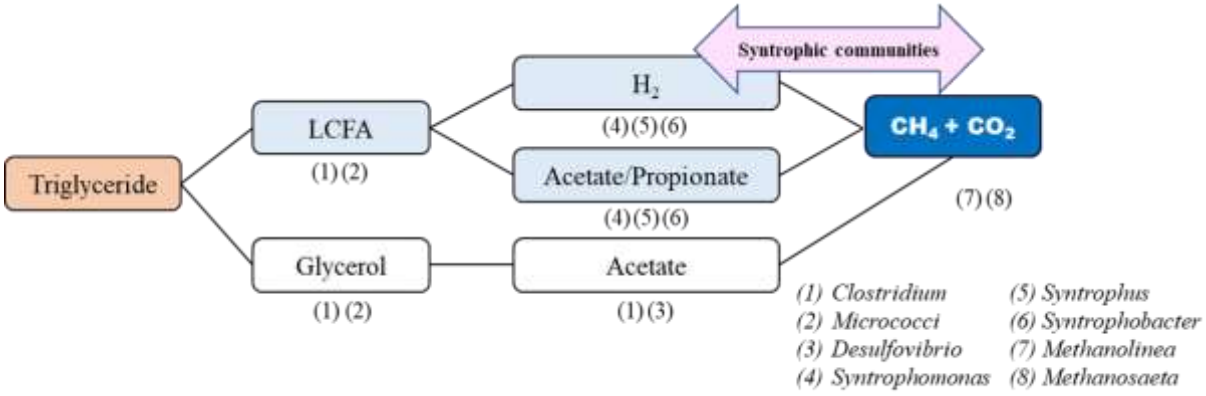
single amino acids (LING et al., 2022). The Stickland reaction is the most common amino acid oxidation reaction in anaerobic digestion, although 60% of an amino acid (from casein) degradation involves uncoupled amino acids (amino acids that do not serve as electron acceptors) (HASSAN; NELSON, 2012).

The main proteolytic bacteria include species in the genera *Clostridium, Bacillus, Micrococcus, Staphylococcus, Alcaligenes, Pseudomonas, Flavobacterium, Brevibacterium* (ERKMEN, 2022)*, Candidatus Cloacimonas, Aminobacterium colombiense* (SIKORA et al., 2017), *Acidaminococcus fermentans, Acidaminobacter hydrogenoformans, Megasphaera elsdenii,* and *Eubacterium acidaminophilum* (RAMSAY; PULLAMMANAPPALLIL, 2001).

1.6 Lipids Degradation

Biochemically, triglycerides are hydrolyzed to glycerol and long-chain fatty acids (LCFA) by lipases from acidogenic bacteria, such as *Clostridium* and *Micrococci* (HASSAN; NELSON, 2012). During acidogenesis, glycerol is converted to acetate inside the bacterial cell by microorganisms such as *Clostridium* and *Desulfovibrio* (NAKASAKI et al., 2020). LCFA are degraded via β-oxidation by *Syntrophomonas*, *Syntrophus*, and *Syntrophobacter*, producing acetate, H_2 and short-chain fatty acids (RASIT et al., 2015; SOUSA et al., 2007). Subsequently, these metabolites are converted to CH_4 by methanogenic Archaea (e.g., *Metanolinea* and *Metanosaeta)* (**Figure I.4**).

Figure I.4 - Metabolic steps pathway in the anaerobic digestion of lipids



Adapted from Marchetti et al. (2020)

According to Marchetti et al., 2020 the degradation of LCFA under methanogenic conditions demands the collaborative interaction of LCFA-degrading

anaerobic bacteria and hydrogenotrophic methanogens (e.g., *Metanolinea)*. The oxidation of LCFA is unfavorable thermodynamically unless the consumption of reducing equivalents (such as hydrogen and/or formate) is coupled with oxidation. Therefore, the process depends on the capacity of hydrogenotrophic methanogens to utilize the H_2 generated during fatty acid oxidation.

Lipid-rich wastes like expired dairy products are advantageous substrates for anaerobic digestion. Theoretically, lipids degradation can produce higher methane yield (1000 mL CH_4/g VS) when compared with proteins (480 mL CH_4/g VS) or carbohydrates (373 mL CH_4/g VS) (AWE et al., 2018). However, lipids can cause physical and chemical inhibition in anaerobic digestion systems (SAGE; DAUFIN; GESAN-GUIZIOU, 2008). β-oxidation is a limiting step due to its slow degradation, which may result in the accumulation of LCFA and consequent instability and failure in the anaerobic system due to the microorganism's inhibition (TIAN et al., 2018; WARE; POWER, 2017).

Additionally, physical inhibition may occur in the biological system due to fat adsorption to the microorganism's cellular membrane; this prevents access to substrates and nutrients, leading to the loss of adequate cellular functions. Furthermore, it causes inoculum flotation, which may lead to loss of active biomass, mainly in UASB-type reactors (*Upflow anaerobic sludge blanket*) and EGSB (*Expanded granular sludge bed*) (ELSAMADONY et al., 2021; HASSAN; NELSON, 2012).

Alternatives to overcome the instabilities and inhibitions caused by the high amounts of carbohydrates and lipids can use techniques such as co-digestion and anaerobic digestion in two stages, which will be further detailed.

1.7 Anaerobic co-digestion and two-stage system

Traditionally, anaerobic digestion corresponded to a single substrate with a single-purpose treatment. Recently studies demonstrated that anaerobic digestion becomes more stable when a diversity of substrates are applied simultaneously. (MARAGKAKI et al., 2017).

According to Kurahashi et al. (2017), anaerobic co-digestion (AcoD) can increase the activity of anaerobic microorganisms, considering that an appropriate mix of residues provides complementary effects. AcoD also compensates for the lack of carbon in some substrates, diluting harmful or excessive substances that may inhibit

microbial activity. In addition, it results in better biogas production, improving the production from 25% to 400% under the mono digestion of the same raw materials, with an improvement in the removal of organic matter.

EDP contains a high organic matter that is easily degradable, possibly leading to fast acidogenesis. An alternative would be to mix them with waste with opposite characteristics to obtain a synergistic and stable fermentation. In this scenario, Wu et al. (2011) evaluated the impact of incorporating milk waste into the anaerobic digestion process of cow manure. This investigation utilized lab-scale batch digesters (500 mL, active volume: 300mL) at 37 ºC during 28d. The findings revealed that co-digestion of milk waste and dairy manure increased biogas productivity compared with the control (without milk addition). The increases between 7 and 18% were observed when milk waste was added to the digested manure at 1 and 3% levels, respectively. However, it was noted that the inclusion of milk waste resulted in a decrease in the methane (CH_4) content of the produced biogas, dropping from 66.5% in the control group to 63.5% when milk waste was added at a level of 19%. This reduction suggested a potential rise in carbon dioxide (CO_2) production and highlights the need to limit milk waste addition to percentages of liquid volume (v/v) below 3% to avoid the decrease of CH_4 production.

Furthermore, Shofie et al. (2015), investigated the co-digestion of milk waste with coffee waste and municipal sludge under thermophilic conditions (55 ºC). The CSTR anaerobic reactor (15 L) with a working volume of 12 L was fed with a mixture substrate with TS 70 g/L. The system was operated for 225 days, with a steady state at HRT of 30 days and OLR 4.0 kg-COD/m^3/d. The average biogas production was 1.54 ± 0.14 L/L-digester/d with ~61% of CH_4 in biogas composition and yield of 432.9 ml CH_4/g VS_{added}. This study confirmed the effectiveness of anaerobic co-digestion of this waste mixture.

Several industrial organic residues can be used in anaerobic co-digestion; however, evaluating the availability and need for treatment of the residues that will be used is essential.

Domestic sewage is a global problem. According to Rajasulochana and Preethy (2016), approximately 1.1 billion people worldwide use water from contaminated sources. In Brazil, 9.1 thousand tons of sewage are generated daily, and only 42.6% are collected and properly treated. In addition, 39% of the organic matter is removed from the sewage before the effluent is released into receiving water bodies (ANA,

2017). This value corresponds to 21% less than the value required by the Brazilian National Environment Council (CONSELHO NACIONAL DO MEIO AMBIENTE - CONAMA, 2011). Improper disposal of effluents compromises the quality of water in the receiving body, which makes it impossible to use water resources, causing damage to the environment and the population (ANA, 2017).

Domestic sewage consists almost entirely of water (99.9%) and solids (0.1%). In addition to solubilized organic matter, solids present in sewage can be divided into organic (proteins, carbohydrates, lipids, and microorganisms) and inorganic (e.g., ammonia, nitrates, orthophosphates) (VON-SPERLING, 2014).

When compared to other industrial effluents or waste, such as EDP (185.3-297.2 g COD L^{-1}) (MARIN et al., 2022), sewage can be considered poor in organic matter, as it has COD around 0.35 g L^{-1} (ADAMES et al., 2021; RODRIGUES, 2021). That makes it advantageous for co-digestion, as it provides dilution in the residues with a high concentration of COD and other advantages, such as having nutrients (nitrogen and phosphorus), which are important for the anaerobic reactors' microbiota (HUSSAIN; KUMAR; MEHROTRA, 2015). In addition, co-digestion of the two residues simultaneously would bring multiple benefits, such as environmental, social, and economical, since they can be treated in the same facility, sharing the same digester and process costs.

Rodrigues et al. (2020) co-digested crude glycerol (CG) with sanitary sewage. The CG had a high organic matter (2 kg COD L^{-1}) and impurities like methanol, soaps, and free fatty acids. The authors concluded that the co-digestion with sewage facilitated the degradation of CG, mainly due to the dilution of the organic matter and potentially toxic compounds such as methanol, avoiding the microorganism’s inhibition.

Likewise, Adames et al. (2022) evaluated the co-digestion of CG with domestic sewage from horizontal anaerobic reactors with fixed bed (HARFB). The authors inferred that co-digestion combined with the series reactors (more than one stage system) were efficient strategies in removing CG (consumptions of 99.9%) and avoiding inhibition of the microbiota due to high organic matter content.

Allied with co-digestion, another strategy to improve the anaerobic digestion performance is using two-stage AD (TSAD). In this system, acidogenesis and methanogenesis occur in two separate reactors (AN; XU; DAI, 2023). In the first reactor, acidogenic microorganisms hydrolyze and ferment organic matter, producing hydrogen and volatile fatty acids. In the second reactor, methanogenic microorganisms

convert the volatile fatty acids produced in the acidogenic reactor into methane and carbon dioxide (CREMONEZ et al., 2021).

TSAD has been used as an alternative to optimizing the conditions for each group of microorganisms since they differ widely in terms of physiology, nutritional needs, growth kinetics, and sensitivity to environmental conditions (DEMIREL; YENIGÜN, 2002). Therefore, the separation of acidogenesis and methanogenesis in two-stage processes can offer many advantages, such as the stability and efficiency offered to the system, consequently leading to more outstanding biogas production, and resulting in high energy recovery (SRISOWMEYA; CHAKRAVARTHY; NANDHINI DEVI, 2020).

Sakarika et al. (2020) compared the single-stage (SAD) and two-stage co-digestion of end-of-life dairy products (milk, yogurt, and cheese) with a mixture of agro-industrial wastes (pig manure, liquid cow manure, cheese whey, poultry waste, and slaughterhouse waste) using a continuously stirred tank reactor (CSTR). Both systems were operated simultaneously, testing various operational parameters such as hydraulic retention time (HRT) and organic loading rate to evaluate their impact on the production of bio-hydrogen and bio-methane. The methane productivity of the single-stage system was 0.64 L_{CH4} L_r^{-1} d^{-1}, while the two-stage system yielded 0.42 LH_2 L_r^{-1} d^{-1} and 0.69 L_{CH4} L_r^{-1} d^{-1}. Consequently, the energy productivity of the two-stage system amounted to 32.9 kJ L_r^{-1} d^{-1} (5.39 kJ L_r^{-1} d^{-1} from H_2 and 27.5 kJ L_r^{-1} d^{-1} from CH_4), which is 30% higher than that of the single-stage system (25.4 kJ L_r^{-1} d^{-1} from CH_4).

Pham Van et al. (2020) also compared the performance between SAD and TSAD systems for vegetable waste treatment. In the SAD, only 17.8–22.3% of the initial carbon was converted into biogas (equivalent to 91–110 NmL gVS_{added}^{-1}) with a low methane content (44.1–48.7%). On the other hand, the TSAD achieved a conversion of 41.67% of the initial carbon into biogas (equivalent to 299.0–374.6 NmL gVS_{added}^{-1}) with a high methane content (71.68–81.0%). These results demonstrated that the TSAD system outperforms the SAD system in terms of stability and overall efficiency.

Although the amounts of expired dairy products are significant, their inappropriate management has serious environmental consequences. In this sense, until the present study was carried out, few works in the literature proposed a sustainable alternative for its treatment and enhancement. In addition, no previous

study has been done to investigate the co-digestion of the EDP with sewage and testing both applying it in a two-stage system. The data obtained in this study will contribute to fulfilling the lack of information regarding applying these wastes in anaerobic processes.

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CHAPTER II

AUTOCHTHONOUS MIXED CULTURE FROM EXPIRED DAIRY PRODUCTS APPLIED TO BIOPRODUCTS PRODUCTION

ABSTRACT

Expired dairy products (EDP) can be a source of fermentative microorganisms capable of generating bioproducts. Therefore, an autochthonous mixed culture was obtained from EDP using a triplicate of anaerobic batch reactors (100 mL) fulfilled with 50 mL of *All Purpose Tween* (APT) culture medium and 50 mL of headspace (N_2 99.99%), kept at 37 ºC for 48 hours. The autochthonous mixed culture removed 93% of the glucose with productions of 8.3 g L^{-1} of lactic acid (LA) (main soluble metabolite) and yields of 0.92 LA g g^{-1}. *Lactobacillus* was identified as the genus with higher relative abundance with 78.4%. The autochthonous mixed culture growth in different sugars (xylose, arabinose, fructose, maltose, glucose, rhamnose, lactose, and sucrose) and wastes (EDP and banana processing wastewater (BPW)), with the highest carbohydrate removal verified in rhamnose (97.0%). The main metabolic pathway was directed toward LA production, for all substrates tested. The highest LA concentration was obtained from glucose (7.85 g L^{-1}) and the highest yield of LA was from BPW (0.98 g g^{-1}). In addition, acetic acid, ethanol, isovaleric acid, isobutyric acid, H_2, and CO_2 were also detected as metabolites in all substrates tested. Thus, the autochthonous mixed culture demonstrated biotechnological potential to produce lactic acid as a major metabolite.

Keywords: Fermentation; lactic acid bacteria; 16S rRNA; anaerobic batch reactors.

CHAPTER II

1 INTRODUCTION

Climate change, environmental degradation, and resource depletion have been increasing the pursuit of sustainable bioprocesses to produce biochemicals and biofuels from renewable biomass sources. Anaerobic processes, such as dark fermentation (DF) and anaerobic digestion (AD), have been investigated and applied for their potential to produce hydrogen, methane, and valuable organic acids such as lactate, acetate, and butyrate (LUONGO et al., 2019).

The dairy industry is a major waste generator throughout its production chain, such as wastewater, cheese whey, and expired dairy products (EDP) (AHMAD et al., 2019; AWASTHI et al., 2022; HAGOS et al., 2017). EDP appears as a promising source of fermentative microorganisms, such as Lactic acid bacteria (LAB), since they already have indigenous microorganisms in their composition resulting from the fermentation of expired products (ESKIN; SHAHIDI, 2013; NOBLECOURT et al., 2018). These microorganisms are adapted to substrates with a high organic matter and acidic environments, making them advantageous for producing value-added bioproducts, mainly lactic acid (LA).

LA is particularly noteworthy due to its wide application range in different industry sectors, such as the chemical industry (green solvents, neutralizers, raw material in the production of biodegradable plastics), pharmaceuticals industry (drug delivered system, mineral formulation, chiral synthesis, as an ingredient in cosmetics preparation), and food industry (fermentation and flavoring agent, preservative, pH regulator) (ABEDI; HASHEMI, 2020; TANG et al., 2016).

The LA production by fermentation is characterized by the biological degradation of different carbohydrates sources, which can occur by both prokaryotic microorganisms such as bacteria and cyanobacteria, as well as eukaryotic fungi and microalgae (SONG et al., 2022). However, approximately 90% of the research on lactic acid production centers around bacterial fermentation, focusing on *Lactobacillus, Streptococcus, Leuconostoc, and Enterococcus* genus (JOHN; NAMPOOTHIRI; PANDEY, 2007). LAB can be divided into homofermentative and heterofermentative based on the metabolites produced during fermentation. Homofermentative LAB degrades glucose predominately to lactic acid, while heterofermentative LAB metabolizes glucose into lactic acid, acetic acid, ethanol, and CO_2 (KOMESU et al., 2017).

Lactic acid production from LAB fermentation offers several benefits compared to chemical syntheses, including a low-cost substrate, process-reduced energy consumption, and lower temperature requirements (JOHN; NAMPOOTHIRI; PANDEY, 2007). Furthermore, the economic and operational advantages can be potentialized from mixed culture utilization once waste can be used as a substrate with no sterilization needs (POLICASTRO et al., 2021). In addition, more than one LAB species often occurs in mixed culture, which helps balance individual strains' strengths and weaknesses, enhancing the lactic acid production yield (CHEN et al., 2020).

Some authors verified production of lactic acid from some dairy substrates in different configurations of reactors. Plessas et al. (2008) studied the LA production from the fermentation of Kluyveromyces marxianus (IFO 288), Lactobacillus delbrueckii ssp. bulgaricus (ATCC 11842), and Lactobacillus helveticus (ATCC 15009) using single and mixed culture with cheese whey (CW) as a substrate. The authors verified the highest production of LA (8.7 g/L and 0.34 g LA/g lactose added) with the mixed culture formed by all three microorganisms in an operation of anaerobic batch reactors (200 mL) with 36 g/L diluting CW. The authors attributed the increase in LA production to a possible synergy between these cultures.

Luongo et al. (2019) investigated CW fermentation using heat-pre-treated mixed culture obtained from the full-scale treatment plant used in a co-digestion of CW and buffalo manure. The experimental tests were conducted using a duplicate of semi-continuous batch reactors (2L), fed with the substrate-to-inoculum ratio (F/M) of 1.9 gVS/gVS, operated for 136 days, under constant stirring (250 rpm), at 35 ºC and uncontrolled pH. The authors verified that the higher values of LA production (20.1 g/L) occurred progressively due to the prevalence of autochthonous bacteria present in the CW and the consequent decrease of acetogenic microorganisms present in the external culture (mixed culture). They highlighted that the autochthonous bacteria were more adapted to the substrate and more acid-tolerant, which probably resulted in a better LA yield production (0.37g/g fed COD).

The presence of LAB in dairy products and dairy waste has been widely studied in the literature (ESKIN; SHAHIDI, 2013; GOMES et al., 2015; JIN et al., 2021; ROY; SARKAR, 2022). However, few studies use autochthonous cultures to valorize and treat dairy waste by lactic acid production. Therefore, due this still scarce information about autochthonous LAB, this study aimed to obtain, characterize, and identify a fermentative autochthonous microbial culture from EDP. The efficiency of organic

acids, alcohols, and biogas (H_2 and CH_4) production was verified using simple sugars and real wastes as substrates to evaluate the biotechnological potential of the autochthonous mixed culture obtained.

2 MATERIALS AND METHODS

2.1 Autochthonous culture source, culture medium, substrates

2.1.1 Autochthonous mixed culture source

The autochthonous mixed culture was obtained from an EDP mixture (milk, milk cream, and yogurt) collected from a dairy company in Descalvado – SP – Brazil. It contained (g L^{-1}); tC - total Carbohydrates (78.5); tCOD - total Chemistry Oxygen Demand (297.2); VS - Volatile Solids (144.5); TS - Total Solids (150.9); fats and oils (12.4) and pH 5.5. The mixture was stored in plastic flasks and kept at -20 °C until its use.

2.1.2 Cultivation conditions

It was used an adapted *All Purpose Tween* (APT) culture medium to enrich the autochthonous mixed culture used for the cultivation of heterofermentative lactic acid-producing bacteria containing (in g L^{-1}): Enzymatic Digest of Casein (10.0); Yeast extract (7.5); Sodium chloride (5.0); Potassium phosphate (5.0); Sodium citrate (5.0); Dextrose (10.0); “Tween 80” (0.2); Magnesium sulfate (0.8); Manganese chloride (0.14); Ferrous sulfate (0.04); Sodium carbonate (1.25); final pH 6.7 (EVANS; NIVEN, 1951).

2.1.3 Substrates

The adapted APT culture medium was used as a substrate to determine the cell growth of the autochthonous mixed culture, carbohydrate removal, pH change, and bioproduct production. Mono- and di-saccharides were used to evaluate the autochthonous mixed culture and evaluate the removal efficiency of several sugars (laboratory-grade): xylose, arabinose, fructose, maltose, glucose, rhamnose, lactose, and sucrose. Furthermore, two organic wastes were also used as a substrate for the evaluation of the autochthonous culture:

1) The same EDP mixture (milk, milk cream, and yogurt) and used to obtain the autochthonous mixed culture;

2) The banana processing wastewater (BPW) from a banana processing company in Tapiratiba – SP – Brazil. It comes from the washing of the cooking containers process, which produces banana candy bars. It contained (g L^{-1}); tC (9.6); tCOD (14.9); VS (10.1); (10.4); and pH 4.3. The BPW was stored at -20 °C until its utilization.

2.2 Autochthonous culture obtainment

Triplicate anaerobic batch reactors (100 mL) were assembled with 50 mL of APT (pH 6.7), and headspace (50 mL) was filled with N_2 (99.99 %). Subsequently, the reactors were closed with a rubber cap and aluminum seal, and then they were submitted to a sterilization step using an autoclave (20 min, 120 °C). After sterilization, 0.5 mL of EPD mixture was added to each reactor using a sterile syringe and needle under aseptic conditions. After that, the reactors were maintained at 37 ºC for 48 hours.

2.3 Cellular enrichment and culture augmentation

Cellular enrichment was performed using the serial dilution technique, where 1mL of the culture liquid fraction obtained was added to 9 mL of APT medium with successive dilutions from 10^{-1} to 10^{-5} (MAINTINGUER et al., 2017). The aseptic conditions and parameters such as pH, anaerobiosis, temperature, and incubation time were the same used for autochthonous culture obtainment.

The culture augmentation was carried out from the flask with the highest dilution (10^{-5}). The liquid fraction was centrifuged (8000 rpm for 4 min.), the supernatant was discarded, and the microbial culture was added to a 100 mL flask containing 50 mL APT medium. The parameters and aseptic conditions were the same as previously adopted. The culture augmentation step was repeated several times until the cellular amount obtained was sufficient to perform subsequent tests.

2.4 Evaluation of autochthonous mixed culture in APT medium

Duplicates of anaerobic batch reactors (1000 mL) were assembled with 750 mL of APT culture medium (pH 6.7) previously added to the reactors under N_2 flow (10 min) and autoclaved (20 min, 120°C). Subsequently, 0.75 gVS L^{-1} from the obtained culture was inoculated into each reactor separately. Inoculation was done under the same aseptic conditions described in previous procedures. After inoculation, the reactors were kept at 37°C, in static mode, for 33 hours.

The cell growth, carbohydrate removal, pH change, biogas, volatile fatty acids (VFA), alcohols, and lactic acid production were evaluated during the test. At the end of the test, an aliquot of the sample was used to characterize the autochthonous culture by Gram stain test, bacterial enumeration (*Pour plate* technique), and molecular biology analysis.

2.5 Autochthonous mixed culture assays using different substrates

Eight sugars and two organic wastes were evaluated separately as carbon sources. The assays were performed in duplicates of anaerobic batch reactors (50 mL) with a 30 mL reaction medium filled with yeast extract (5 g L^{-1}), potassium phosphate (5 g L^{-1}), sodium carbonate (1.25 g L^{-1}), and 10.0 g L^{-1} of the sugars mentioned above (xylose, arabinose, fructose, maltose, glucose, rhamnose, lactose, and sucrose) or waste (EDP and BPW), at initial pH 7. The reaction medium was previously added to the reactors under N_2 flow (10 min) and autoclaved (20 min, 120°C). After that, 0.75 gVS L^{-1} from the obtained culture was inoculated into each reactor under the same aseptic conditions described previously. The reactors were kept at 37°C in static mode for 6 days. A control reactor was assembled without substrate, in the same conditions described previously.

Analyzes were carried out until the stabilization of the reactors regarding the generation of biogas, being evaluated volatile fatty acids, lactic acid and alcohol productions. The final pH and carbohydrate removal were also evaluated.

2.6 Analytical methods

The cell growth was determined by measuring the optical density at 600 nm using a spectrophotometer (Bioespectro SP-22) and pH according to (APHA, 2012). The colorimetric phenol-sulfuric acid method was used to determine total carbohydrate concentration (DUBOIS et al., 1956). The biogas components (H_2, N_2, CH_4, and CO_2) were measured by a Shimadzu® gas chromatograph (GC-2014) equipped with a thermal conductivity detector (TCD) and Carboxen 1010 PLOT column. The column flow was 5.0 mL min^{-1} with ultra-pure argon as the carrier gas. The injector and detector temperatures were 220 °C and 230 °C, respectively. Column temperature ramp was 120 °C (1 min), 40 °C/min up to 200 °C (3 min), and 50 °C/min up to 230 °C (0.5 min), with a total run time of 7.1 minutes (MAINTINGUER et al., 2008). The soluble metabolites (volatile fatty acids (VFAs) and alcohols) were determined using a GC

(Shimadzu® GC-2030) equipped with a flame ionization detector (FID), HP-INNOWAX capillary column (30m x 0.25mm x 0.25 µm) and AOC 6000 Plus autosampler (ADORNO; HIRASAWA; VARESCHE, 2014). The lactic acid quantification was performed in the pre-filtered samples (0.22 µm pore size filter) using High-Performance Liquid Chromatography (HPLC). The refractive index detector (Waters 2014) was maintained at 40ºC. The analytical column Aminex® HPX-87H (300 × 7.8 mm) was held at 50°C with a flow rate of 0.6 mL/min, using sulfuric acid 0.005 M as a mobile phase (PERIMENIS et al., 2018).

2.7 Microbiological analysis

Morphological characteristics of autochthonous culture obtained from EDP were performed by light under an Olympus microscope with objective lens magnification (1000x), using the Gram staining technique to differentiate Gram+ and Gram- microorganisms (RODRIGUES et al., 2016).

Bacterial enumeration was performed in triplicate, where 100µL of the sample was used in serial dilutions from 10^{-1} to 10^{-5} in phosphate buffer saline solution (PBS). The dilutions were spread over Petri dishes containing specific APT solid culture media to quantify colonies of fermentative bacteria (SONG et al., 2012). The colonies' quantification was realized by the Pour plate technique. The Petri dishes were incubated for 6 days at 37°C under anaerobic conditions. After this period, the colonies were counted manually.

2.8 Microbial community analysis

The analysis was performed at the end of the evaluation of autochthonous culture in APT medium. DNA extraction was made using the modified phenol-chloroform method (GRIFFITHS et al., 2000). After the extraction procedure, tests were carried out to verify the integrity of the extracted DNA by the ratio of 260/280 nm > 1.8, measured by an ND-2000 spectrophotometer (Nanodrop Inc., Wilmington, DEA).

Microbial characterization was performed at GenOne Biotechnologies (Rio de Janeiro, Brazil) from an NGS Illumina MiSeq PE250 platform. The sequencing process involved 2 × 250 bp cycles, producing 100k reads per sample. The specific primers used for high throughput amplicon sequencing of the V3–V4 regions from the 16S rRNA gene were 515F (5'-barcode-GTGCCAGCMGCCGCGG-3′) and 806R (5'-

GGACTACHVGGGTWTCTAAT-3'). The initial bioinformatic processing was performed by GenOne Biotechnology Solutions, utilizing the QIIME (V1.7.0) framework (CAPORASO et al., 2010). The clustering of the data was accomplished at a 97% identity threshold, employing Uparse (v7.0.1001) (EDGAR, 2013). For the taxonomic assignment, the Mothur software was used, and it referenced the SSUrRNA database of SILVA (http://www.arb-silva.de/) (WANG et al., 2007) with an 80% threshold.

The sequences obtained were submitted to National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), in the project accession number PRJNA643935, under the sample accession number: SAMN 15438765 (SRR 14252959).

2.9 Experimental data fitting

The average of experimental data obtained during the evaluation of autochthonous mixed culture in APT medium was fitted by the modified Gompertz function using the software Statistic® (version 10.0) according to Equation (1) (LAY; LI; NOIKE, 1998).

$$LA = P * exp\left\{-exp\left[\left(\frac{(R_m * e)}{P}\right) * (\lambda - t) + 1\right]\right\} \quad (1)$$

LA - Cumulative lactic acid (g L^{-1}),

P - LA production potential (g L^{-1}),

Rm - Maximum rate of LA production (g L^{-1} h^{-1}),

λ - Start time of LA production (h),

e - 2,718281828,

t - Period of incubation (h).

3 RESULTS AND DISCUSSION

3.1 Autochthonous mixed culture evaluation in APT medium

3.1.1 Soluble metabolites generation

Figure II.1 illustrates microbial growth (optical density), glucose removal, and lactic acid generation during the operation of anaerobic batch reactors. Glucose was readily removed within the first hours of operation, concomitantly with culture growth and lactic acid generation (Appendix A).

After 33 hours of operation, 9.0 grams of glucose was removed, representing 93.0% of glucose added initially (**Table II.1**). The mainly soluble metabolite produced was LA with a final concentration of 8.3 g L^{-1} and yield of 0.92 g LA g removed glucose^{-1} (g g^{-1}). Data obtained by Gompertz adjustment demonstrated the production rate of 1.4 g LA L^{-1} h^{-1} and an adaptation phase of 3.8 hours. Beyond the LA production, acetic acid, propionic acid, and ethanol were produced with final concentrations of 1.32 g L^{-1}, 0.53 g L^{-1}, and 0.18 g L^{-1}, respectively. The acid production, mainly LA, justifies the initial pH reduction by 4.6. Only the presence of CO_2 from fermentation was detected.

Figure II.1 – Temporal variation of microbial growth, glucose removal, LA production of autochthonous mixed culture in APT culture medium

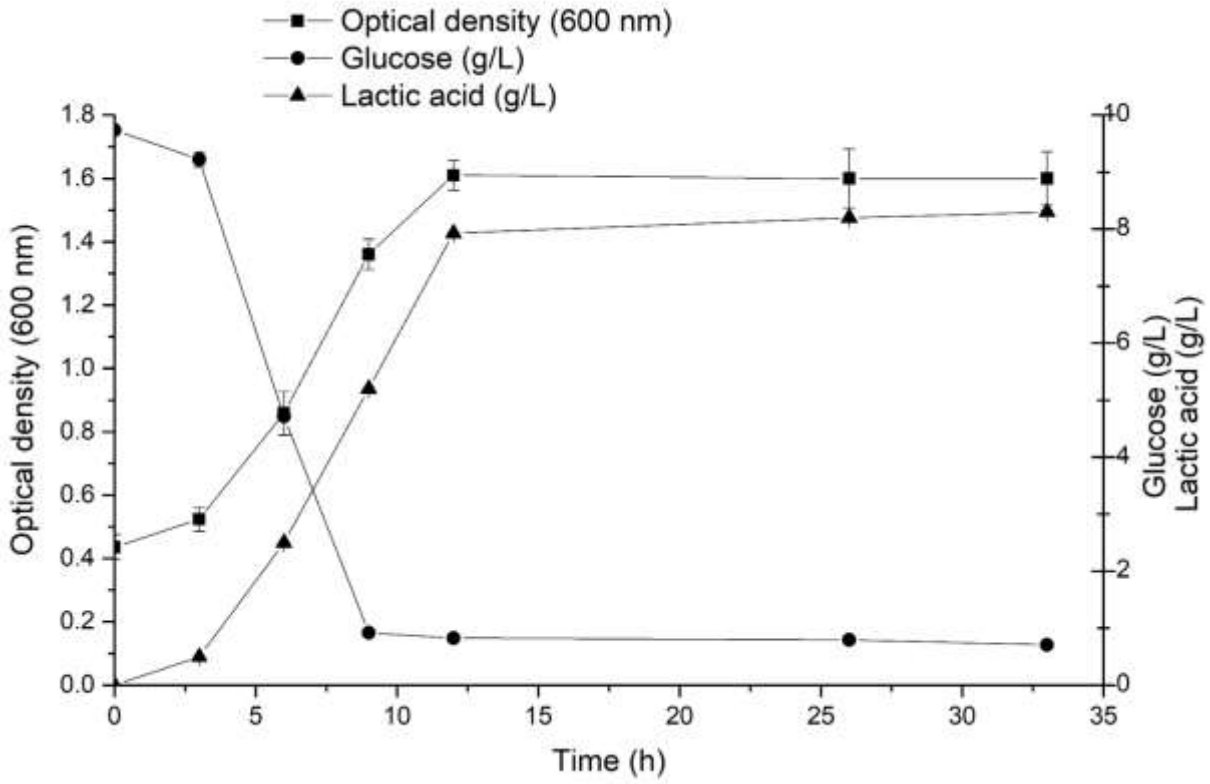


Table II.1 - Autochthonous culture performance using APT medium as substrate

Parameters	Autochthonous Culture Performance
Final pH	4.6
Initial glucose (g L^{-1})	9.7
Glucose removal (%)	93
LA yield (g g^{-1})	0.92
Gompertz function of LA production	
P (g LA L^{-1})	8.3
Rm (g LA L^{-1} h^{-1})	1.4
λ (h)	3.8
Other soluble metabolites (g L^{-1})	
Acetic acid	1.32
Propionic acid	0.53
Ethanol	0.18

P: production potential (g L^{-1}); Rm: maximum rate production (g L^{-1} h^{-1}); λ: the start time production (h).

3.1.2 Microbiological analysis of autochthonous mixed culture

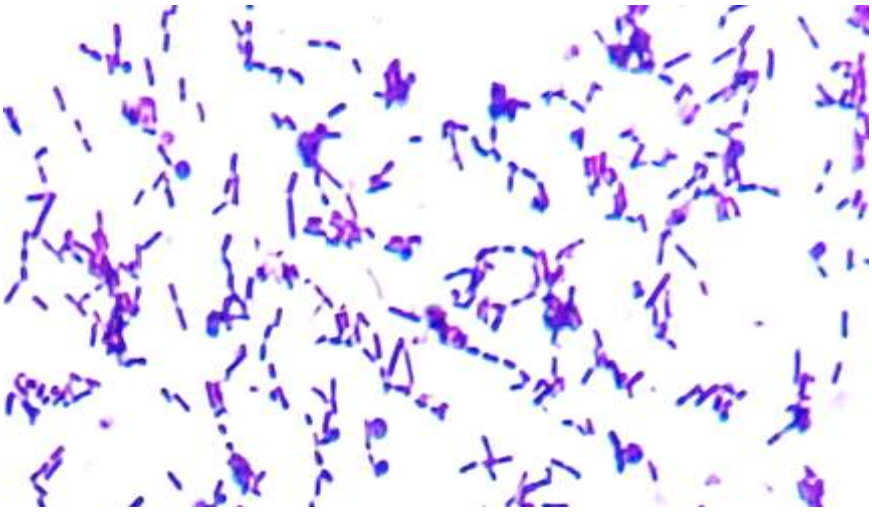
Colony growth was observed in the APT solid culture media with a counting of 1.4 10^8 CFU mL^{-1}, confirming that the autochthonous mixed culture obtained had fermentative bacteria involved in the LA production from glucose (**Figure II.2**).

Furthermore, the predominance of Gram-positive bacilli (blue staining) was verified in the autochthonous mixed culture (**Figure II.3**). The initial pH of the EDP (5.5) allied to a complex nutritional culture medium (APT), which is specific for the fermentation, favored the permanence of fermentative bacteria in Gram-positive bacilli morphologies (ROMBOUTS et al., 2020).

Additionally, Gram-positive bacteria are often found and isolated from fermented foods, such as dairy products, meats, and vegetables. Fevria and Hartanto (2019) isolated the bacteria responsible for fermenting sauerkraut using the De Men, Rogrosa and Sharpe medium (MRS) with streak plate methods. Gram-positive bacteria with bacilli cell form were verified, similar to those in the present work. Yi et al. (2019) isolated the bacteria from natural fermented yak yogurt with an MRS culture medium and detected a Gram-positive bacillus.

Figure II.2 – Colony counting (CFU mL^{-1}) with autochthonous mixed culture in specific APT culture media.

Figure II.3 - Morphology of Gram-positive bacilli from autochthonous mixed culture (x1000)



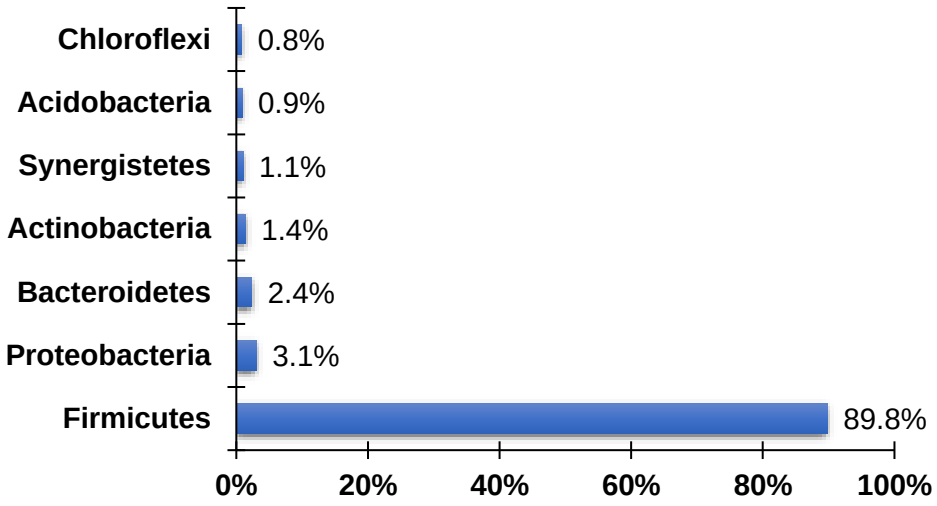
3.1.3 Molecular analysis of autochthonous mixed culture

The autochthonous mixed culture presented a predominant relative abundance for the Domain Bacteria at 99.88% and Domain Archaea at only 0.08%.

The phylum with the highest relative abundance verified was Firmicutes with 89.8%. The phyla Proteobacteria (3.1%), Bacteroidetes (2.4%), Actinobacteria (1.4%),

Synergistetes (1.1%), Acidobacteria (0.9%), and Chloroflexi (0.8%) were observed with lower relative abundance (**Figure II.4**).

Figure II.4 - Relative Abundance of Phyla.



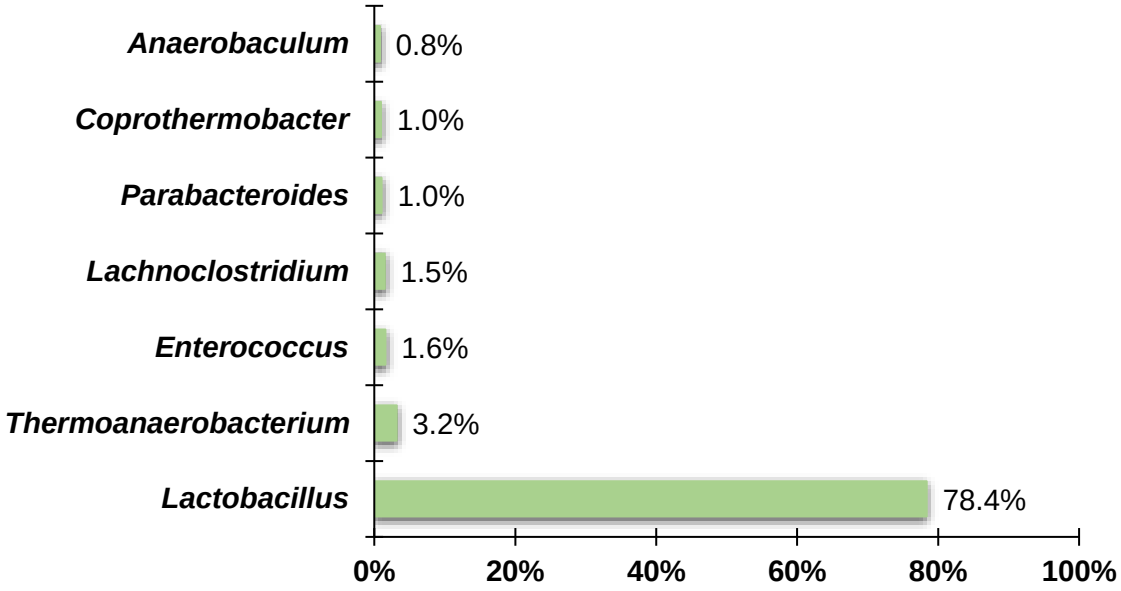
Lactic acid bacteria (LAB) can produce lactic acid from carbohydrates degradation. These bacteria are found in two distinct phyla, Firmicutes and Actinobacteria (HORVATH et al., 2009), and both were observed in the autochthonous mixed culture. The presence of these phyla was already expected since the autochthonous mixed culture was obtained from EDP using APT medium, which is a suitable medium for LAB cultivation.

Regarding genus, a higher relative abundance was observed for *Lactobacillus*, with 78.4%. The genera *Thermoanaerobacterium*, *Enterococcus*, *Lachnoclostridium*, *Parabacteroides*, *Coprothermobacter*, and *Anaerobaculum* were also observed. However, they were verified with lower relative abundances than *Lactobacillus*, corresponding to 3.2%, 1.6%, 1.5%, 1.0%, 1.0%, and 0.8%, respectively. These data complement the Gram stain and colony counting test results since the genera detected with the most relative abundance (*Lactobacillus*, *Thermoanaerobacterium*, *Enterococcus*, and *Lachnoclostridium*) belong to the Gram-positive fermentative microorganisms' group (DANDACHI et al., 2021; LEE et al., 1993; PANKRATOVA et al., 2018) (**Figure II.5**).

Moreover, the genera of *Lactobacillus* and *Enterococcus* belong to the LAB group, which is known to compete for substrate with hydrogen producers' bacteria (e.g., *Thermoanaerobacterium*) when the nutritive conditions are favorable

(CASTELLÓ et al., 2018). The APT culture medium is specific for LAB's growth; therefore, it is a rich source of peptides, amino acids, B vitamins, and carbohydrates, which makes it a favorable environment for the dominant growth of *Lactobacillus* (ALONSO et al., 2010). In addition, the rapid decrease in pH (to 4.6) also favors the growth of the LAB genus since they can grow at extremely low pH, being more acid-tolerant than other fermentative bacteria (LUONGO et al., 2019).

Figure II.5 - Relative Abundance of Genera.



The LAB are categorized as homofermentative and heterofermentative lactic acid bacteria species. The homofermentative species have a major interest in lactic acid production on a commercial scale, mainly because it can produce LA as the only product of glucose fermentation, with a theoretical yield of 2 mol of lactic acid per mol of removed glucose or 1 g g^{-1}. The heterofermentative LAB can metabolize glucose into LA, acetic acid, ethanol, and CO_2, with a LA theoretical yield of 0.5 g g^{-1} or 1.0 mol mol^{-1} (ABDEL-RAHMAN; TASHIRO; SONOMOTO, 2013; CASTILLO MARTINEZ et al., 2013).

The presence of LA, acetic acid, ethanol, and CO_2 was verified in the present study, which can be related with the heterofermentative pathway. However, a theoretical yield greater than 0.5 g g^{-1} was observed (0.92 g g^{-1}), proving that homofermentative bacteria, which can provide theoretical yields of 1 g g^{-1}, also participated in the fermentation process.

3.2 Autochthonous mixed culture essays using different substrates

The growth of autochthonous mixed culture was verified in all substrates, with carbohydrate removal above 85% for rhamnose (97.0%), fructose (96.0%), glucose (95.2%), arabinose (93.9%), EDP (90.1%), BPW (88.0%), and lactose (86.2%) (**Table II.2**). Lower removals were observed for maltose, sucrose, and xylose, with 57.6%, 67.3%, and 72.3%, respectively.

The pH decay was observed in all tests, which did not affect the autochthonous mixed culture growth. The microorganisms were already adapted to being tolerant to the acidic pH range with productions of VFA's, ethanol, and mainly LA. Such results follow the Pour plate quantification technique, applied for specific culture media of fermentative microorganisms, which corroborates with verified results (**Table II.2**).

The presence of soluble metabolites at the beginning of assays was observed only for the wastes, with the following concentrations (g L^{-1}): EDP: 0.6 LA, 0.1 acetic acid, and 0.1 ethanol; BPW: 0.11 LA, 0.03 acetic acid, and 0.01 ethanol. Such a presence was already expected due to the waste fermentation by their indigenous microorganisms.

Table II.2 - Final reactors operation results fed with different substrates.

Substrate	Carbohydrates removal (%)	Final pH	Ethanol (g/L)	Acetic acid (g/L)	Isobutyric acid (g/L)	Isovaleric acid (g/L)	LA (g/L)	LA yield (g/g)*
Control	-	7.0	n.d.	0.50	n.d.	n.d.	0.30	-
Glucose	95.2	4.0	1.13	0.48	0.03	0.03	7.85	0.84
Sucrose	67.3	4.1	0.50	0.40	0.08	0.09	2.90	0.49
Lactose	86.2	4.2	0.70	0.47	0.08	0.09	5.31	0.57
Maltose	57.6	4.3	0.31	0.36	0.03	0.05	2.40	0.40
Fructose	96.0	4.3	0.33	0.76	0.08	0.09	4.22	0.47
Xylose	72.3	4.4	0.21	1.12	0.06	0.09	2.51	0.28
Arabinose	93.9	3.9	0.12	1.17	0.07	0.01	3.23	0.54
Rhamnose	97.0	4.5	0.02	0.43	n.d.	n.d.	4.03	0.54
EDP	90.1	4.0	0.40	0.30	0.01	n.d.	6.43	0.82
BPW	88.0	3.8	0.51	0.28	n.d.	n.d.	7.72	0.98

n.d. – not determined; * g LA g^{-1} removed carbohydrate; EDP: expired dairy products; BPW: banana processing wastewater.

The metabolic pathway was directed towards the LA production for all substrates tested. However, higher results of LA production were achieved for glucose, BPW, EDP, lactose, fructose, and rhamnose with (g L^{-1}) 7.85, 7.72, 6.43, 5.31, 4.22, and 4.03, respectively. The high concentration of LA obtained from these substrates may be related to the adaptation of the microorganisms in the autochthonous mixed culture from the dairy waste, since sugars are present in the EDP composition. In the same way, the good performance regarding organic waste as a substrate is also justified since the microorganisms were already adapted to EDP. According to Chan-Blanco et al., (2003), BPW is reported as a waste rich in high amounts of sucrose, glucose, and fructose in its composition, which could have been used by microorganisms for the growth and generation of metabolites in particular LA.

For tests using sugars as a substrate, the second major soluble metabolite was acetic acid at concentrations of 1.17 g L^{-1} (arabinose), 1.12 g L^{-1} (xylose), 0.76 g L^{-1} (fructose), 0.47 g L^{-1} (lactose), 0.48 g L^{-1} (glucose), and ~ 0.40 g L^{-1} (sucrose, maltose, and rhamnose). Ethanol was also generated in high amounts in the degradation of glucose (1.13 g L^{-1}), lactose (0.70 g L^{-1}), and sucrose (0.50 g L^{-1}). Concentrations lower than 0.3 g L^{-1} of ethanol were verified for maltose, fructose, xylose, arabinose, and rhamnose. Isobutyric and isovaleric acids were also produced in concentrations below 0.10 g L^{-1} (except for rhamnose).

Regarding tests using organic wastes, besides lactic acid, ethanol was the second major soluble metabolite detected with concentrations of 0.51 and 0.40 g L^{-1} for BPW and EDP, respectively. Acetic acid was also detected at ~0.3 g L^{-1} for both residues. Biogas analyses were performed at the end of tests for all substrates. CO_2 was detected as the predominant gas and H_2 in no significant concentration (data not showed).

In terms of lactic acid yield, the tests using BPW, glucose, EDP, and lactose achieved higher results (0.98, 0.84, 0.82, and 0.57 g LA g^{-1}, respectively) than the other substrates tested, which also can be attributed to the culture adaptation. In addition, the higher LA yield obtained for organic residues may be related to the greater availability of nutrients on these substrates compared to tests involving only single sugars (ASTUDILLO et al., 2023; NZETEU et al., 2022), proving that for the concentration tested in this study, the use of organic waste was beneficial for microorganisms.

3.3 Comparative study in LA production from fermentation

Table II.3 describes an overview of some studies using different substrates to produce LA by fermentation compared with the present study. Kim et al. (2009) studied carbohydrate removal by a fermentation process using *L.brevis ATCC 14869 strain* as inoculum. The tests were conducted in 500mL flasks with MRS medium (100mL) containing a mixture of glucose (10.5 g L^{-1}) and xylose (11.1 g L^{-1}). The fermenters were kept under agitation mode (100 rpm), at 30 ºC, with initial pH of 6.0. LA was the main soluble metabolite (12.5 g L^{-1}), followed by acetate (4.5 g L^{-1}) and ethanol (0.7 g L^{-1}). The LA yield was 0.57 g g^{-1}, corresponding to 38.0% and 32.1% less than the yield found in the present study when the APT medium and glucose were used as a substrate, respectively.

Alonso et al. (2010) evaluated the LA production from expired yogurt whey as substrate using batch fermentation by *L. casei ATCC 393 strain*, which was precultured in two conditions (without and with 2.5 g L^{-1} of yeast extract supplementation). The anaerobic reactors operated without pH control were assembled with a final working volume of 1L and kept at 37 ºC in an orbital shaker (100 rpm). They achieved the LA yield (g g^{-1}) of 0.71 and 0.72 for the assays using preculture inoculum without and with yeast extract supplementation, respectively. These yields were lower than the present study with similar waste (EDP).

Table II.3 - Comparative results of lactic acid (LA) production

References	Microrganism	Substrate	LA (g L^{-1})	LA yield (g g^{-1})
Kim et al. (2009)	*L.brevis ATCC 14869*	MRS medium (glucose + xylose)	12.5	0.57
Alonso et al., (2010)	*L. casei ATCC 393*	Expired yogurt whey	12.25 - 14.5	0.71 -0.72
Tashiro et al., (2011)	*Lactobacillus delbrueckii subsp. lactis QU 41*	MRS medium	20.1	1.01
Tang et al., (2017)	Indigenous microbiota from fresh food waste	Food waste	28.4	0.46
The present study (1)	Mixed Culture	APT medium	8.30	0.92

The present study (2)	Mixed Culture	Several substrates	2.40 – 7.85	0.28 – 0.98

Tashiro et al. (2011) isolated and characterized a *Lactobacillus delbrueckii subsp. lactis QU 41* from grime in the sink. They investigated the effects of operation parameters on LA production using batch fermentation carried out with a working volume of 0.4 L that included 10% (v/v) inoculum, MRS medium (20 g L^{-1} or 55– 100 g L^{-1} glucose) under agitation mode (200 rpm). The pH levels were controlled between 5.0 and 6.5, in temperatures between 37 - 55 °C. The optimal LA production (20.1 g L^{-1}) was achieved with 20.0 g L^{-1} of glucose, pH controlled at 6.0, at 43°C. The yield was 1.01 g g^{-1}, near the present work (0.92 g g^{-1}) with the culture medium (APT) as a substrate. However, it is important to mention that the present study used a mixed culture, which can be advantageous in terms of costs and overcoming contamination problems in the fermentation process.

Tang et al. (2017) evaluated the effect of pH (uncontrolled 4, 5, and 6) on LA production from food waste fermentation. An indigenous microbiota from fresh food waste was used as inoculum. The batch reactors (250 mL) were assembled with 85% sterilized food waste slurry and 15% inoculum (dry weight), maintained at 37 ºC, under agitation (120 rpm) for 7 days. The optimal LA concentration (28.4 g L^{-1}) and yield (0.46 g g^{-1}) were achieved at pH 5. Microbial community analysis demonstrated that *Lactobacillus* was the predominant genus (98.5%) when the reactors achieved their highest LA yield (120h). Despite a high LA concentration, their LA yield was lower than the present study, mainly compared with EDP (0.82 g g^{-1}) and BPW (0.98 g g^{-1}) as a substrate.

4 CONCLUSIONS

A new autochthonous mixed culture was obtained from the expired dairy products (EDP) using anaerobic batch reactors filled with *All Purpose Tween* (APT) culture medium as a substrate. The autochthonous mixed culture removed 93.0% of glucose in the APT culture medium, producing 8.3 g L^{-1} lactic acid, corresponding to a yield of 0.92 g g^{-1}. Furthermore, acetic acid (1.32 g L^{-1}), propionic acid (0.53 g L^{-1}), and ethanol (0.18 g L^{-1}) were also produced as soluble metabolites. CO_2 was the only biogas generated. The *Lactobacillus* genus was identified with a higher relative abundance (78.4%). The autochthonous mixed culture could grow by consuming

different substrates, such as xylose, arabinose, fructose, maltose, glucose, rhamnose, lactose, sucrose, EDP, and banana processing wastewater (BPW) (57.6-97.0%). LA was the major soluble metabolite from all substrates tested (2.40-7.85g L^{-1}). The highest yield of LA was obtained from BPW with 0.98 g g^{-1}. In addition, acetic acid (1.17-0.28 g L^{-1}), ethanol (1.13-0.02 g L^{-1}), isovaleric acid (0.09-0.01 g L^{-1}), isobutyric acid (0.08-0.01 g L^{-1}), H_2, and CO_2 were also detected as metabolites. These results indicate that the autochthonous mixed culture is a promising alternative for bioproduct production, mainly lactic acid from different substrates.

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CHAPTER III

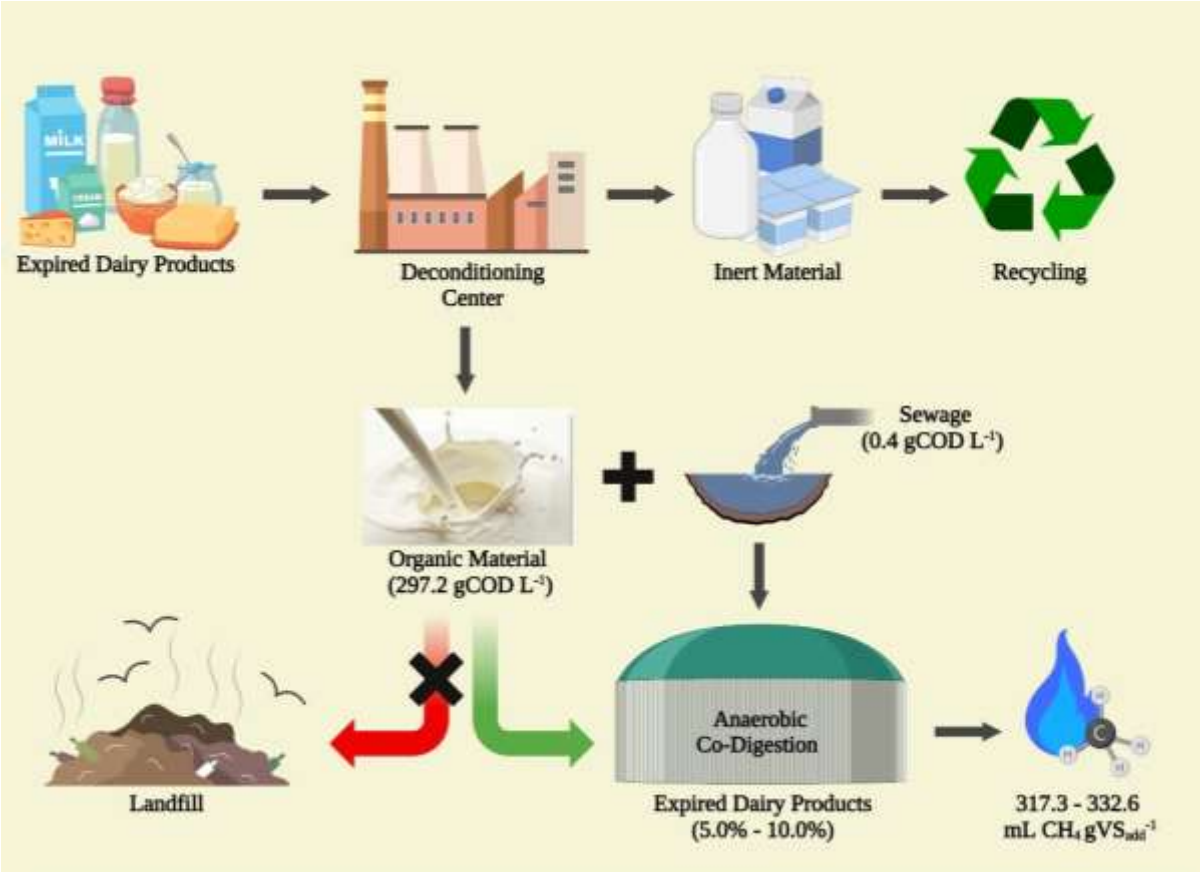
EVALUATION OF BIOMETHANE PRODUCTION FROM ANAEROBIC CO-DIGESTION OF EXPIRED DAIRY PRODUCTS WITH SYNTHETIC SEWAGE

ABSTRACT

Substantial amounts of expired dairy products (EDP) are generated due to their high perishability. They are rich in organic matter, and their landfill disposal negatively impacts the environment. An alternative to reduce and recover this waste would be its utilization as substrate in anaerobic digestion, aiming at biogas production. This study investigated the co-digestion of EDP with synthetic sewage (SS) in assays composed of EDP percentage in substrates mixture of (1) 0%, (2) 5.0%, (3) 7.5%, (4) 10.0%, and (5) 15.0% (v/v) to evaluate the effect of dairy waste increasing in the digester performance. Duplicates of anaerobic batch reactors (1000 mL) were assembled with a working volume of 500 mL filled with 19 g VS L^{-1} of granular sludge from a UASB reactor, 5 g L^{-1} $NaHCO_3$, initial pH of 8.2, performed at 37 °C, in a static mode. COD removals were 81.4%, 83.6%, 80.0%, 89.2%, and 14.3% for the assays from 1 to 5, respectively. The cumulative CH_4 productions were (mL L^{-1}) 716.8, 3353.8, 5326.6, 6583.6, and 1156.4 for the assays from 1 to 5, respectively. Assay 5 (15% EDP) was inhibited by VFA accumulation. However, assays 2, 3, and 4 (from 5.0% to 10.0% EDP) showed high methane yields (mLCH_4 gVS_{add}^{-1}) 318.5, 332.6, and 317.3, respectively, proving the feasibility of the co-digestion of EDP with SS. Acetoclastic (*Methanosaeta*) and hydrogenotrophic (*Methanolinea, Methanoregula,* and *Methanobacterium*) archaea were identified in assays 2, 3, and 4 (from 5.0% to 10.0% EDP), which probably contributed to the high organic matter removal efficiency and high methane production.

Keywords: Expired dairy products; Methane; Anaerobic Digestion; Anaerobic Microbial Community.

GRAPHICAL ABSTRACT



1 INTRODUCTION

The population and economic growth, combined with the improvement in the quality of life, have led to an increase in global demand for food. Dairy is one of the most relevant parts of the food industry, representing an essential role in the global economy, producing 906 million tons of milk in 2020 (FAO, 2020).

High milk production contributes to supplying the nutritional needs of the population. However, the dairy industry is known for being a massive waste generator from the entire supply chain (TOSTIVINT et al., 2017). It has been reported that around 18% of the production is wasted for diverse reasons, resulting in significant waste stream generation (SAR et al., 2022).

Expired dairy products (EDP) are considered unfit for consumption after expiration and are characterized as a residue (SAKARIKA et al., 2020). Part of them is generated due to the high perishability of dairy products, which have a consequently short shelf life. EDP management currently uses a small portion to feed animals, and the most significant amount is disposed in landfills (ADGHIM et al., 2020).

The disposal of EDP can be harmful to the environment since they are rich in organic components, such as carbohydrates (57.51%), lipids (28.43%), and proteins (14.05%) (SLOPIECKA et al., 2022), readily biodegradable by microorganisms responsible for greenhouse gases release (AHMAD et al., 2019; USMANI et al., 2022). Several studies demonstrate the adverse effects caused by food waste disposal and highlight the necessity to convert waste into resources using sustainable methods (BRUNKLAUS; REX; CARLSSON, 2018; USMANI et al., 2022).

Anaerobic digestion (AD) has been considered an alternative and suitable process for waste treatment. This biological process has a vast potential in waste recovery, transforming it into feedstock for biogas generation, which can be employed as a renewable energy resource. Furthermore, AD reduces waste organic matter, avoiding environmental degradation (HUNTER; BLANCO; BORRION, 2021). However, only a few studies report EDP applied in AD for biogas production.

Slopiecka et al. (2022) characterized and evaluated the biochemical potential of methane (BPM) of various expired food waste to improve this waste utilization in anaerobic digestion plants. These residues were divided into fifteen main categories containing common food products worldwide. The dairy products category (DP) evaluated cheese, milk, baby milk, yogurt, cottage cheese, and butter. The BPM yield of dairy products ranged from 231 to 660 mL CH_4/g VS, with the lowest result for milk and the highest for butter, respectively. The authors attributed the high BPM values to the high lipid content of the dairy substrate.

Although the anaerobic digestion process has several advantages, its application to waste containing large amounts of easily biodegradable compounds, such as EDP, can result in the rapid formation and accumulation of volatile fatty acids (VFAs) and long-chain fatty acids (LCFAs), leading to biological process failure (AHMAD et al., 2019; XU et al., 2018).

Anaerobic co-digestion (AcoD) has been reported as a simple alternative to overcome instability and failures caused by mono-digestion (single substrate) once it treats, simultaneously, two or more substrates with complementary characteristics (MATA-ALVAREZ et al., 2014). AcoD can be advantageous since it dilutes inhibitory compounds while enhancing the balance of nutrients in a biological system. It results in a synergistic effect between substrates providing a stable environment for anaerobic microorganisms, which improves biogas yield (VOLSCHAN JUNIOR; DE ALMEIDA; CAMMAROTA, 2020; XU et al., 2018).

In Brazil, 9.1 k tons of sewage are generated daily, and only 42.6% is appropriately collected and treated. In addition, 39% of the organic matter is removed from sewage before the effluent is released into receptor water bodies (ANA, 2017), corresponding to 21% less than the value required by Brazilian National Environment Council (CONSELHO NACIONAL DO MEIO AMBIENTE - CONAMA, 2011). The inadequate effluent discharge affects the water quality in the receiving body, which makes the water resources infeasible, damaging the environment and population (ANA, 2017).

In this perspective, sewage can be a promising co-substrate to solve problems caused by a large amount of organic matter present in dairy waste because due to its low Chemical Oxygen Demand (COD) and high content of water (99.9%) (VON-SPERLING, 2014), it can be applied as an efficient diluent. Additionally, sewage has nutrients such as phosphorus and nitrogen, essential during biological processes (HUSSAIN; KUMAR; MEHROTRA, 2015).

Therefore, co-digestion of both residues simultaneously would bring environmental, social, and economic benefits since they can be treated at the same facility, sharing the same digester and process costs. In addition, no previous study has been done to investigate the co-digestion of these residues, which can significantly contribute to the lack of information regarding the application of expired dairy products as a substrate in anaerobic processes.

In this sense, this study evaluated the co-digestion of expired dairy products (EDP) with synthetic sewage (SS) from different EDP percentages addition, using anaerobic batch reactors to determine the effect of dairy waste increasing in biological process performance.

2 MATERIALS AND METHODS

2.1 Inoculum source and substrates

The methanogenic inoculum was obtained from a mesophilic granular sludge from a UASB (Upflow Anaerobic Sludge Blanket) reactor used to treat poultry slaughterhouse wastewater (Tietê – SP – Brazil). It was maintained at 4 °C until its utilization.

The expired dairy products were collected from a dairy company (Descalvado – SP – Brazil), and they comprised a mixture composed of milk, milk cream, and yogurt

containing (g L^{-1}); TS - Total Solids (150.9); VS - Volatile Solids (144.5); tCOD - total Chemistry Oxygen Demand (297.2); tC - total Carbohydrates (78.5), fats, and oils (12.4), and pH 5.5. It was stored in plastic flasks to preserve the mixture properties and kept at -20 °C until its use.

The synthetic sewage was prepared according to Martín-González et al. (2010) with the same macro and micronutrient composition as real domestic sewage. It contained (g L^{-1}); TS (0.3); VS (0.2); tCOD (0.4); tC (<0.01), and pH 6.8.

The same substrates were used for all assays to minimize errors associated with variations from different collection or preparation times.

2.2 Experimental setup

The anaerobic batch reactors were assembled in duplicate using 1000 mL flasks with a working volume of 500 mL. The EDP percentage in the substrate mixture (EDP + SS) was 0.0% (control), 5.0%, 7.5%, 10.0%, and 15.0% (v/v), corresponding to a total COD concentration of 0.4, 9, 15, 21, and 30 g COD L^{-1}, in the assays 1, 2, 3, 4, and 5, respectively. Assays 2, 3, 4, and 5 were consecutively set up (sequential batches) to adapt the inoculum to the increasing addition of dairy waste. All reactors were set with 19 g VS L^{-1} of inoculum (160 mL) and 5 g L^{-1} buffer ($NaHCO_3$). The initial pH was adjusted to 8.0 with 1M NaOH or 1M HCl. After assembly, each reactor was sealed with rubber stoppers and purged with N_2 (99.9%) to create an anaerobic environment, and they were subsequently maintained in mesophilic conditions (37°C) at static mode. The methane production from the inoculum was monitored in blank reactors (inoculum and water), and its results were used to subtract from the production of the batch anaerobic reactors. All anaerobic reactors were operated until the stabilization of the biogas production or until the coefficient of variation between the last three records of cumulative biogas was lower than 5% (ANGELIDAKI et al., 2009).

2.3 Analytical methods

The TS, VS, tCOD and soluble chemical oxygen demand (sCOD), pH, fats, and oils were measured as described in "*Standard Methods for the Examination of Water and Wastewater*" (APHA, 2012).

Total carbohydrate concentration was determined by colorimetric method using phenol-sulfuric acid (DUBOIS et al., 1956).

The biogas composition analysis was performed using a Shimadzu® gas chromatograph (GC-2014) equipped with a thermal conductivity detector (TCD) and Carboxen 1010 PLOT column. The column flow was 5.0 mL min^{-1} with ultra-pure argon as the carrier gas. The injector and detector temperatures were 220 °C and 230 °C, respectively. The column temperature was: 120 ºC (1 min), 200 ºC (3 min), 40 ºC $min^{-1,}$ and 230 ºC (0.5 min) 50 ºC min^{-1}, with a total run time of 7.1 minutes (MAINTINGUER et al., 2008). The biogas volume was recorded daily through water displacement (ZUO et al., 2020), and the values were normalized to 273 K and 1013 hPa (VDI 4630, 2006).

The soluble metabolites (volatile fatty acids (VFAs), acetone, and alcohols) were determined using a GC (Shimadzu® GC-2030) equipped with a flame ionization detector (FID), HP-INNOWAX capillary column (30m x 0.25mm x 0.25 µm) and AOC 6000 Plus autosampler (ADORNO; HIRASAWA; VARESCHE, 2014).

2.4 Experimental data fitting

The experimental data obtained during the biogas production were adjusted using the software Statistica® (version 8.0) according to a modified Gompertz [Equation (1)] proposed by Kim et al. (KIM; HAN; SHIN, 2003). This model uses two terms and is recommended for curves with diauxic behavior.

$$BMP = P_1 \times exp\left(-exp\left(\frac{R_1 \times e}{P_1}(\lambda_1 - t) + 1\right)\right) + P_2 \times exp\left(-exp\left(\frac{R_2 \times e}{P_2}(\lambda_2 - t) + 1\right)\right) \quad [1]$$

Where BPM is cumulative CH_4 production (mL CH_4 L_r^{-1}); P1 and P_2 are the maximum CH_4 production (mL CH_4 L_r^{-1}) for the first and exponential phases s, respectively; R_1 and R_2 maximum CH_4 production rate mL CH_4 L_r^{-1} d^{-1}), for the first and second exponential phases, respectively; λ_1 and λ_2 time to start the CH_4 production (days), for the first and second exponential phases, respectively; t is the operation time (days), and e is the Euler's number.

2.5 Statistical analysis

The averages referring to the removal efficiency values of carbohydrates, tCOD, sCOD, TS, and VS in the assays were evaluated statistically through analysis of variance (ANOVA) applying the Tukey test at the 5% significance level.

2.6 Microbial diversity analysis

Molecular biology analyses were performed on inoculum *in natura* and for assays 2, 3, and 4, with 5.0%, 7.5%, and 10.0% EDP, respectively, which obtained the highest methane production. DNA extraction was made using the modified phenol-chloroform method (GRIFFITHS et al., 2000). After the extraction procedure, tests were carried out to verify the integrity of the extracted DNA by the ratio of 260/280 nm > 1.8, measured by an ND-2000 spectrophotometer (Nanodrop Inc., Wilmington, DEA).

Microbial characterization was performed at GenOne Biotechnologies (Rio de Janeiro, Brazil) from an NGS Illumina MiSeq PE250 platform. The sequencing process involved 2 × 250 bp cycles, producing 100k reads per sample. The specific primers used for high throughput amplicon sequencing of the V3–V4 regions from the 16S rRNA gene were 515F (5'-barcode-GTGCCAGCMGCCGCGG-3′) and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The initial bioinformatic processing was performed by GenOne Biotechnology Solutions, utilizing the QIIME (V1.7.0) framework (CAPORASO et al., 2010). The clustering of the data was accomplished at a 97% identity threshold, employing Uparse (v7.0.1001) (EDGAR, 2013). For the taxonomic assignment, the Mothur software was used, and it referenced the SSUrRNA database of SILVA (http://www.arb-silva.de/) (WANG et al., 2007) with an 80% threshold.

The sequences obtained were submitted to National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), in the project accession number PRJNA643935, under the sample accession number: SRX10614891 (inoculum *in natura*); and SRX10809359, SRX10809556; SRX10809585 for the inoculum at the end of the tests with EDP percentages of 5.0%, 7.5% and 10.0% (v/v), respectively.

3 RESULTS AND DISCUSSION

3.1 Operation and removal efficiency

The operation time increased proportionally with increasing organic matter in assays from 1 to 5 (from 0.0% to 15.0% EDP) with 14.0, 20.7, 23.8, 24.0, and 24.0 days, respectively (**Table III.1**). The increased metabolite concentrations produced during hydrolysis and acidogenesis lead to prolonged consumption in the following steps (acetogenesis and methanogenesis) in assays with high quantities of EDP.

The carbohydrates and TS removals were most efficient for assay 4 (10.0% EDP), with 98.0% and 59.7%, respectively. Regarding sCOD, the best removal

efficiency (88.9%) occurred for assay 2, with 5.0% of EDP, while the VS removal was higher for assay 3, with 7.5% EDP. Although the sCOD and SV values were not higher for assay 4, the difference between the results was not statistically significant.

The removal of tCOD was also most efficient (89.2%) for assay 4, which corresponds to 7.8%, 5.6%, 9.2%, and 74.9% more than for assays 1, 2, 3, and 5, respectively. It was observed that the increase in the amount of EDP up to 10.0% (assay 4) provided more efficient removal results than the lower concentrations from 0% to 7.5% (assays 1, 2, and 3). However, despite assay 5 (15.0% EDP) achieving high carbohydrate degradation (95.7%), it showed low COD, TS, and VS removal (14.3%, 14.6%, and 37.1%, respectively), demonstrating that the soluble metabolites forming during acidogenesis step were not consumed by microorganisms, which will be discussed further.

Table III.1 - Anaerobic reactors performance

Parameters	Assays (% EDP)				
	1 (0.0)	**2 (5.0)**	**3 (7.5)**	**4 (10.0)**	**5 (15.0)**
Operation time (d)	14.0	20.7	23.8	24.0	24.0
Initial concentration (g L^{-1})					
Carbohydrates	<0.1	2.4	3.6	4.8	6.0
tCOD	0.4	9.1	15.0	21.3	30.2
sCOD	-	4.5	7.0	9.1	15.5
TS	0.3	8.9	11.0	14.8	21.3
VS	0.2	5.2	7.7	10.0	15.8
Removal (%)					
Carbohydrates	−	96.5^{bc}	97.2^{ab}	98.0^{a}	95.7^{c}
tCOD	81.4^{bc}	83.6^{b}	80.0^{c}	89.2^{a}	14.3^{d}
sCOD	−	88.9^{a}	87.2^{a}	87.5^{a}	5.3^{b}
TS	42.5^{c}	43.1^{c}	56.8^{b}	59.7^{a}	14.6^{d}
VS	79.1^{b}	79.4^{b}	87.7^{a}	86.7^{a}	37.1^{c}

Means followed by the same letter within the same line do not differ according to Tukey's Test at 5% of significance.

3.2 Methane and soluble metabolites production

The increase in EDP concentrations improved the methane production compared to assay 1 (0.0% EDP) with total volume (P1 + P2) of 716.8, 3353.8, 5326.6, 6583.6, and 1156.4 mL L_r^{-1} from assay 1 to 5, respectively (**Table III.2)**

Table III.2 - Methane production determined by fitting the modified Gompertz equation.

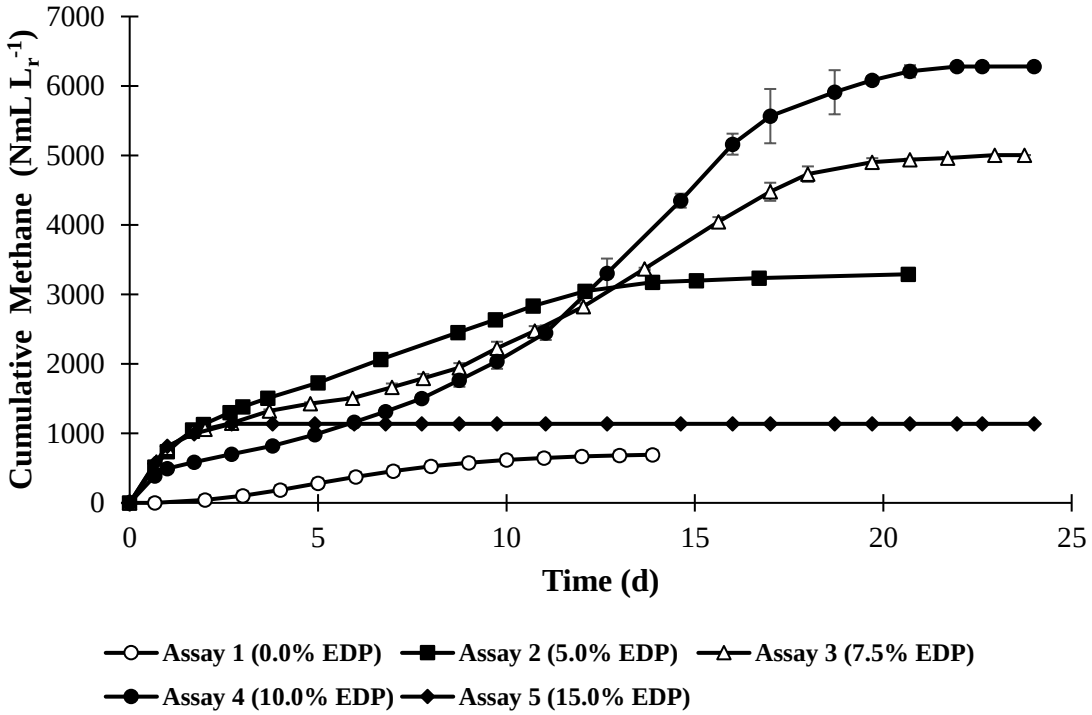
	Assays (% EDP)				
Parameters	**1 (0.0)**	**2 (5.0)**	**3 (7.5)**	**4 (10.0)**	**5 (15.0)**
P_1 (mL L_r^{-1})	716.8	3353.8	1435.2	2933.3	1156.4
P_2 (mL L_r^{-1})	−	−	3891.4	3650.3	−
R_1 (mL L_r^{-1} d^{-1})	97.2	286.1	350	185.9	427.1
R_2 (mL L_r^{-1} d^{-1})	−	−	569.9	513.9	−
λ_1 (days)	2.1	0	0	0	0
λ_2 (days)	−	−	7.7	10.5	−

P_1, P_2: the production potential of CH_4 for the 1st and 2nd exponential phases, respectively; R_{M1}, R_{M2}: the maximum production rate of CH_4 for the 1st and 2nd exponential phases, respectively; λ_1, λ_2: the lag phase time for the 1st and 2nd exponential phases, respectively.

The methane production started immediately in all EDP loading reactors (5.0% - 15.0%), indicating prompt substrate utilization. However, it was observed that methane was produced at a single rate for assay with 5.0% EDP. In contrast, the assays with 7.5% and 10.0% EDP presented curves with diauxic behavior (two exponential phases) (**Figure III.1**), with the second exponential phase occurring within 7.7 and 10.5 days, respectively. This happened because the increase in dairy waste concentration consequently increased the amount of the metabolites, which required more time to be consumed, probably due to the need for microorganisms' adaptation (Appendix B).

The high organic matter clearly inhibited methane production for assay 5 (15.0% EDP), which ceased on the third day of operation. Despite this, the reactors were maintained at a controlled temperature of 37°C for up to 24 days to verify a viable microorganism's adaptation and production resumption, which did not occur. Furthermore, it was identified that the average methane content in biogas sharply decreased in assay 5 (18.5% of CH_4) when compared with assays 1, 2, 3, and 4, with average values of 70.2%, 64.7%, 61.5%, and 59,8% of CH_4, respectively.

Figure III.1 - Cumulative methane production.



Low concentrations of VFAs and ethanol were verified only at the operation start of assays with the EDP addition (from assay 2 to 5, respectively) (**Table III.3**). These metabolites were already present in the dairy waste, probably produced due to its fermentation by endogenous microorganisms during its transportation.

Analyzes carried out at the end of the assays demonstrated that from 0.0% to 10.0% EDP concentrations (from assays 1 to 4), the acids produced from organic matter degradation were consumed during the anaerobic process. These data justified no variation in the pH of the assays with the high COD removal verified.

However, an accumulation of VFAs was detected at the end of Assay 5 (15.0% EDP), mainly from acetic (3712.8 mg L^{-1}), butyric (3548.5 mg L^{-1}), and propionic acid (751.1 mg L^{-1}). The accumulation of VFAs could explain the low COD removal efficiency and consequent failure in methane production.

To the anaerobic digestion work without limitations, the four stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) must occur in equilibrium with an equalization between the production and consumption of metabolites. The excess organic matter, such as verified in assay 5, can lead to process destabilization due to acidogenic activity occurring faster than consumption causing saturation of acetogenic

and methanogenic microorganisms (ANGELIDAKI et al., 2009; SIKORA et al., 2017; VÁZQUEZ-FERNÁNDEZ; SUÁREZ-OJEDA; CARRERA, 2022).

Table III.3 - *Soluble metabolites (initial and final).*

		Soluble Metabolites (mg L^{-1})								
Assays (% EDP)		**pH**	**Ethanol**	**Acetic Acid**	**Propionic Acid**	**Isobutyric Acid**	**Butyric Acid**	**Isovaleric Acid**	**Valeric Acid**	**Caproic Acid**
1 (0.0)	Initial	8.0 ±0.00	<10.0	<10.0	<10.0	<10.0	<10.0	N.D	N.D	N.D
	Final	8.0 ±0.01	<10.0	<10.0	<10.0	<10.0	<10.0	N.D	N.D	N.D
2 (5.0)	Initial	8.0 ±0.00	N.D	124.9 ±2.3	27.7 ±0.5	13.1 ±0.5	83.7 ±0.5	N.D	N.D	33.8 ±0.8
	Final	7.3 ±0.03	<10.0	160.4 ±3.3	30.9 ±0.5	11.0 ±0.7	68.5 ±3.8	<10.0	<10.0	33.4 ±1.4
3 (7.5)	Initial	8.0 ±0.00	31.0 ±0.5	75.9 ±2.2	22.5 ±5.5	<10	34.2 ±2.4	<10.0	N.D	15.3 ±0.8
	Final	7.9 ±0.04	<10.0	116.2 ±29.4	29.8 ±0.5	11.3 ±0.3	33.3 ±0.1	<10.0	<10.0	14.8 ±1.1
4 (10.0)	Initial	8.0 ±0.00	25.2 ±0.3	118.5 ±0.1	37.9 ±0.4	13.8 ±0.5	113.7 ±0.9	<10.0	N.D	47.0 ±0.76
	Final	7.9 ±0.01	<10.0	216.4 ±1.1	90.0 ±0.5	13.4 ±0.3	12.0 ±0.4	<10.0	35.1 ±0.6	<10.0
5 (15.0)	Initial	8.0 ±0.00	86.8 ±3.3	91.0 ±2.2	22.1 ±0.5	<10.0	148.5 ±6.9	N.D	N.D	56.7 ±1.1
	Final	6.9 ±0.00	<10.0	3712.8 ±559.2	751.1 ±0.5	291.4 ±94.6	3548.5 ±477.4	374.2 ±7.1	16.3 ±0.6	87.1 ±9.1

N.D – not detected.

Angelidaki et al. (1993) suggested that each AD process has its own “suitable” levels of VFAs, determined by the composition of substrate input into anaerobic reactors. Therefore, specifying VFAs limits responsible for causing reactors failure is unreasonable.

Thus, based on the data obtained from this study, the concentration of 15.0% of EDP (assay 5) led to the complete inhibition of the methanogenic process and the inefficient use of organic matter. However, co-digestion of EDP with SS at concentrations from 5.0% to 10.0% EDP (assays 2, 3, and 4) was effective and presented a high methane production yield. The highest CH_4 yield of 332.6 mL gVS_{add}^{-}

^{1} was obtained for assay 3, with 7.5% EDP. Assays with 5.0% and 10.0% EDP had yields of 318.5 and 317.3 mL gVS_{add}^{-1}, respectively.

Table III.4 describes an overview of recent studies with dairy waste as a substrate in anaerobic digestion processes compared with the present study. Wu et al. (2011) investigated the co-digestion of waste milk with manure using batch reactors (500 mL, active volume: 300mL) at 37 ºC during 28d. They tested different milk waste contents in feedstock 1-19% (w/w) and obtained lower methane yield (224.2 – 324.1 mL CH_4 gVS_{add}^{-1}) and COD removal (49.7-77.8%) values than the present study (317.3-332.6 mL CH_4 gVS_{add}^{-1} and 80.0-89.2%, respectively).

Sivakumar et al. (2012) treated 100% w/w of spoiled milk (200 gCOD L^{-1}) using Anaerobic Sequential Batch Reactor (ASBR) with 100 L of operational volume. The reactor was operated in mesophilic conditions (32 ºC) for 30 days. The authors reported a COD removal efficiency of 92.8% and a biogas yield of 396.7 mL CH_4 gVS_{add}^{-1}. Their results were slightly higher than those achieved in this study, probably due to the authors adding only spoiled milk to the reactors, which contain fewer carbohydrates and fats than the mixture of expired dairy products tested in the present study.

Kopsahelis et al. (2018) studied the co-digestion of End-of-Life Dairy Products (milk, yogurt, and white cheese) with agro-industrial wastes (pig manure, liquid cow manure, cheese whey, poultry wastes, and slaughterhouse wastes) using a pilot-scale CSTR reactor with an active volume of 1.8 m^3 at HTR of 37 days. For a methanogenic reactor (single-stage system), they achieved removal efficiency of 71.9% COD and 83.5% for carbohydrates, which correspond to 17.3% and 14.5% less than the values obtained in this present study, respectively. In addition, the maximum methane yield obtained was lower (236.2 mL CH_4 gVS_{add}^{-1}) than the one obtained in the present study. The authors inferred a possible inhibition of methanogenic microorganisms caused by unsaturated free fatty acids produced via hydrolysis of lipids present in the EoL-DPs mixture. The high- fat content contained in the EDP mixture combined with the high percentage of the EoL-DPs (19.5%) added to the CSTR reactor may have contributed to such inhibition.

Similarly, Sakarika et al. (2020) studied the co-digestion of EoL-DPs with agro-industrial waste. They evaluated the gradual increase of EoL-DPs from 4.5% to 19.5% using a CSTR reactor (working volume: 2.8L), operated with uncontrolled pH (range

between 7.5 to 8.3), with HTR of 37 days at mesophilic conditions (37ºC). The yields for the methanogenic reactor (single-stage system) were 205, 241, and 282 mL CH_4 gVS_{add}^{-1} for tests from the lowest to the highest EoL-DPs percentages, respectively. Regarding removal efficiency, the percentages were 33.0, 44.8, and 30.5%. Both yield and removal values reported by the authors were lower than the ones found in the present study.

Table III.4 - Performance of anaerobic systems using dairy waste as substrate.

Authors	Reactor	Substrate	CH_4 yield (mL/gVS_{add})	tCOD rem. (%)
Wu et al. (2011)	Batch	Waste milk (1 -19% v/v) + dairy manure	224.2-324.1	49.7-77.8
Sivakumar et al. (2012)	Batch (ASBR)	Spoiled milk (100% w/w)	396.7	92.8
Kopsahelis et al. (2018)	Continuous (CSTR)	End-of-Life dairy products (19.5% w/w) + agro-industrial wastes	236.2	71.9
Sakarika et al. (2020)	Continuous (CSTR)	End-of-Life dairy products (4.5-19.5% w/w) + agro-industrial wastes	205.0-282.0	30.5-44.8
Present study	Batch	Expired dairy products (5 -10% v/v) + synthetic sewage	317.3-332.6	80.0-89.2

3.3 Microbial diversity of assays with high CH_4 yields

The OTU (operational taxonomic unit) and Chao-1 index represent microbial community richness (**Table III.5**). The highest number of OTUs (1636) was observed in the *in natura* inoculum and the lowest (687) in assay 4 with the highest percentage of the substrate (10.0% EDP). The increase in substrate concentration inhibited some populations by the reduction in Shannon index with decreases of 8.058 for *in natura* inoculum to 6.141, 5.557, 5.698 for the assays with 2, 3, and 4, respectively. The reduction in microbial and diversity populations suggested that the characteristics and concentration of substrate could affect the diversity of microbial community bringing on the selection and adaptation of some populations able to resist the inhibitory conditions of dairy waste.

Table III.5 - Quantitative and Statistical values of microbial biodiversity.

Samples	Richness index		Diversity index
	OTUs	Chao I	Shannon
In natura inoculum	1636	1.763.898	8.058
Assay 2 (5.0% EDP)	771	988.882	6.141
Assay 3 (7.5% EDP)	791	1.020.174	5.557
Assay 4 (10.0% EDP)	687	876.181	5.698

The richness of the microbial community given by the Chao-1 index was also reduced in assay 4, with the highest substrate amount (876.181) compared to the *in natura* inoculum (1.763.898).

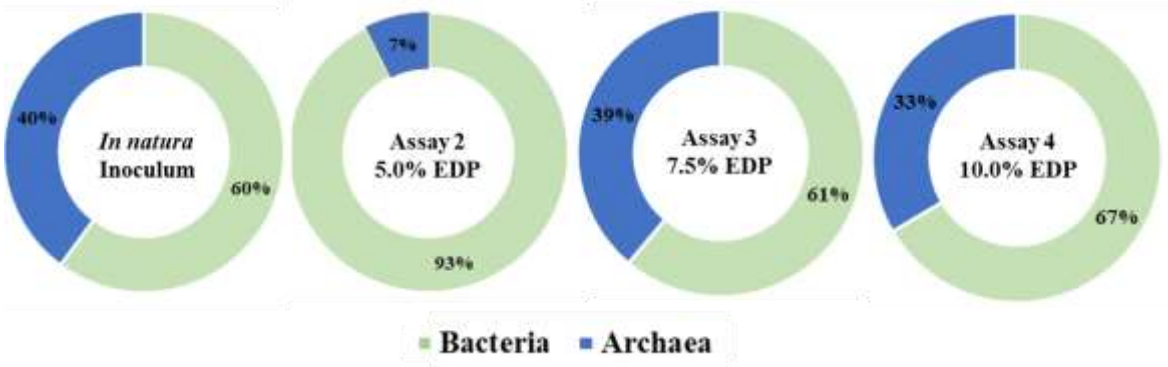
Patil et al. (2021) studied the anaerobic co-digestion of food waste (FW) with sewage sludge using batch reactors with a volume of 1L, kept in a shaking incubator at 37 °C and 150 rpm. They tested seven ratios prepared by FW (0%, 2%, 4%, 6%, 8%, and 10% v/v) and sewage sludge in the substrate mixture. They observed similar tendencies in the OTU and Shannon index of the microbiome from anaerobic co-digestion of food waste (FW) with sewage sludge. The authors reported that the increase in FW ratio (0% to 10%) led to a decrease in OTU and Shannon indexes, with the highest numbers found in reactors with 0% FW (18909 and 6.58) and the lowest (8161 and 4.86) in reactors 10% FW, respectively. They attribute this behavior to overload in anaerobic digestion caused by the exhaustion of substrates required for microbial growth, as observed in the present study.

Figure III.2 illustrates the result of taxonomic analysis for the Bacteria and Archaea domains in terms of relative abundance. *In natura* inoculum presented a relative abundance percentage of 60% and 40% for the Bacteria and Archaea domains, respectively. For assay 2 (5.0% EDP), a reduced abundance percentage was observed for the Archaea Domain (7%) and an increase for the Bacteria Domain (93%). These differences were probably due to the *in natura* inoculum being adapted to the poultry slaughterhouse wastewater treatment, which is rich in protein and lipids (DELFORNO et al., 2017).

Although these components are present in the composition of EDP, more than half of its composition are carbohydrates (SLOPIECKA et al., 2022), which are more easily hydrolyzed, leading to a rapid pH drop in the medium. The pH change favors

bacteria growth during the acidogenic process of AD once they are more tolerant and adaptable to an acid environment. However, the inocula from assays 3 and 4 (7.5% and 10.0% EDP) showed relative abundances close to the *in natura* inoculum, with values of 61% and 67% for the Bacteria Domain and 39% and 33% for the Archaea Domain, respectively, demonstrating that a gradual increase of the EDP (from 5% to 10%) was efficient for the inoculum adaptation to the substrate.

Figure III.2 - Relative abundance of Domains.

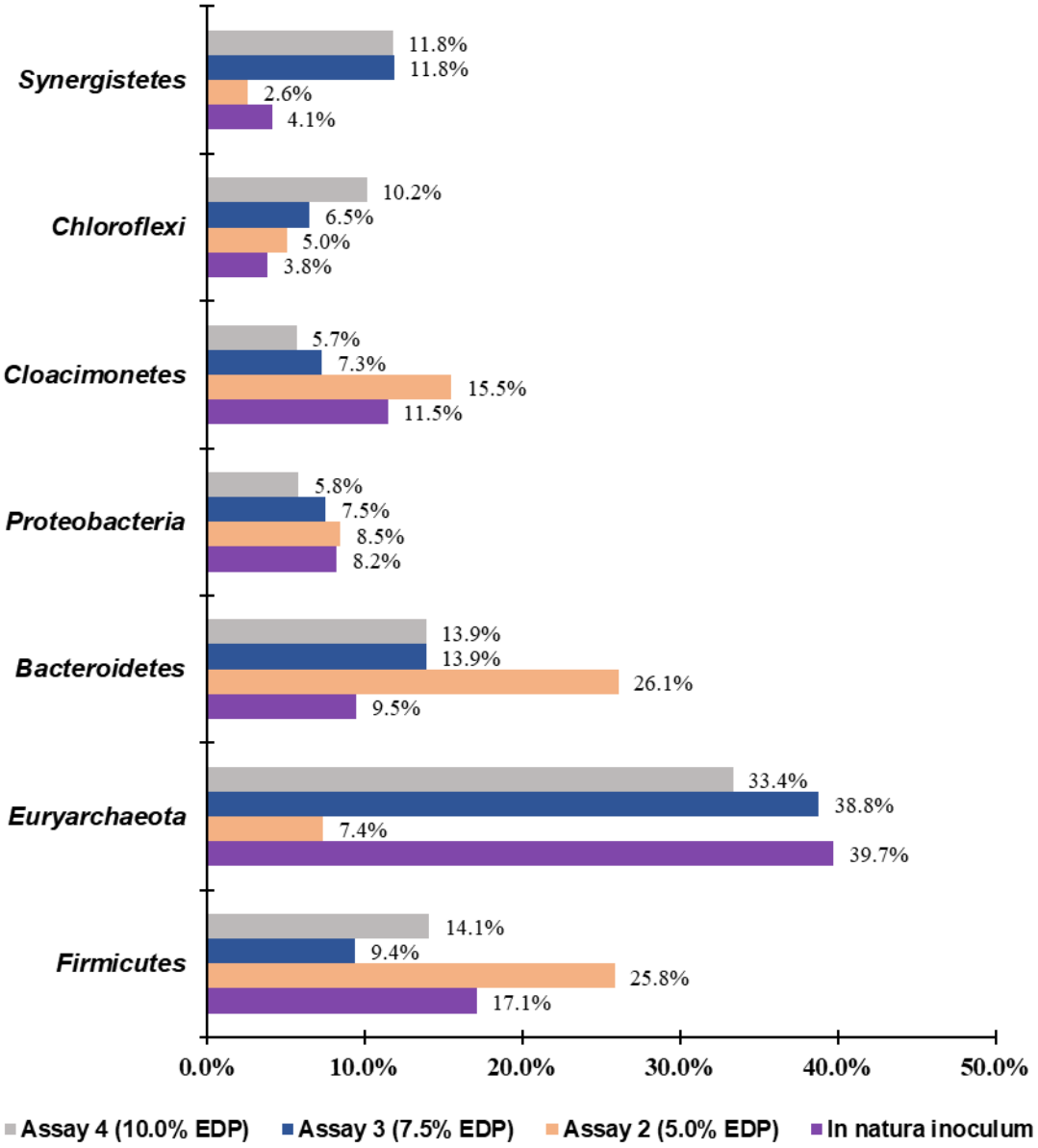


In terms of the relative abundance of phyla, similar results were found for the *in natura* inoculum and the assays 3 and 4 (7.5 and 10.0% EDP), which showed a predominance of the Euryarchaeota phylum with 39.7%, 38.8%, and 33.4%, respectively. This phylum was already expected since the *in natura* inoculum comes from an anaerobic reactor (UASB) and is associated with abundant methanogenic microorganisms in the sample (DELFORNO et al., 2017; LIU et al., 2020). However, in assay 2 (5.0% EDP), it was detected with an abundance of only 7.4%. Euryarchaeota is an archaea domain phylum, and its decrease was also related to the inoculum's adaptation to the dairy substrate (**Figure III.3**).

The other phyla with the highest relative abundance observed in all inocula were: Firmicutes (17.1%; 25.8%; 9.4%, and 14.1%), Bacteroidetes (9.5%; 26.1%; 13.8%, and 13.9%), Synergistetes (4.1%; 2.6%; 11.8% and 11.8%), Proteobacteria (8.2%; 8.5%; 7.5% and 5.8%) and Chloroflexi (3.8%, 5.0%, 6.5%, and 10.2%) considering the percentages from the *in natura* inoculum to assays 2, 3, and 4 (5.0%, 7.5%, and 10.0% EDP). Berninghaus and Radniecki (BERNINGHAUS; RADNIECKI, 2022) studied microbiome dynamics in an anaerobic digester fed with recycled activated sludge (RAS) and fats, oils, and greases (FOG). They also observed the predominance of Firmicutes, Bacteroidetes, Synergistetes, Proteobacteria, and

Chloroflexi phyla. The authors associated it with the addition of lipid-rich co-substrate. Furthermore, Delforno et al. (2017) studied the microbial diversity of the UASB reactor applied to the treatment of poultry slaughterhouse wastewater. They reported the predominance of these phyla, such as Bacteroidetes (26%), Proteobacteria (20%), Firmicutes (18%), and Euryarchaeota (8.7%), for the dataset obtained from the Amplicon_PS_M5RNA technique.

Figure III.3 - Relative Abundance of Phyla.



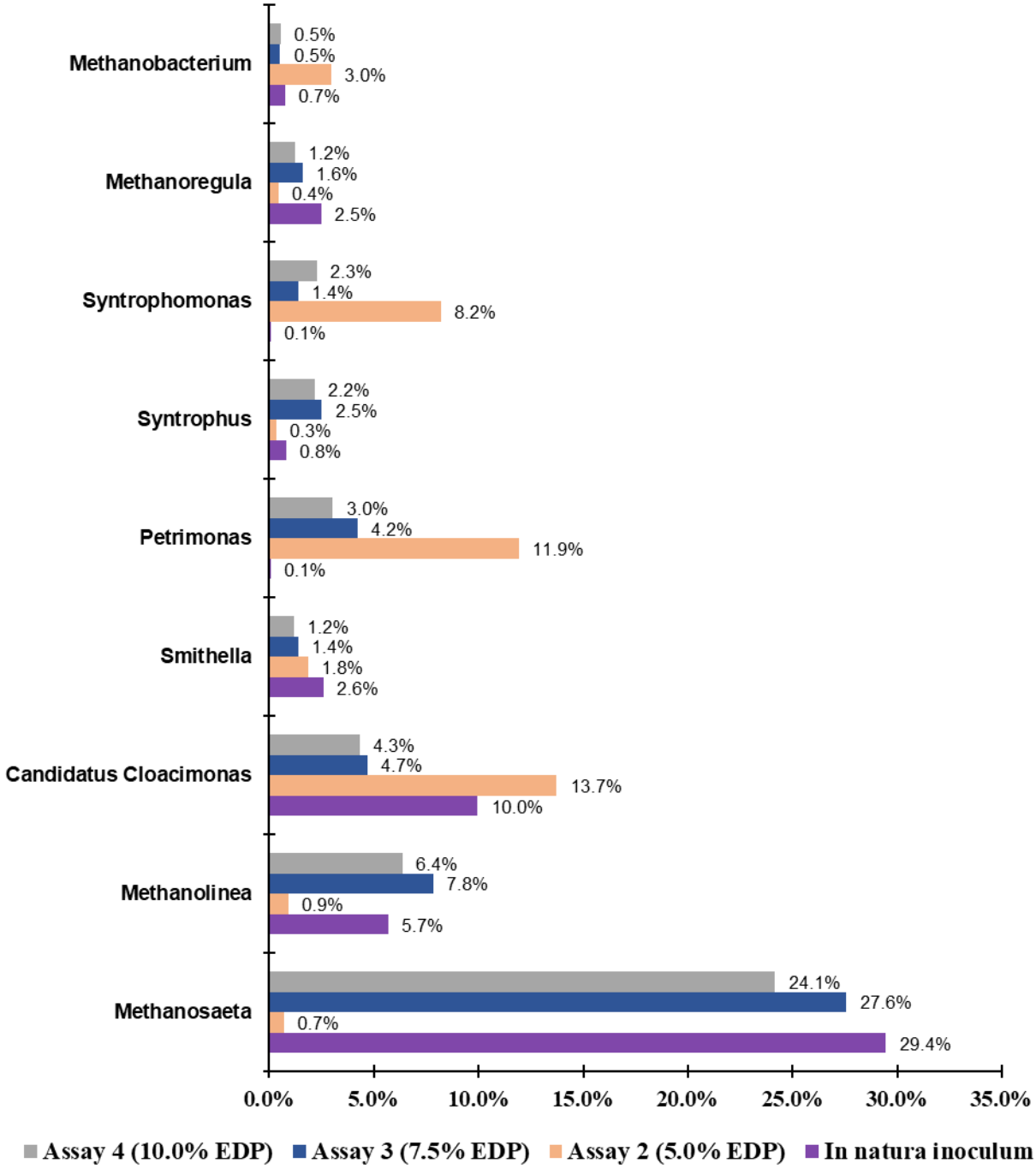
Regarding genus, a high relative abundance was observed for the archaea *Methanosaeta*, which only presented a percentage below 24% for assay 2 (5.0% EDP).

These archaea are classified as acetoclastic microorganisms, as they produce methane from the consumption of acetate, considered the most crucial precursor of methane production and the source of up to 70% of the methane produced in reactors (VÁZQUEZ-FERNÁNDEZ; SUÁREZ-OJEDA; CARRERA, 2022). In addition, Delforno et al. (2017) also described the prevalence of Methanosaeta. However, despite the predominance of the *Methanosaeta* genus, a slight decrease in the relative abundance was observed compared to the *in natura* inoculum. Probably, it was caused by the substrate changes (from poultry slaughterhouse wastewater to EDP) (**Figure III.4**).

The opposite was observed for the *Methanolinea* genus, which was the second most abundant genus of archaea verified, with relative abundances of 5.7%, 0.9%, 7.8%, and 6.4% for *in natura* inoculum, and from assays 2, 3, and 4 (5.0%, 7.5%, and 10.0% EDP), respectively. A slight increase in the relative abundance of this genus was observed when comparing the inoculum of assays 3 and 4 to the *in natura* inoculum. It is responsible for reducing CO_2 with H_2 to produce CH_4 (hydrogenotrophic pathway) (LACKNER et al., 2018). Other hydrogenotrophic archaea, *Methanoregula,* and *Methanobacterium* genera were also identified in all samples from the present study. This occurrence suggested that the EDP substrate could favor a synergy between hydrogenotrophic and acetoclastic genera, contributing to the high methane yield obtained in this study.

The synergy between different genera could be observed among several microorganisms identified in this study. For example, the proteins present in expired dairy products (casein, albumin, and globulin) were probably metabolized by the genus *Candidatus Cloacimonas*, which is capable to extracts most of its carbon source, producing H_2 and CO_2, which is extremely important for the maintenance of methanogenesis by the hydrogenotrophic pathways (PELLETIER et al., 2008; SOLLI et al., 2014). Whereas the different carbohydrates, such as lactose from expired milk, glucose, and fructose from expired fruit yogurt, can be metabolized by the genus *Petrimonas*. This genus can metabolize these sugars producing acetate and hydrogen, which are used as substrates in both methanogenic pathways (GRABOWSKI et al., 2005).

Figure III.4 - Relative Abundance of Genera.



Syntrophus, Syntrophomonas, and *Smithella* are responsible for the metabolism of fatty acids produced from the hydrolysis of lipids. These microorganisms are essential in the anaerobic digestion of fatty residues, such as dairy products, once they avoid the inhibition caused by the accumulation of these acids in the reactors (ELSAMADONY et al., 2021). In addition, several studies have observed the coexistence and cooperation of these genera with methanogenic microorganisms, associating better efficiency in the degradation of long-chain fatty acids with an

increase in methane generation (AGHASA et al., 2022; KURADE et al., 2019; SINGH et al., 2019).

4 CONCLUSIONS

The anaerobic co-digestion of EDP with synthetic sewage enhanced methane production from 716.8 mL L_r^{-1} (assay 1 - 0% EDP) to 6583.6 mL L_r^{-1} (assay 4 -10.0% EDP). However, higher addition of EDP (assay 5 - 15.0%) caused a build-up VFAs (mainly acetic, butyric, and propionic acid), leading to a complete anaerobic process inhibition. Nevertheless, this study demonstrated high organic matter removal (80.0-89.2%) and high methane yield (317.3-332.6 mL gVS_{add}^{-1}) for assays 2, 3, and 4 (from 5.0% to 10.0% EDP), which were probably achieved by the synergy between acetoclastic archaea (*Methanosaeta* genus) and hydrogenotrophic ones *(Methanolinea*, *Methanoregula*, and *Methanobacterium*, genera). The results of this study encourage EDP utilization as a substrate in the anaerobic digestion process. However, more studies must be conducted to increase its concentration above 10.0%, overcoming methanogenesis suppression and biological process failure.

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CHAPTER IV

REUSE OF EXPIRED DAIRY PRODUCTS AND SEWAGE IN THE PRODUCTION OF BIOGAS BY ANAEROBIC DIGESTION

ABSTRACT

The dairy industry is a prominent sector of the agro-industry, with global production of 906 million tonnes of milk. Several dairy products are wasted due to their high perishability. Anaerobic digestion is a sustainable alternative for reducing this waste aimed at biogas production. This study evaluated the co-digestion of expired dairy products (EDP) with synthetic sewage from two assays using two types of dairy waste, separately: Assay I (EDP mixture of 80% of milk + 15% of yogurt + 5% of milk cream) and Assay II (industrial raw mixture of EDP). Both assays were composed of 20 g COD L^{-1} (Chemical Oxygen Demand per Liter) using anaerobic batch reactors (1.0 L) assembled in duplicates with 0.5 L headspace (N_2) and working volume of 0.5 L with initial pH 8.2, at 37°C, in a static mode. The cumulative CH_4 productions were 2722.5 NmL in 31 days of operation (Assay I) and 3140.0 NmL in 25 days (Assay II). Equivalent CH_4 yields were obtained for both assays with ~330 NmLCH_4 g COD_{rem}^{-1}. Carbohydrates and COD removals were 96.8% and 98.0%; 87.3% and 89.4%, respectively. The co-digestion of EDP with synthetic sewage was effective for CH_4 production with efficient organic matter removal. These results encourage new strategies for the reuse of expired dairy products by the use of anaerobic digestion.

Keywords: Expired dairy products; Anaerobic digestion; Reverse logistics; Methane; Biogas;

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CHAPTER IV

1 INTRODUCTION

The dairy industry has stood out in the agro-industrial sector, reaching a global production of 906 million tons of milk in 2020 (FAO, 2021). In the same year, Brazil set a record with the production of 35.4 million tons of milk (CNA, 2021). However, it has been reported that the annual dairy waste residue is around 4–11 million tons worldwide (USMANI et al., 2021).

Wastewater and cheese whey are known to be potential dairy industry residues (AHMAD et al., 2019). Furthermore, expired dairy products (EDP) are included in this context due to their high perishability and short shelf life. After the expiration date, they are considered unfit for consumption and are usually disposed of in landfills (SAKARIKA et al., 2020).

EDP contains large amounts of carbohydrates (45-50 g L^{-1}), proteins (6-30 g L^{-1}), and lipids (4-8 g L^{-1}) (STAVROPOULOS et al., 2016). Therefore, they are characterized as organic matter rich-compounds (COD 121-191 g L^{-1}) (KOPSAHELIS et al., 2018; SAKARIKA et al., 2020; WU et al., 2011), which makes their disposal in landfills harmful to the environment. Furthermore, this practice is contrary to Brazil's National Solid Waste Policy instituted by Law nº 12.305 of August 2nd, 2010, since this law aims to reduce waste disposal from its reuse, recycling, and treatment, as a way to minimize environmental impacts (VERZOLA, 2018).

The reverse logistic can be another way to overcome this problem, which is an alternative allied to the Brazilian laws, where expired products can be sent to a deconditioning center, going through a de-packaged process with consequent separation of inert material (packaging) and organic material (expired dairy product) (NOBLECOURT et al., 2018). After that, the packaging can be recycled, while organic material can be used as a substrate in anaerobic digestion (AD), instead of being disposed of in the landfill.

Reuse of dairy waste by anaerobic digestion (AD) has been a promising alternative for treating and valuing them (ADGHIM et al., 2020; WU et al., 2011) since AD is able to degrade organic matter to produce biogas, which is an emerging and essential biofuel for the world energy matrix (SAR et al., 2022). Sakarika et al. (2020) reported a maximum yield of 0.757 mol H_2 mol^{-1} carbohydrates consumed and 318 mL CH_4 g VS_{add}^{-1} using the co-digestion of expired dairy products with a mixture of agroindustrial wastes (pig manure, liquid cow manure, cheese whey, poultry wastes,

and slaughterhouse wastes) as substrates in a pilot-scale two-stage mesophilic (37ºC) anaerobic system.

Although AD has many advantages, its application for EDP treatment, which contains high amounts of carbohydrates, lipids, and proteins, can result in the rapid formation of volatile fatty acids (VFAs) and long-chain fatty acids (LCFAs), leading to methanogenesis suppression with consequent inhibition of biogas formation (HASSAN; NELSON, 2012; XU et al., 2018).

Therefore, anaerobic co-digestion (ACoD) is a good option to overcome the mono digestion problem (LV et al., 2021). ACoD is frequently used to enhance anaerobic digestion and biogas production performance from substrates with complementary characteristics providing nutrient balance, synergistic effects of microorganisms, and diluting harmful or excessive substances (XU et al., 2018).

Several industrial residues can be used in anaerobic co-digestion; however, it is important to evaluate the availability and costs of residues that will be used (SIDDIQUE; WAHID, 2018). In this scenario, sewage can be considered a good co-substrate for dairy waste, due to its high treatment requirements and low cost (ANA, 2017). Furthermore, sewage is basically composed of water (99.9%), acting as a good diluent (VON-SPERLING, 2014). Additionally, it contains important nutrients, such as nitrogen and phosphorus, essential for the growth of microorganisms in the biological process (MARTÍN-GONZÁLEZ et al., 2010). Therefore, feeding bioreactors with a mixture of both wastes is an exciting approach (ADAMES et al., 2021; MARTÍN-GONZÁLEZ et al., 2010).

Thus, the present study evaluated the co-digestion of expired dairy products with sewage in anaerobic batch reactors, operated with different mixtures of dairy wastes, to reuse them in biogas production.

2 MATERIAL AND METHODS

2.1 Inoculum source and substrates

The methanogenic granular sludge was collected from a UASB (Upflow Anaerobic Sludge Blanket) reactor, and it was applied to the treatment of poultry slaughterhouse wastewater (Tietê – SP – Brazil). The inoculum was stored in plastic bottles and kept in the refrigerator (Consul, model FFE24) at 4ºC until its utilization.

Dairy waste A consisted of a mixture of expired dairy products (80% of milk + 15% of yogurt + 5% of milk cream). These proportions were based on the study of KOPSAHELIS et al. (2018), and they were also similar to the proportion of expired products in the market that return to a dairy factory. The dairy waste A was composed of (g L^{-1}) Total Solids - TS (124.3); volatile solids - VS (116.8); total Chemical Oxygen Demand- tCOD (185.3); total carbohydrates - tC (52.3) and pH 6.2.

Dairy waste B consisted of raw dairy waste composed of a mixture of expired dairy products containing milk, milk cream, and yogurt collected from a dairy company (Descalvado – SP – Brazil). It was composed of (g L^{-1}) TS (150.9), VS (144.5), tCOD (297.2), tC (78.5), and pH 5.5.

Synthetic sewage was prepared according to the methodology described by MARTÍN-GONZÁLEZ et al. (2010) (**Table IV.1**) with the same nutrient composition as domestic sewage. It was composed of (g L^{-1}) TS (0,3), VS (0.2), tCOD (0.4), tC (<0.01), and pH 6.8.

2.2 Batch Reactors Setup

Two assays were assembled (Assay I and Assay II) in duplicates of anaerobic batch reactors (1000 mL) and a reaction medium (composed of substrates + inoculum) of 500mL. The reactors were set up in both assays to get a substrate mixture with a COD concentration of 20 g COD L^{-1}. Therefore, in Assay I, the substrate mixture was composed of 16% of Dairy Waste A + 84% of synthetic sewage, which corresponded to a total volume of 340 mL. In Assay II, the mixture was composed of 10% of Dairy Waste B + 90% of synthetic sewage, also corresponding to a total volume of 340 mL. The amount of 19 g VS L^{-1} of inoculum (160 mL) and 5 g L^{-1} buffer solution ($NaHCO_3$) were added to all reactors. The initial pH was adjusted to 8.2 with 1M NaOH or 1M HCl additions. The headspace of the reactors (500mL) was filled with N_2 (99.9%) to create an anaerobic environment, and they were capped with butyl rubber stoppers, wrapped, and kept in a static mode, at mesophilic conditions (37°C) using a dry oven (Solab, SL-101). Control reactors (without substrates addition) were operated to determine the background methane production from the inoculum. The methane production was calculated by subtracting the control reactors' methane production from the batch anaerobic reactors (ANGELIDAKI et al., 2009). The anaerobic reactors were operated until no biogas production was detected.

Table IV.1 - *Composition of Synthetic Sewage*

Macronutrient solution		Micronutrient solution	
Compound	**[mg L^{-1}]**	**Compound**	**[mg L^{-1}]**
Starch	200	$FeCl_3.4H_2O$	1000
Ovoalbumine	21.0	$CoCl_2.6H_2O$	1000
Sunflower oil	13.1*	$MnCl_2.4H_2O$	250
Urea	13.0	$CuCl_2.2H_2O$	15
KH_2PO_4	5.26	$ZnCl_2$	25
$CaCl_2.2H_2O$	22.05	H_3BO_3	25
$MgSO_4.7H_2O$	0.43	$(NH_4)_6Mo_7O_{24}.4H_2O$	45
KCl	21.3	$NaSeO_3.H_2O$	50
$NaHCO_3$	8.76	$NiCl_2.6H_2O$	35
Yeast extract	100	EDTA	500
Micronutrients	1.0*	HCl 36%	1.0*
		Resarzurin	250

* Amount expressed in mL L^{-1}

2.3 Analytical Methods

The tCOD, pH, TS, and VS were analyzed according to “Standard Methods for the Examination of Water and Wastewater” (APHA, 2012).

Total carbohydrate concentration was determined by colorimetric method using phenol-sulfuric acid. The measurements were performed using a Nanocolor VIS II spectrophotometer (Macherey-Nagel) at the wavelength of 490 nm (DUBOIS et al., 1956; HERBERT, D.; PHILIPPS, O.S.; STRANG, 1971).

The biogas components (H_2, N_2, CH_4, and CO_2) were measured using a Shimadzu® gas chromatograph (GC-2014), equipped with a thermal conductivity detector (TCD) and Carboxen 1010 PLOT column. The column flow was 5.0 mL min^{-1} with ultra-pure argon as the carrier gas. The injector and detector temperatures were 220°C and 230°C, respectively. The column temperatures were: 120 °C (1 min), 40 °C/min up to 200 °C (3 min), and 50 °C/min up to 230 °C (0.5 min), with a total run time of 7.1 minutes (MAINTINGUER et al., 2008). The biogas volume was measured daily by the water displacement method according to ZUO et al. (2020) methodology, and

the values were normalized to 273 K and 1013 hPa according to VDI 4630 (2006) methodology.

2.3 Statistical analysis of experimental data

Statistical analysis was performed using the average values obtained by ANOVA and Tukey's test at a 5% significance level, considering a completely randomized design. The tests were applied to all CH_4 production parameters (cumulative production, maximum production rate, and yield).

3 RESULTS AND DISCUSSION

The bioreactor operation data are presented in **Table IV.2**. For both tests, the final pH values (8.0 and 7.9, respectively) were close to the initial ones (8.2), indicating that all acids formed during the anaerobic digestion were consumed.

Table IV.2 - Bioreactors Operation Data and Removal Efficiency

Parameters	Unit	Assay I (Dairy Waste A)	Assay II (Dairy Waste B)
Operation time	days	31	25
pH (initial – final)	–	8.2 – 8.0	8.2 – 7.9
Carbohydrates (tC) removal	%	96.8	98.0
COD removal	%	87.3	89.4
VS removal	%	87.0	86.4

In terms of removal of tC, COD, and VS, both assays succeeded with percentages above 85%. However, Assay II obtained tC and COD removal percentages slightly higher (98.0% and 89.4%) than Assay I (96.8% and 87.3%). However, VS removal was 0.6% higher for Assay I than Assay II.

The removal of COD and VS obtained in this present study were higher than the results achieved by WU *et al.* (2011), which were 59.1% COD and 28% VS with the anaerobic co-digestion of milk waste with dairy manure using batch reactors (19.8 g COD L^{-1}) at 37°C during 28 days.

The average volume of methane produced by the control reactors was 472.1 NmL, which was discounted from the methane volume produced in Assays I and II. Significant differences were observed for the parameters of cumulative CH_4 production

and maximum CH_4 rate, demonstrating that the best results were achieved for Assay II (3140.0 NmL and 251.2 NmL L_r^{-1} d^{-1}) than for Assay I (2722.5 NmL and 177.1 NmL L_r^{-1} d^{-1}), respectively. In contrast, no significant differences were observed in CH_4 yield, which was close to 330 NmL CH_4 g COD_{rem}^{-1} for both assays (**Table IV.3**).

Table IV.3 - Average Values of Cumulative CH_4 production, maximum CH_4 production rate and CH_4 yield.

Analysis of variance	Cumulative CH_4 production (NmL)	Maximum CH_4 Rate (NmL L_r^{-1} d^{-1})	CH_4 Yield (NmLCH_4 g COD_{rem}^{-1})
F treatments	18.71*	185.05 **	0
C.V. (%)	3.30	3.50	3.22
Tukey's Test (5%):			
Assay I	2722.50[b]	177.07[b]	330.40[a]
Assay II	3140.04[a]	251.20[a]	330.53[a]

Means followed by the same letter within the same row do not differ (p>0.05) by Tukey Test. ** Significant at 1% probability level (p<0.01); * Significant at 5% probability level (p<0.05). C.V.: coefficient of variation.

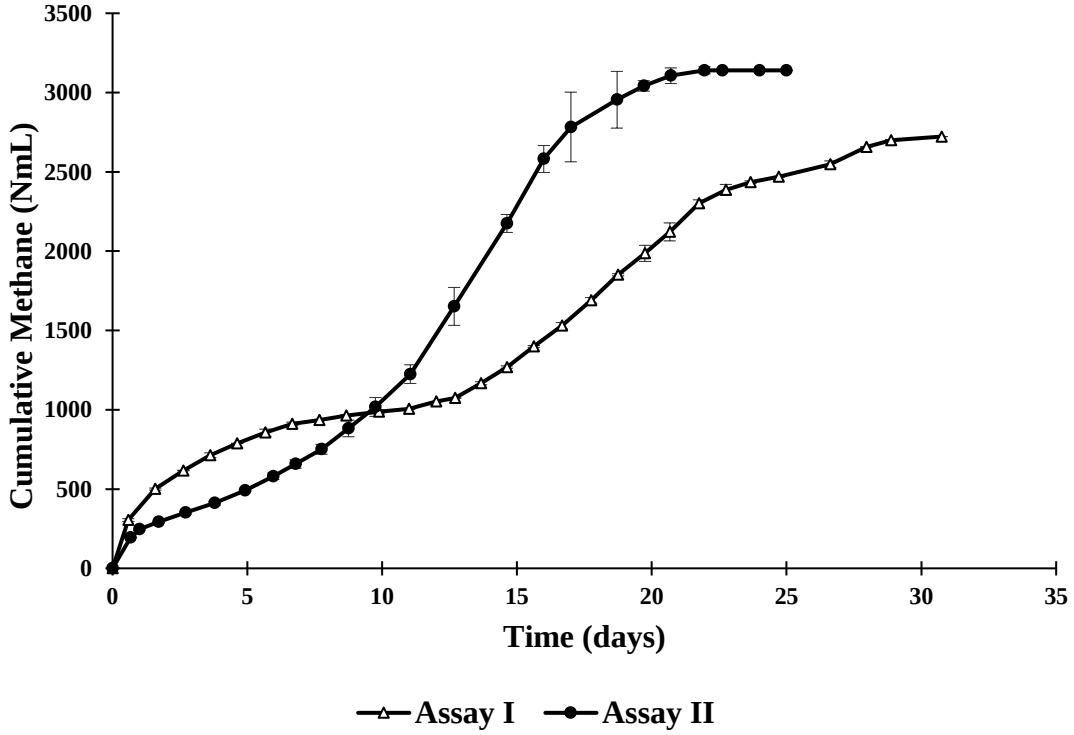
The average cumulative biogas volume produced is presented in **Figure IV.1**. A degradation pattern was identified in both assays, characterized by two exponential phases. In this pattern, methane was produced in the initial phase, followed by decreasing in its production, which can be considered an adaptation phase. Thereafter, a significant increase in CH_4 production was verified, even higher than that observed in the initial phase, until it reached the stationary phase (Appendix C).

The adaptation phase in Assay I was longer, remaining for 12.8 days, while for Assay II, this period was 10.5 days. The longer adaptation time in CH_4 production verified in Assay I may have contributed to a lower production rate observed.

The methane production pattern observed in this study had already been reported in other studies as a typical behavior of residues with high lipids content, such as those from dairy products. HANAKI; MATSUO; NAGASE (1981) evaluated the inhibitory effect of long-chain fatty acids (LCFAs) (product of fat hydrolysis) on anaerobic digestion using batch experiments from synthetic substrates, such as powdered whole milk, sodium oleate, and fatty acids mixture. They verified that the accumulation of LCFAs were linked with an increase in the lag phase and with a decrease in methane yield at the beginning of the reactor's operation.

Similarly, SAGE; DAUFIN; GESAN-GUIZIOU (2008) studied the anaerobic degradation steps of milk fat, evaluating the kinetics of fat degradation in comparison with other milk components (lactose, proteins). The experiments were conducted using different substrates (Thermized cream, anhydrous milk fat, commercial UHT skim milk) evaluated “in natura” and after alkaline hydrolyze. The reactors were assembled with 250mL of active volume fulfilled with 5 kg_{VSS}/m^3 of anaerobic biomass and substrate to biomass ratio of 0.1 to 10 $kg_{COD}/kg_{VSS,}$ at 35ºC, under agitation (60 rpm), for 70 days. The authors verified that the milk fat was degraded after several days of lag phase, mainly due to unsaturated free fatty acids (FFA).

Figure IV.1 - Cumulative Methane Production in Assay I (Dairy waste I) and Assay II (Dairy waste II)



Therefore, lipids can be considered a limiting waste component in AD due to their slow degradation caused by the accumulation LCFAs. This behavior leads to an increase in the adaptation phase and, consequently, a decrease in the initial methane production. This fact was verified by WU et al. (2011), who observed a decrease of CH_4 in biogas due to the increase of milk residue (from 66.5% for control to 63.9% for the assay with 19% of milk residue), which could be associated with more lipid content added to reactors. Despite that, no inhibition was observed, and the authors obtained a methane yield of 324.1 mL $gCOD_{rem}^{-1}$.

The same phenomena were verified in the present study. Despite a long adaptation phase mainly for Assay I, lipids degradation was not an inhibitory factor for none of the assays, since good results were achieved.

Sivakumar *et al.* (2012) studied the anaerobic digestion of 100% w/w of spoiled milk (200 g COD L^{-1}) using an ASBR reactor fed daily for 30 days. The authors achieved lower values of the methane yield (220 mL CH_4 g COD_{rem}^{-1}) and of COD removal efficiency (92.8%) than the values got to Assay I and II.

Kopsahelis *et al.* (2018) investigated co-digestion of End-of-Life Dairy Products (~ 20% w/w) with agro-industrial wastes (composed of Pig Manure, Liquid Cow Manure, Cheese Whey, Poultry Wastes, and Slaughterhouse Wastes) using mesophilic CSTR reactor with HRT of 37 days. The maximum methane yield achieved for a methanogenic reactor (single-stage system) was 230 mL CH_4 $gCOD_{rem}^{-1}$ with 71.9% of COD removal efficiency. These results were lower than those verified in the present study (with 330.4 and 330.5 NmL CH_4 g COD_{rem}^{-1}; and COD removal of 87.3% and 89.4% for Assay I and Assay II, respectively).

Thus, the results of this study demonstrated that the co-digestion of expired dairy products with synthetic sewage was efficient in the COD concentration proposed (20 g L^{-1}). Furthermore, satisfactory results of COD removal and methane production were obtained in both evaluated assays, especially when compared to previous studies with similar dairy wastes tested.

4 CONCLUSIONS

The anaerobic digestion of expired dairy products was effective, despite the longer adaptation phase in the initial methane production observed in both assays.

The anaerobic co-digestion of expired dairy products with synthetic sewage was effective in the production of biogas.

New approaches for the reuse of dairy waste using anaerobic digestion are encouraged.

Further studies should be undertaken in order to evaluate the effect of the increase of dairy wastes on the anaerobic digester performance.

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CHAPTER V

APPLICATION OF TWO-STAGE ANAEROBIC SYSTEMS FOR RECOVERY OF EXPIRED DAIRY PRODUCTS AND BIOGAS PRODUCTION

ABSTRACT

Expired dairy products (EDP) are produced in significant quantities due to their perishable nature. These products contain high amounts of organic matter, and their disposal in landfills has adverse environmental effects. A promising solution to mitigate waste and recover resources is to use EDP as a substrate for anaerobic digestion, aiming for biogas production (H_2 and CH_4). This study investigated the EDP consumptions in co-digestion with synthetic sewage (SS) in assays composed of (v/v): (1) 10% EDP + 90% SS, (2) 15% EDP + 85% SS, and (3) 20% EDP + 80% SS to evaluate the hydrogen (H_2) and methane (CH_4) production from a two-stage anaerobic system. In the acidogenic stage, duplicates of anaerobic batch reactors (1000 mL) were assembled with a working volume of 500 mL filled with 19 g VS L^{-1} of acidogenic inoculum, 5 g L^{-1} $NaHCO_3$, initial pH of 6.5, performed at 37 °C, in a static mode. In the methanogenic stage, the effluents generated at the end of the acidogenic stage from assays 1, 2, and 3 were used as substrates, corresponding to assays 4, 5, and 6, respectively. All reactors were set with 19 g VS L^{-1} of methanogenic inoculum and 5 g L^{-1} of $NaHCO_3$. The initial pH was adjusted to 7.5 at 37°C in a static mode. In the acidogenic stage, the carbohydrates removals were 86.8%, 90.1%, and 92.3%, and the H_2 yields were 1.5, 1.9, and 1.7 mol mol^{-1} carbohydrate removed for the assays from 1 to 3, respectively. *Paraclostridium* was the genus with high relative abundance (55.7-82.7%) identified in all assays of the acidogenic stage. In the methanogenic stage, the COD removals were 91.8%, 94.7%, and 16.4% for the assays from 4 to 6, respectively. Assay 6 (20% EDP + 80% SS acidified) was inhibited by VFA accumulation. However, assays 4 and 5 (10% EDP + 90% SS acidified and 15% EDP + 85% SS acidified) showed high methane yields ($mLCH_4$ gVS_{add}^{-1}) 323.0 and 259.2, respectively, demonstrating the viability of EDP's co-digestion with synthetic sewage in a two-stage system. Acetoclastic archaea (*Methanosaeta*) were identified with high archaea relative abundance at 60% and 62% for Assays 4 and 5, respectively, which possibly favored a high COD removal and a positive methane yield.

Keywords: Expired dairy products; Two-stage system; Hydrogen; Methane; Anaerobic Microbial Community.

1 INTRODUCTION

In Brazil, the dairy industry is among the pillars of the economy in the food sector. In 2021, Brazil was the sixth largest milk producer in the world, with a production of almost 36 million tons (FAO, 2022). Milk is currently one of the six most important products of Brazilian agriculture. It is considered essential in the supply of food and the generation of employment and income for the population in all stages of its production chain (EMBRAPA, 2019). However, the dairy industry generates a large amount of waste. It is estimated that 18% of milk production is wasted for several reasons (SAR et al., 2022).

Expired dairy products (EDP) are among the wastes generated by the dairy industry. Most of this waste is disposed of in landfills (KOPSAHELIS et al., 2018). Despite not having toxic compounds in their composition, they are characterized by high Chemical Oxygen Demand (297 g COD L^{-1}) (MARIN et al., 2022), and when disposed of improperly, cause serious problems to the environment (AHMAD et al., 2019).

Therefore, anaerobic digestion (AD) can be applied to EDP to produce biofuels such as H_2 and CH_4. Biogas utilization is rising due to its distinct benefits, including energy preservation, greenhouse gas reduction, and social advantages (DE CARVALHO; MAINTINGUER, 2022). Despite the benefits, the high organic matter of EDP, and its high amount of carbohydrates (57.5%) (SLOPIECKA et al., 2022), cause rapid waste biodegradation resulting in acidification and instability of anaerobic reactors (XU et al., 2018).

The synergy between acidogenic and methanogenic microorganisms is essential for the stability of the biological process. Methanogenic archaea are extremely sensitive to sudden changes in pH and acid environment (CREMONEZ et al., 2021). Thus, the EDP mono-digestion becomes impracticable, requiring the application of anaerobic co-digestion with residues of opposite characteristics. In this case, sewage is an excellent co-substrate since it has low COD (0.35 g COD L^{-1}) and high-water content (99.9%); it can be used as an excellent diluent (VON-SPERLING, 2014).

Additionally, phase separation in anaerobic digestion processes offers an alternative to optimize reaction conditions for different groups of microorganisms. The two-stage anaerobic digestion (TSAD) offers significant advantages for highly fermentable residues. It ensures process stability by eliminating limitations in the hydrolysis phase. These systems can tolerate higher organic loading rates, enabling efficient organic waste treatment. The ability to adjust conditions in each stage optimizes the performance of microorganisms, resulting in optimal biodegradation. The enhanced substrate conversion efficiency in the two-stage system leads to increased production of methane and hydrogen gases, contributing to sustainable utilization of organic waste in AD (CREMONEZ et al., 2021; KOPSAHELIS et al., 2018; XU et al., 2018).

In this sense, this study tested the generations of hydrogen and methane in a two-stage anaerobic digestion from expired dairy products (EDP) co-digested with synthetic sewage (SS) using batch reactors. In the first stage (acidogenic reactors), the EDP + SS was acidified, producing H_2. Thus, the liquid effluent generated in the first stage was used in methanogenic reactors (second stage) to remove its high organic matter, producing CH_4. The co-digestion of EDP with SS using TSAD system has never been studied before. Therefore, this study presents promising results in this area in order to recover EDP by mitigating the environmental impacts that this waste can cause due to its inappropriate disposal.

2 MATERIALS AND METHODS

2.1 Substrates

The expired dairy products were collected from a dairy company (Descalvado – SP – Brazil). They are composed of milk, milk cream, and yogurt containing (g L^{-1}); TS - Total Solids (150.9); VS - Volatile Solids (144.5); tCOD - total Chemistry Oxygen Demand (297.2); tC - total Carbohydrates (78.5), fats, and oils (12.4), and pH 5.5. It was stored in plastic flasks to preserve the mixture properties and kept at -20 °C until its use.

The synthetic sewage was prepared according to Martín-Gonzáles et al. (2010) with the same macro and micronutrient composition as real domestic sewage. It contained (g L^{-1}); TS (0.3); VS (0.2); tCOD (0.4); tC (<0.01), and pH 6.8.

2.2 Inoculum source and pretreatment conditions

2.2.1 Acidogenic Inoculum

The inoculum was obtained from a mesophilic granular sludge from a UASB (Upflow Anaerobic Sludge Blanket) reactor used to treat poultry slaughterhouse wastewater (Tietê – SP – Brazil). It was submitted to heat pre-treatment (100 ºC for 15 min), followed by immersion in an ice bath until reaching a temperature of 25ºC, to inhibit methanogenic archaea (MAINTINGUER et al., 2008).

2.2.2 Methanogenic Inoculum

The same inoculum obtained from mesophilic granular sludge from a UASB (Upflow Anaerobic Sludge Blanket) reactor used to treat poultry slaughterhouse wastewater (Tietê – SP – Brazil) was used without pretreatment (*in natura*).

2.3 Experimental Setup

2.3.1 First Stage - Acidogenic Reactors

Three assays (Assay 1, Assay 2, and Assay 3) were assembled in duplicates of anaerobic batch reactors (1000 mL) with a working volume of 500 mL. The assays were composed of a substrate mixture of (v/v): (1) 10% EDP + 90% SS, (2) 15% EDP + 85% SS, and (3) 20% EDP + 80% SS (v/v), corresponding to a total COD concentration of 20, 30, and 40 g COD L^{-1} in the assays 1, 2, and 3, respectively. All reactors were set with 19 g VS L^{-1} of acidogenic inoculum (160 mL) and 5 g L^{-1} buffer ($NaHCO_3$). The initial pH was adjusted to 6.5 with 1M NaOH or 1M HCl (**Table V.1**). After assembly, each reactor was sealed with rubber stoppers and purged with N_2 (99.9%) to create an anaerobic environment. They were subsequently maintained in mesophilic conditions (37°C) at static mode. The anaerobic reactors were operated until either the biogas production reached a stable state or the coefficient of variation between the last three cumulative biogas records fell below 5% (ANGELIDAKI et al., 2009). Endogenous control was assembled in previously described conditions fed with deionized water and inoculum without substrates.

2.3.2 Second Stage - Methanogenic Reactors

The liquid effluents generated at the end of the first stage (acidified EDP + SS mixture) were separated from the acidogenic inoculum by a sieving process. After that,

the liquid fraction was used as substrates in the methanogenic reactors, corresponding to assays 4, 5, and 6, respectively. All reactors were set with 19 g VS L^{-1} of methanogenic inoculum (160 mL) and 5 g L^{-1} buffer ($NaHCO_3$). The initial pH was adjusted to 7.5 under the same operating conditions as the acidogenic reactors (temperature and headspace). Endogenous control and the operation time were the same as adopted for the first stage.

Table V.1 - Setup of acidogenic and methanogenic reactors.

	1st stage			**2nd stage**		
	Acidogenic Reactors			**Methanogenic Reactors**		
Parameters	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 6**
Working volume (mL)	500	500	500	500	500	500
Inoculum condition	Acidogenic Inoculum	Acidogenic Inoculum	Acidogenic Inoculum	Methanogenic Inoculum	Methanogenic Inoculum	Methanogenic Inoculum
Inoculum (gVS L^{-1})	19	19	19	19	19	19
Substrate	10% EDP + 90% SS	15% EDP + 85% SS	20% EDP + 80% SS	10% EDP + 90% SS acidified	15% EDP + 85% SS acidified	20% EDP + 80% SS acidified
Buffer (g L^{-1})	5	5	5	5	5	5
Initial pH	6.5	6.5	6.5	7.5	7.5	7.5
Temperature (°C)	37	37	37	37	37	37

EDP: Expired dairy products; SS: Synthetic Sewage.

2.4 Analytical methods

The TS, VS, COD, and pH were measured as described in "*Standard Methods for the Examination of Water and Wastewater*" (APHA, 2012). The colorimetric method used phenol-sulfuric acid determined total carbohydrate concentration (DUBOIS et al., 1956).

The biogas components (H_2, N_2, CH_4, and CO_2) were measured using a Shimadzu® gas chromatograph (GC-2014) equipped with a thermal conductivity detector (TCD) and Carboxen 1010 PLOT column. The column flow was 5.0 mL min^{-1} with ultra-pure argon as the carrier gas. The injector and detector temperatures were 220°C and 230°C, respectively. The column temperatures were: 120 °C (1 min), 40 °C/min up to 200 °C (3 min), and 50 °C/min up to 230 °C (0.5 min), with a total run time of 7.1 minutes (MAINTINGUER et al., 2008). The biogas volume was recorded daily through volume displacement (AQUINO et al., 2007), and the values were normalized to 273 K and 1013 hPa (VDI 4630, 2006).

The soluble metabolites (volatile fatty acids (VFAs) and alcohols) were determined using a GC (Shimadzu® GC-2030) equipped with a flame ionization detector (FID), HP-INNOWAX capillary column (30m x 0.25mm x 0.25 µm) and AOC 6000 Plus autosampler (ADORNO; HIRASAWA; VARESCHE, 2014). The lactic acid quantification was performed in the pre-filtered samples (0.22 µm pore size filter) using High-Performance Liquid Chromatography (HPLC). The refractive index detector (Waters 2014) was maintained at 40ºC. The analytical column Aminex® HPX-87H (300 × 7.8 mm) was held at 50°C with a flow rate of 0.6 mL/min, using sulfuric acid 0.005M as a mobile phase (PERIMENIS et al., 2018).

2.5 Experimental data fitting

The experimental data obtained during the H_2 and CH_4 production were adjusted using the software Statistica® (version 8.0) according to a modified Gompertz [Equation (1)] (LAY; LI; NOIKE, 1998).

$$BPM = P * exp\left\{-exp\left[\left(\frac{(R_m * e)}{P}\right) * (\lambda - t) + 1\right]\right\} \quad [1]$$

BPM is cumulative H_2 or CH_4 production (mL L_r^{-1}); P is the maximum H_2 or CH_4 production (mL L_r^{-1}) R_m maximum H_2 or CH_4 production rate (mL L_r^{-1} d^{-1}), λ is the time to start H_2 or CH_4 production (days), t is the operation time (days), and e is the Euler's number.

For a curve with diauxic behavior (two exponential phases), it was applied a modified Gompertz [Equation (2)], which uses two terms, as proposed by Kim et al. (2003).

$$BMP = P_1 \times exp\left(-exp\left(\frac{R_1 \times e}{P_1}(\lambda_1 - t) + 1\right)\right) + P_2 \times exp\left(-exp\left(\frac{R_2 \times e}{P_2}(\lambda_2 - t) + 1\right)\right) \quad [2]$$

Where BPM is cumulative CH_4 production (mLCH_4 L_r^{-1}); P1 and P_2 are the maximum CH_4 production (mLCH_4 L_r^{-1}) for the first and second exponential phases, respectively; R_1 and R_2 maximum CH_4 production rate mLCH_4 L_r^{-1} d^{-1}), for the first and second exponential phases, respectively; λ_1 and λ_2 time to start the CH_4 production

(hours) for the first and second exponential phases, respectively; t is the operation time (hours), and e is the Euler's number.

2.6 Microbial diversity analysis

Molecular biology analyses were performed for in natura inoculum; at the end of first stage for Assay 1 (10% EDP + 90% SS), 2 (15% EDP + 85%), and 3 (20% EDP + 80% SS); and at the end of second stage for assays 4 (10% EDP + 90% SS acidified) and 5(15% EDP + 85% acidified). DNA extraction was made using the modified phenol-chloroform method (GRIFFITHS et al., 2000). Posteriorly, tests were performed to verify the integrity of the extracted DNA by the ratio of 260/280 nm > 1.8, measured by an ND-2000 spectrophotometer (Nanodrop Inc., Wilmington, DEA).

After that, the Facility *ByMyCell* located at Ribeirão Preto (São Paulo, Brazil) (https://bymycell.com.br/) performed microbial characterization on the Illumina HiSeq PE250 Platform. The primers used in the polymerase chain reaction (PCR) were: 341F/806R (V3-V4 16S rRNA region). Sequences containing low-quality bases were eliminated. Chimeras were identified and removed using the DADA2 pipeline in QIIME2 (CALLAHAN et al., 2016). Amplicon Sequence Variants (ASVs) with a similarity of ≥97% underwent taxonomic classification. The SILVA SSU 123 rRNA Database Project (McLaren and Callahan, 2021) was utilized for taxonomic assignment.

The sequences obtained were submitted to National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), in the project accession number PRJNA643935, under the sample accession number: SRX19935667 for *in natura* inoculum (before the reactor's operation); SRX19946468, SRX19946588; SRX19946594 for Assay 1 (10% EDP + 90% SS), 2 (15% EDP + 85%), and 3 (20% EDP + 80% SS), respectively; and SRX19939163 and SRX19946384 for assays 4 (10% EDP + 90% SS acidified) and 5 (15% EDP + 85% acidified), respectively.

3 RESULTS AND DISCUSSION

3.1 First Stage - Acidogenic Reactors

The effects of EDP increasing percentage were evaluated on AD during 107 h. The results obtained are presented in **Table V.2**. The carbohydrate removal increased proportionally with increasing EDP in assays 1, 2, and 3 with 86.8%, 90.1%, and

92.3%, respectively. It confirms that the increase in EDP positively affected the acidogenic microorganisms.

Table V.2 - Performance of acidogenic reactors.

Parameters	Assay 1 (10% EDP + 90% SS)	Assay 2 (15% EDP + 85% SS)	Assay 3 (20% EDP + 80% SS)
pH (initial – final)	6.5 - 6.2	6.5 - 5.7	6.5 - 5.5
Operation time (h)	107	107	107
Initial Carbohydrates (g L^{-1})	3.3	4.6	7.1
Carbohydrates Removal (%)	86.8	90.1	92.3
Initial COD (g L^{-1})	23.1	30.8	40.3
COD Removal (%)	9.9	9.5	11.6
H_2 in biogas (%)	30.7	37.6	43.5
H_2 yield (mol H_2 mol^{-1} carbohydrates removed)	1.7	1.9	1.5
Gompertz Adjustment			
P (mL L_r^{-1})	652.6	1023.4	1309.3
Rm (mL H_2 L_r^{-1} h^{-1})	77.6	122.5	185.8
λ (h)	8.1	8.3	8.8

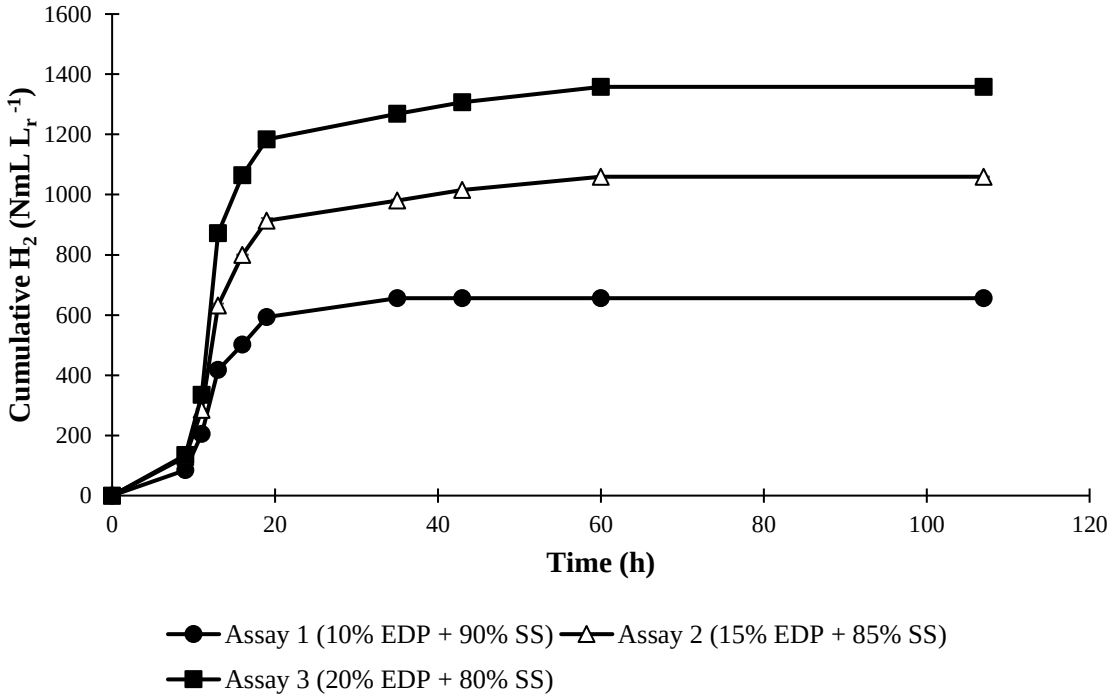
P_1: the production potential of H_2; R_m: the maximum production rate of H_2; and λ: the lag phase time.

All assays presented H_2 in biogas composition, which was directly associated with the degradation of carbohydrates present in EDP. **Figure V.1** shows the temporal variation of cumulative hydrogen production in the acidogenic reactors on assays 1, 2, and 3 (Appendix D). The amount of total accumulated H_2 increased equivalently with increasing organic matter in assays 1 (10% EDP + 90% SS), 2 (15% EDP + 85% SS), and 3 (20% EDP + 80% SS) with production values of 652.6, 1023.4, and 1309.3 mL L_r^{-1}, respectively. The equivalent increase regarding organic matter was also observed for values of maximum production rate with were 77.6, 122.5, e 185.8 mL H_2 L_r^{-1} h^{-1} for assays 1, 2, and 3, respectively. The lowest H_2 production start phase was verified for the assay with the lowest percentage of EDP (Assay 1) at 8.1 hours. However, Assay 3 (20% EDP + 80% SS) had the H_2 production start at 8.8 hours. This phenomenon

probably occurred in function of the greater amount of organic matter in the reactors that caused by the longer period of microorganism's adaptation.

The increase in the percentage of EDP was positive about the average percentage of H_2 in the biogas, with an increase of approximately 13% comparing Assay 1 with Assay 3. In addition to H_2 generation, it was verified only the presence of CO_2 in the biogas composition, indicating that only the fermentative stage was active and thus proving the effectiveness of the pre-treatment applied to the inoculum.

Figure V.1 - Cumulative hydrogen production.



The production of H_2 occurred simultaneously with the generation of different soluble metabolites. The final pH values were 6.2, 5.7, and 5.5 in assays 1 (10% EDP + 90% SS), 2 (15% EDP + 85% SS), and 3 (20% EDP + 80% SS) respectively, proving that the increase in acidity was proportional to the amount of EDP. It was already expected due to the high amount of substrate available for fermentation with consequent organic acids and alcohols generation. Despite the reduction, the pH values were still considered within the optimal range expected for hydrogen generation between 4.0 – 6.5 (STAVROPOULOS et al., 2016).

Figure V.2 illustrates the relative production of soluble metabolites generated during the 107h of operation of the acidogenic reactors. It was observed lactic acid as the predominant metabolite in all assays at the operation start. The relative

abundances of lactic acid were 35%, 53%, and 54% (94, 173, 250 mg L^{-1}) for assays 1, 2, and 3, respectively. In addition, acetic acid (8-22%) (36.4-60.3 mg L^{-1}), ethanol (12-17%) (32.0-80.5 mg L^{-1}), butyric acid (13%) (34.2 -61.4 mg L^{-1}), caproic acid (5-6%) (15.4-24.5 mg L^{-1}), and isobutyric acid (2-4%) (9.5-10.2 mg L^{-1}) were also detected in all assays. The presence of metabolites at the start of operation could be due to the waste fermentation by indigenous microorganisms (NOBLECOURT et al., 2018). Therefore, the predominance of lactic acid was expected due to the abundant presence of lactic acid bacteria (LAB) in dairy products and dairy waste (ESKIN; SHAHIDI, 2013; GOMES et al., 2015; JIN; CAI; YANG, 2021; ROY; SARKAR, 2022).

During the exponential phase of H_2 production (11h of operation), the main soluble metabolite in all tests was acetic acid, with relative production of 42% for assays 1 and 2 and 38% for assay 3. At the same time, butyric acid was generated with percentages of 28%, 29%, and 32% for assays 1 to 3, respectively. With an operating time of 16h, acetic acid remained the main metabolite in assays 1 and 2, with relative production of 39% and 34%, followed by butyric acid with 28% and 29%, respectively. For the assay 3, the pathway was favored for butyric acid production (35%), followed by acetic acid (34%).

At the end of the operation (107h), butyric acid was the main soluble metabolite in all assays with relative production of 42%, 50%, and 55%, which corresponds to concentrations of (mg L^{-1}): 1695.1, 2384.8, and 2557.1, for assays 1, 2, and 3, respectively (**Table V.3**). Acetic acid was the second major final soluble metabolite with relative production of 32% (1268.7 mg L^{-1}), 26% (1258.8 mg L^{-1}), and 23% (1083.4 mg L^{-1}), for assays 1, 2, and 3, respectively. In addition, in all assays, ethanol (5-7%) and the following acids were also observed in smaller amounts: propionic (6-9%), isobutyric (2-5%), isovaleric (2-6%) and caproic (2-4%). Lactic acid was observed only at the end of assay 3 with a relative abundance of 3% (142.5 mg L^{-1}), demonstrating that it was consumed in all assays during the acidogenesis stage.

In general, the H_2 yield depends on the combination of fermentative metabolic pathways that occur in the bioreactor. The correlation between the hydrogen yield and the final metabolic products can be established based on stoichiometry, where the fermentation of glucose to acetic acid exhibits the highest theoretical yield of 4 mol of H_2 mol^{-1} of glucose (**Equation I**), while the conversion to butyric acid leads to a yield of 2 mol of H_2 mol^{-1} of glucose (**Equation II**). It is also possible that there is consumption of the H_2 produced when the metabolite is propionic acid (**Equation III**).

The simultaneous production of hydrogen is facilitated by the generation of acetic and butyric acids (ANTONOPOULOU et al., 2008).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (I)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (II)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (III)$$

Figure V.2 – Relative production of soluble metabolites generated during the operation of acidogenic reactors: Assay 1 (a), Assay 2 (b), and Assay 3 (c).

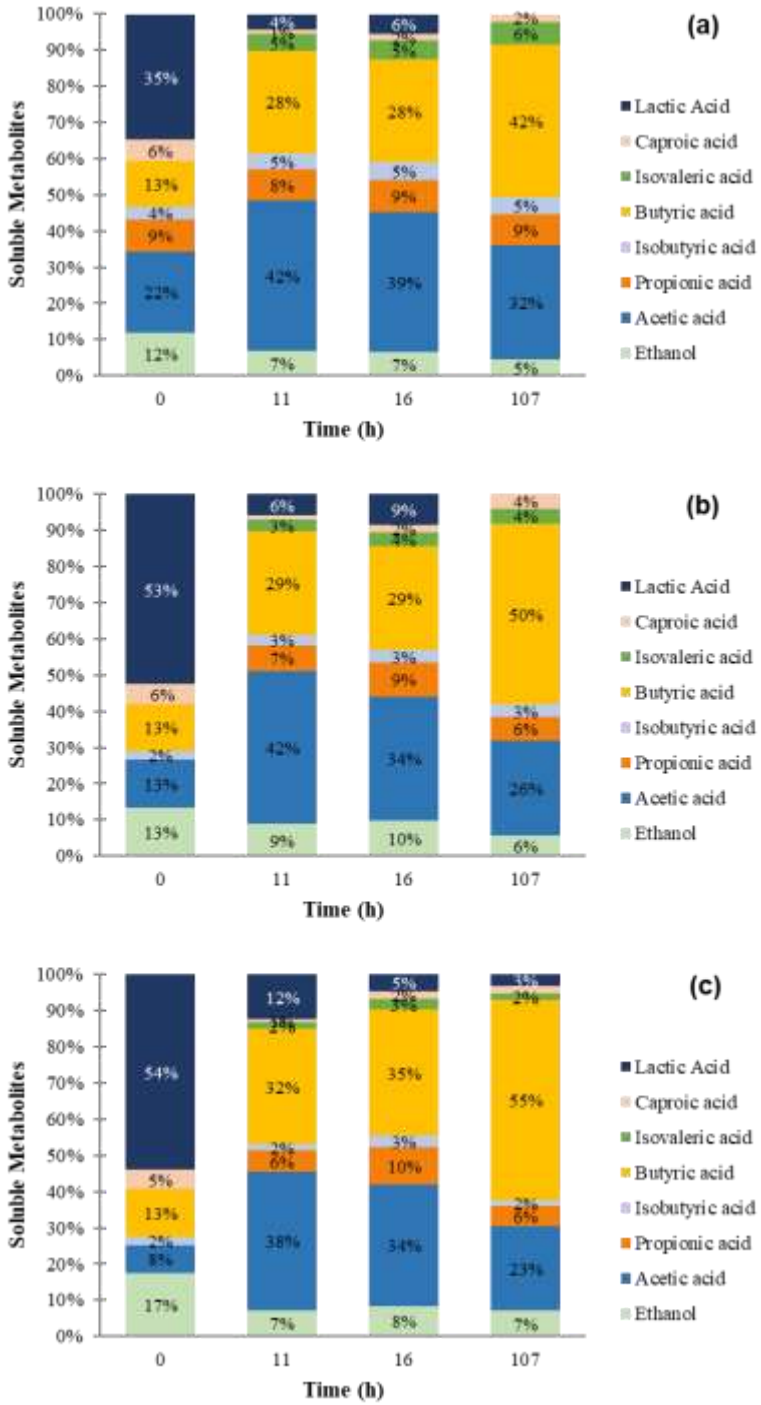


Table V.3 - The final soluble metabolites production in acidogenic reactors.

Soluble metabolites (mg L^{-1})	Assay 1 (10% EDP + 90% SS)	Assay 2 (15% EDP + 85% SS)	Assay 3 (20% EDP + 80% SS)
Ethanol	181.6 ± 18.8	270.4 ± 97.8	327.7 ± 0.3
Acetic acid	1268.7 ± 289.3	1258.8 ± 14.6	1083.4 ± 262.1
Propionic acid	342.7 ± 42.3	307.9 ± 78.0	257.3 ± 13.3
Isobutyric acid	185.9 ± 37.2	166.6 ± 8.1	74.3 ± 22.4
Butyric acid	1695.1 ± 196.6	2384.8 ± 33.7	2557.1 ± 279.0
Isovaleric acid	246.8 ± 62.1	204.8 ± 8.6	87.0 ± 11.9
Caproic acid	88.2 ± 7.3	190.1 ± 11.7	99.8 ± 3.7
Lactic acid	N.D.	N.D.	142.5 ± 13.0
Total	4009.0 ± 653.5	4783.4 ± 329.8	4629.6 ± 592.8

N.D – not detected.

In the present study, the presence of acetate and butyrate pathways, mainly in the H_2 exponential phase, may have contributed to the high H_2 yield. The highest yield was seen for Assay 2 (15% EDP + 85% SS) with 1.9 mol mol^{-1} carbohydrate removed, followed by Assay 1 (10% EDP + 90% SS) and 3 (20% EDP + 80% SS) with yields of 1.7 and 1.5 mol mol^{-1} carbohydrate removed.

Venetsaneas et al. (2009) evaluated the performance of a CSTR (*Continuously Stirred Tank Reactor*) for the production of H_2 and CH_4 in two-stages, using cheese whey as substrate at mesophilic temperature (35 ºC). The acidogenic stage was operated at an HRT of 24 h and organic load rate (OLR) of 30g COD d^{-1} at uncontrolled pH 5.2. The main metabolites produced were butyric, acetic, lactic acids, and ethanol. The yield of H_2 production was 0.78 ± 0.05 mol H_2 mol^{-1} glucose consumed with 86% of carbohydrate removal. Both yield and removal values reported by the authors were lower than the ones found in the present study.

Stavropoulos et al. (2016) investigated the effect of different controlled pH values (4.0; 4.5; 4.7; 5.0; 5.3; 5.7) to determine the best value for hydrogen production from a mix of expired dairy products (93% milk – 5% yogurt – 2% cheese, w/w) using dark fermentation. A CSTR-type reactor was operated under mesophilic conditions with a hydraulic retention time (HRT) of 6 days using mesophilic mixed culture. The test with pH 5 showed the highest H_2 yield (0.84 mol mol^{-1} carbohydrate removed), where 93.1% of the carbohydrates were removed. The yield value obtained by the

authors corresponds between 44.0 - 55.8% less than that found in this study, despite the removal of carbohydrates corresponding to 0.8 - 6.3% more. Butyric acid was the predominant metabolite produced, followed by acetic and lactic acid.

Both previously mentioned authors reported lactic acid as an intermediate metabolite, suggesting the coexistence of H_2-producing bacteria (HPB) (e.g., *Clostridium* and *Enterobacter*) with lactic acid bacteria (LAB) (e.g., *Lactobacillus, Lactococcus, Streptococcus*). LAB are naturally present in numerous types of waste, especially dairy products. They are characterized as homofermentative when lactate is the only product of glucose fermentation and heterofermentative when the products include lactate, acetate, CO_2, and ethanol (SIKORA et al., 2013).

Stavropoulos et al. (2016) considered that the decrease in H_2 productivity was caused by substrate competition between HPB and LAB. This hypothesis is frequently reported in several studies of anaerobic digestion of dairy waste (CASTELLÓ et al., 2018; GOMES et al., 2015; PALOMO-BRIONES et al., 2017). However, the same did not occur in the present study since lactic acid was produced in much lower amounts than other metabolites, in addition to being completely consumed in assays 1 and 2 and remaining with low relative production at the end of Assay 3.

3.2 Second stage - Methanogenic reactors

All methanogenic reactors were conducted for 872 hours, approximately 36 days (**Table V.4**). Among all the assays, the highest removal efficiency for all parameters evaluated was observed in Assay 5 (15% EDP + 85% SS acidified), which achieved removal percentages of 94.7% for COD, 49.9% for TS, and 81.6% for VS. These values correspond to 2.9%, 12.6%, and 6.5% above the values obtained for Assay 4 (10% EDP + 90% SS acidified) for COD, TS, and VS, respectively.

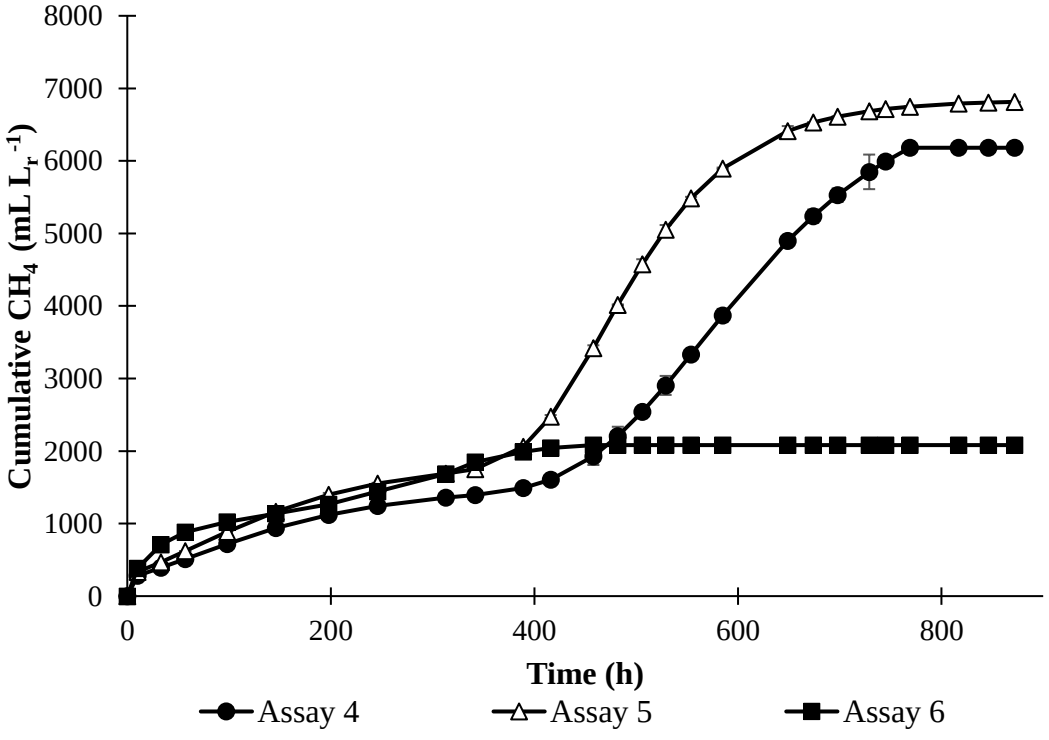
Assay 6 (20% EDP + 80% SS acidified) presented low COD, TS, and VS removal (16.4%, 15.5%, and 40.7%, respectively). These results indicated that the soluble metabolites formed during the acidogenesis stage were not consumed by microorganisms, which will be discussed in more detail. Furthermore, it was observed that the assay 6 was the only one with an acid pH (6.6) at the end of the methanogenic stage.

Table V.4 - Performance of methanogenic reactors.

Parameters	Assay 4 (10%EDP + 90%SS acidified)	Assay 5 (15%EDP + 85%SS acidified)	Assay 6 (20%EDP + 80%SS acidified)
pH (initial – final)	7.5 – 7.2	7.5 – 7.3	7.5 – 6.6
Operation time (h)	872	872	872
Removal (%)			
COD	91.8	94.7	16.4
TS	37.3	49.9	15.5
VS	75.1	81.6	40.7

Figure V.3 presents the cumulative methane production of the methanogenic reactors (Appendix D). The total methane produced was (mL L_r^{-1}) 6180.9, 6813.4, and 2120.7 for assays 4 (10% EDP + 90% SS acidified), 5 (15% EDP + 85% SS acidified), and 6 (20% EDP + 80% SS acidified). In all assays, the methane production started immediately ($\lambda_1 = 0$) (**Table V.5**). However, assays 4 and 5 presented curves with diauxic behavior (two exponential phases), with the second exponential phase occurring within 447.3 hours and 392.7 hours, respectively.

Figure V.3 - Cumulative methane production.



The diauxic behavior curves are a characteristic pattern observed in waste containing components with different biodegradability. In the case of EDP (specific example), carbohydrates and proteins are converted into short-chain fatty acids at a faster pace compared to lipids (KIM; KIM, 2017). In anaerobic digestion, the acidogenic bacteria initiate the hydrolysis of lipids, breaking them down into glycerol and long-chain fatty acids (LCFAs) (SAGE; DAUFIN; GESAN-GUIZIOU, 2008). Glycerol is further converted into acetate through acidogenesis, while the LCFAs are transformed into hydrogen, acetate, and/or propionate via the β-oxidation pathway (WARE; POWER, 2017). However, β-oxidation is known to be a limiting step due to its slow degradation rate, resulting in increases in the lag phase, and in some cases causing acid accumulation in reactors (CIRNE et al., 2007; HASSAN; NELSON, 2012).

Sage et al. (2008) investigated the anaerobic degradation processes of milk fat. They examined the kinetics of fat degradation compared to other milk components, such as lactose and proteins. Various substrates, including thermalized cream, anhydrous milk fat, and commercial UHT skim milk, were analyzed in their original state and after alkaline hydrolysis. The results revealed a lag phase lasting several days, during which the maximal degradation rate of milk fat was found to be 2 to 5 times lower than that of the other milk components. Furthermore, they observed that the lag phase preceding biogas production is primarily associated with unsaturated fatty acids, particularly oleic acid.

In contrast to the previous assays, 6 (20% EDP + 80% SS acidified) exhibited different behavior, with methane production ceasing after 458 hours of operation. Despite maintaining the reactors at a constant temperature of 37°C for up to 872 hours, there was no evidence of microorganisms' adaptation or resumption of production, confirming the system inhibition.

The soluble metabolites analysis was carried out at the end of the operation of the reactors. The assay 6 (20% EDP + 80% SS acidified) was the only one which demonstrated an accumulation of total acids with 11427.9 mg L^{-1}, where 74% of this value corresponds to acetic acid (8455.6 mg L^{-1}). In addition, the following acids were also detected (mg L^{-1}): propionic (1054.4), isovaleric (776.7), isobutyric (643.0), butyric (434.4) and caproic (63.8) (**Table V.6**). These results confirmed that the inhibition of anaerobic digestion in Assay 6 was caused by the quantities of total acids not consumed by acetoclastic microorganisms.

Table V.5 - Methane production determined by modified Gompertz equation.

Parameters	Assay 4 (10%EDP + 90%SS acidified)	Assay 5 (15%EDP + 85%SS acidified)	Assay 6 (20%EDP + 80%SS acidified)
P_1 (mL L_r^{-1})	1495.2	1857.4	2120.7
P_2 (mL L_r^{-1})	4685.7	4978.6	-
R_1 (mL L_r^{-1} d^{-1})	5.1	6.5	5.8
R_2 (mL L_r^{-1} d^{-1})	17.3	24.6	-
λ_1 (hours)	0	0	0
λ_2 (hours)	447.3	392.7	-
R^2	0.99	0.99	0.98

P_1, P_2: the production potential of CH_4 for the 1st and 2nd exponential phases, respectively; R_{M1}, R_{M2}: the maximum production rate of CH_4 for the 1st and 2nd exponential phases, respectively; λ_1, λ_2: time to start the CH_4 production 1st and 2nd exponential phases, respectively.

Table V.6 - The final soluble metabolites production in methanogenic reactors.

Soluble metabolites (mg L^{-1})	Assay 4 (10%EDP + 90%SS acidified)	Assay 5 (15%EDP + 85%SS acidified)	Assay 6 (20%EDP + 80%SS acidified)
Ethanol	N.D.	N.D.	N.D.
Acetic acid	172.8 ± 62.7	58.8 ± 10.5	8455.6 ± 359.0
Propionic acid	N.D.	21.8 ± 8.0	1054.4 ± 40.4
Isobutyric acid	16.7 ± 4.9	N.D	643.0 ± 22.4
Butyric acid	15.2 ± 4.9	N.D	434.4 ± 79.0
Isovaleric acid	34.9 ± 1.0	N.D.	776.7 ± 11.9
Caproic acid	N.D.	N.D.	63.8 ± 3.7
Lactic acid	N.D.	N.D.	N.D.
Total	239.6 ± 73.5	80.6 ± 18.5	11427.9 ± 516.4

N.D – not detected

Karthikeyan and Visvanathan (2013) reported that acetic acid accumulation above 2000 mg L^{-1} and total VFAs above 8000 mg L^{-1} significantly affect the methanogenesis phase. DASA et al. (2016) mentioned that the build-up of VFAs above 4 g L^{-1} in the anaerobic systems digester results in an imbalance of the biological

process. The VFAs and acetic acid values observed in 6 (20%EDP + 80%SS acidified) are well above these mentioned values.

Probably, the combination of acetate production from the conversion of first-stage metabolites, combined with acetate/propionate production from the β-oxidation of LCFAs, caused an imbalance between the steps of anaerobic digestion (acidogenesis, acetogenesis, and methanogenesis). This imbalance may have resulted in the saturation of the microorganisms responsible for converting acetic acid into methane (acetoclastic archaea) in 6 (20%EDP + 80%SS acidified).

The accumulation of acetic acid is related to a kinetic limitation between the microorganisms of the microbial consortium. In particular, acetoclastic archaea exhibit slower growth than acidogenic, acetogenic, and hydrogenotrophic microorganisms. As a result, they are prone to becoming easily saturated from a kinetic perspective (AQUINO; CHERNICHARO, 2005).

In addition, the increasing amount of EDP (from 10% to 20%) and consequent increase in lipid content may have caused a physical limitation in 6 (20%EDP + 80%SS acidified). This limitation can be caused by unsaturated fatty acids (oleic acid), mainly attributed to their absorption into the biomass. This absorption hinders the transfer of substrates and products, resulting in an inhibitory effect on acetate-consuming microorganisms (PEREIRA et al., 2005).

The opposite was observed for Assay 4 (10% EDP + 90% SS acidified) and Assay 5 (15% EDP + 85% SS acidified), which obtained an efficient soluble metabolite removal, corresponding to 94% and 98.3%, respectively when compared with the final concentrations of the acidogenic stage. These results corroborate with the high COD removal values previously discussed, confirming that the concentrations of 10 and 15% EDP were not inhibitory to the biological process. Furthermore, a high methane production yield was achieved for Assay 4 (10% EDP + 90% SS acidified) with 323.0 $mLCH_4\ g^{-1}VS_{add}$ (380.0 $mLCH_4\ g^{-1}\ COD_{rem}$), while the Assay 5 (15% EDP + 85% SS acidified) presented a methane yield of 225.1 $mLCH_4\ g^{-1}VS_{add}$ (259.2 $mLCH_4\ g^{-1}\ COD_{rem}$).

Kopsahelis et al. (2018) studied the co-digestion of End-of-Life Dairy Products (milk, yogurt, and white cheese) with agro-industrial wastes (pig manure, liquid cow manure, cheese whey, poultry wastes, and slaughterhouse wastes) from a two-stage system using CTSR reactors. The acidogenic-CSTR (active volume of 0.9 m^3) was fed with 100% of End-of-Life Dairy Products (EoL-DPs) at an HTR of 6 days, for 200 days

at controlled pH of 5.7. The Methanogenic-CTR (active volume of 1.8 m^3) was fed with a mixture of 19.5% of EoL-DPs (first-stage effluent) with 80.5% of agro-industrial wastes. The methanogenic reactor was operated at an HTR of 35 days, for 200 days at uncontrolled pH (7.93). Both stages were operated at 37 ºC under agitation (30-70 rpm). The acidogenic stage removed 72.7% of carbohydrates with a yield of 22.9 g H_2 $g^{-1}VS_{add}$. These results were lower than those obtained in acidogenic stage of the present study, which obtained carbohydrate removals between 86.8-92.3% and hydrogen yield of 32.8-40.1 99 g H_2 $g^{-1}VS_{add}$. In methanogenic stage the authors reported a COD removal of 62.2% with a yield of 390 mL CH_4 g^{-1} COD_{rem}. Compared to the methanogenic stage, Assays 4 and 5 of the present study obtained higher COD removal (91.8-94.7%), while only Assay 4 achieved a similar yield (380 mL CH_4 g^{-1} COD_{rem}).

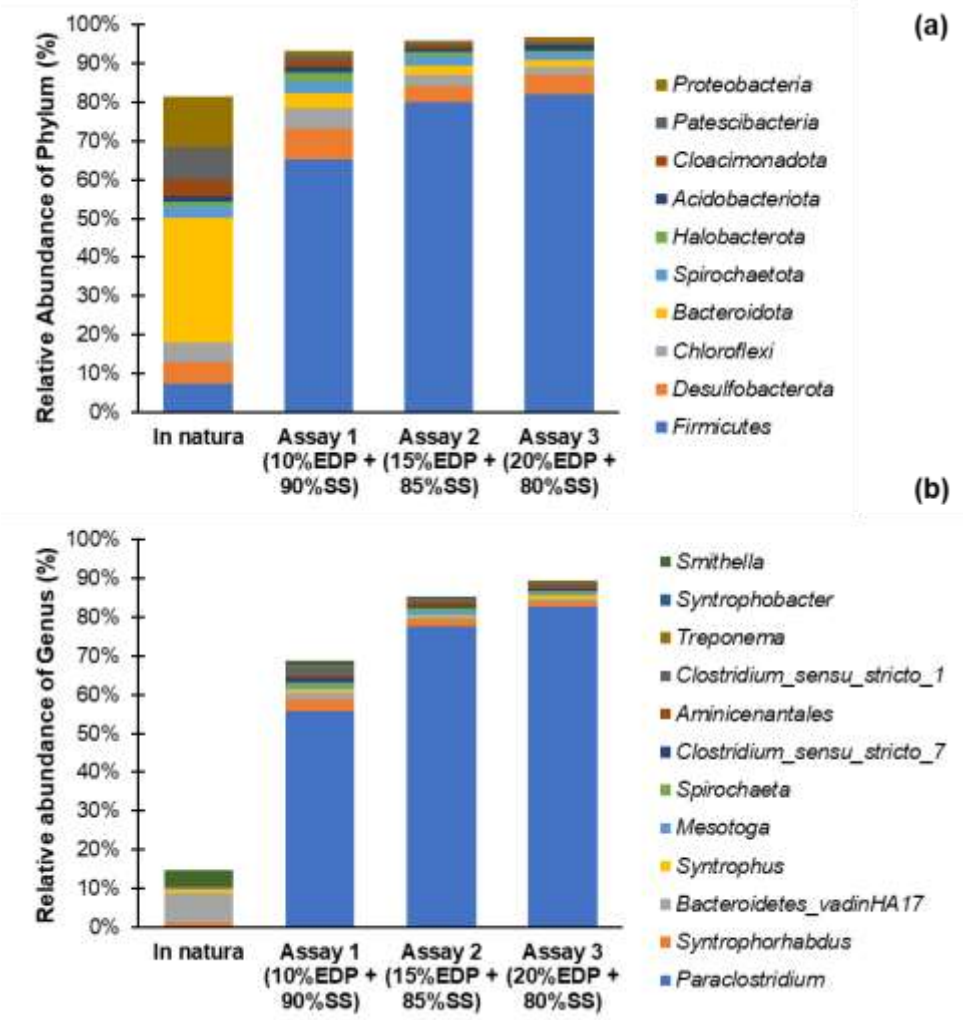
Sakarika et al. (2020) also studied the co-digestion of EoL-DPs with agro-industrial waste from a two-stage anaerobic digestion at mesophilic conditions (37 ºC). The acidogenic-CSTR (working volume of 0.75L) was fed with 100% EoL-DPs at controlled pH of 5.7. The Methanogenic-CTR (working volume of 3.3L) was fed with the acidogenic effluent (EoL-DPs) co-digested with agro-industrial wastes at uncontrolled pH (7.5 – 8.3). Different percentages of EoL-DPs effluents were tested (from 4.5% to 19.5% w/w). The acidogenic stage removed 82.5% of carbohydrates with a yield of 0.757 mol H_2 mol^{-1} carbohydrate removed. In methanogenic stage the authors maximum COD removal value (43.6%) was achieved for test with 4.5% EoL-DPs, while the best yield (318 mL CH_4 $g^{-1}VS_{add}$) was obtained for the test with 19.5% EoL-DPs. All results mentioned were below the values obtained in the present study.

3.3 Microbial diversity

3.3.1 Acidogenic Stage

Figure V.4 demonstrates the taxonomic profile of microbial communities for in natura inoculum and for assays 1 (10% EDP + 90% SS), 2 (15% EDP + 85% SS), and 3 (20% EDP + 80% SS) after 107 h of operation.

Figure V.4 - Relative abundance of microorganisms identified at the (a) phyla and (b) genera levels.



In terms of the relative abundance of phyla (**Figure V.4**a), the microbial community of the in natura inoculum include mainly Bacteroidota (32.3%), Proteobacteria (12.8%), Patescibacteria (8.8%), Firmicutes (7.4%), Desulfobacterota (5.6%), Chloroflexi (4.9%), and Cloacimonadota (4.2%). After 107 h of operation the Firmicutes phylum presented a predominant relative abundance in all assays with 65.3%, 80.0% and 82.1% for assay 1, 2, and 3, respectively.

The high abundance of the phylum Firmicutes may be associated with its capability to form endospores under extreme environmental conditions. This ability allowed these microorganisms to resist the heat pre-treatment applied to the in natura inoculum without damaging its fermentative capacity (LUO et al., 2022). In addition, this phylum is associated with the hydrolysis and fermentation of carbohydrate-rich substrates, such as EDP. Also, they have a significant role in producing the short and

long-chain fatty acids, lactic acid, alcohols, and H_2 produced during acidogenesis (SIKORA et al., 2013).

In genus level, *Paraclostridium* was identified with superior relative abundance of 55.7%, 77.8% and 82.7% in assays 1 (10% EDP + 90% SS), 2 (15% EDP + 85% SS), and 3 (20% EDP + 80% SS), respectively. The proportional growth between *Paraclostridium* and EDP indicating that the increase in organic matter didn't affect the fermentative activity of this genus. This genus includes gram-positive bacteria with ability to form endospores under unfavorable conditions. Therefore, it is often reported with high relative abundance in several studies that apply heat pre-treatment to the fermentative inoculum (DE CARVALHO; MAINTINGUER, 2022; LUO et al., 2022; YANG; YIN; WANG, 2019). This genus is often identified in acidogenic reactors fed with high carbohydrate content since it can metabolize it, producing H_2 as a metabolite (SILVA RABELO et al., 2023; YANG; WANG, 2019). Therefore, it contributes to the high carbohydrate removal efficiencies and high hydrogen yields observed in the present study.

The genus *Clostridium_sensu_stricto_1* was observed with relative abundance of 2.0%, 0.7%, 06% for assays 1, 2, and 3, respectively. Additionally, *Clostridium_sensu_stricto_7* was also identified with relative abundance of 1.3% and 0.8%, and 0.7% for assays for assays 1, 2, and 3, respectively. These genera can utilize many sugars as carbon and energy sources to produce acetate, butyrate, H_2 and CO_2 (LI et al., 2018; ZHAO et al., 2020). Despite presenting lower relative abundances than those of the *Paraclostrium* genus, these microorganisms possibly participated in the metabolization of lactose and glucose from EDP, contributing to the high H_2 yield and soluble metabolites observed in the acidogenic reactors.

Syntrophus, Smithella, and *Synthophobacter* have the capability to metabolize long-chain fatty acids (LCFA) generated from lipid hydrolysis, through the β-oxidation pathway. As a result, they produce acetate, propionate, and H_2 as metabolic byproducts (BECKER et al., 2023; FLORENTINO et al., 2020; SUN et al., 2022). These genera were identified with relative abundances of: *Syntrophus* 1.0%, 0.8%, 0.5%, and 1.0%; *Smithella* 3.8%, 0.6%, 0.2%, and 0.1%*;* and *Synthophobacter* 0.2%, 0.7%, 0.2%, and 0.1% for in natura inoculum and assays 1, 2 and 3, respectively. These relative abundance results demonstrated that there was a low growth of these microorganisms in the acidogenic reactors (first stage).

3.3.2. Methanogenic Stage

The **Figure V.5** demonstrates the taxonomic profile of microbial communities for in natura inoculum and for assays 4 (10% EDP + 90% SS acidified), 5 (15% EDP + 85% SS acidified), both assays achieved high methane production after 872 h of operation.

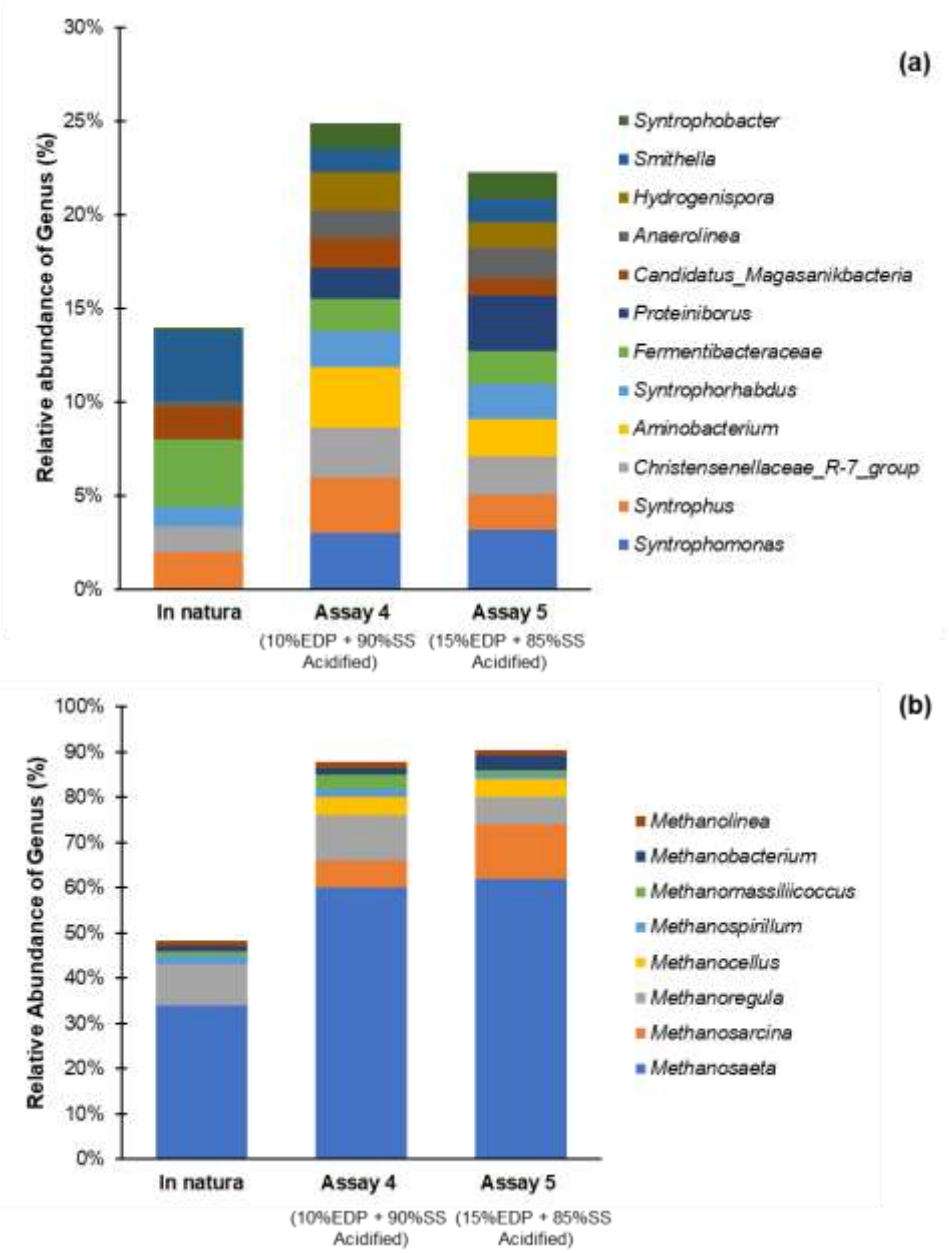
Syntrophomonas are capable of metabolizing LCFA (via the β-oxidation), producing acetate, propionate, and H_2 (SOUSA et al., 2007; ZIELS; BECK; STENSEL, 2017). This genus was identified with relative abundances of 3.0% and 3.2% for assays 4 and 5, respectively. It was not identified in the in natura inoculum, indicating that they probably originated from non-sterile EDP. They were favored in the reactor operational conditions, including, temperature, pH, and substrate availability (**Figure V.5**a).

Syntrophus, Smithella, and *Synthophobacter* also related with metabolization of LCFA as describe previously (Section 3.3.1) were identified with relative abundances of: *Syntrophus* 1.0%, 2.95%, and 1.89%; *Smithella* 3.8%, 1.3%, 1.3%*;* and *Synthophobacter* 0.2%, 1.3%, 1.4% for in natura inoculum and assays 4 and 5, respectively.

An increase in the relative abundance of these four genera was observed in methanogenic reactors (second stage) when compared to acidogenic reactors (first stage) (**Figure V.4**). This finding supports the observation of diauxic behavior during methane production in the second stage (**Figure V.3**). The slower degradation of LCFA (β-oxidation pathway) compared to other metabolites results in methane production occurring in two exponential phases.

Aminobacterium can metabolize EDP proteins, such as casein, albumin, and globulin producing acetic acid, H_2, CO_2, and traces of isovaleric and isobutyric acid (BAENA et al., 2000; BELLA; RAO, 2021). This genus was identified with relative abundances of 3.3% and 2.0% for assays 4 and 5, respectively. In the same way, *Proteiniboru*s can produce acetic acid, H_2, and CO_2 from protein metabolization (HAHNKE; LANGER; KLOCKE, 2018; SINGH et al., 2023). It was observed with relative abundances of 1.7% and 3.0% for assays 4 and 5, respectively; none of these genera was identified in the natura inoculum. Like *Syntrophomonas*, they probably came from unsterilized EDP.

Figure V.5 - Relative abundance of genera of methanogenic stage: (a) bacteria and *(b) archaea.*



In terms of methanogenesis, *Methanosaeta* is characterized as acetoclastic genus, as they generate methane by consuming acetate, which is recognized as the principal precursor for methane production. Acetate is the source of up to 70% of the CH_4 produced in reactors (AQUINO; CHERNICHARO, 2005; VÁZQUEZ-FERNÁNDEZ; SUÁREZ-OJEDA; CARRERA, 2022). It was identified with a relative abundance of 34.0%, 60.0%, and 62.0% for in natura inoculum and assays 4 and 5, respectively. *Methanosarcina* is also classified as an acetoclastic genus, and it was verified with the relative abundance of 0.1%, 6.0%, and 12.0% for in natura inoculum

and assays 4 and 5, respectively (**Figure V.5**b). Both genera had an increase when compared with in natura inoculum. It indicates that the high production of acetic acid produced in the first stage (Assays 1 and 2), associated with acetic acid production from the LCFAs metabolization, favored the acetoclastic pathway in the methanogenic reactors.

In addition, the genera *Methanoregula, Methanocellus, Methanospirillum, Methanomassiliicoccus, Methanobacterium, Methanolinea* were also verified for assays 4 and 5. These genera reduce CO_2 with H_2 to produce CH_4 (hydrogenotrophic pathway) (HASSAN; NELSON, 2012; LIU; WHITMAN, 2008). The coexistence of acetoclastic and hydrogenotrophic archaea suggests that the co-digestion of EDP with SS favored a synergy between these genera, contributing to the high COD removal and positive methane yield achieved in both assays.

4 CONCLUSIONS

The anaerobic co-digestion of EDP with synthetic sewage in two-stages was viable for biogas production (H_2 and CH_4). In acidogenic reactors (first stage), high carbohydrate removal and H_2 production were achieved for Assay 3 (20% EDP + 80% SS). It confirmed that the increase in organic matter was not an inhibitory factor for acidogenic microorganisms. *Paraclostridium* was the genus with the highest relative abundance (55.7%-82.7%) in all assays of the first stage.

In the second stage (methanogenic reactors), the Assay 5 (15% EDP + 85% SS acidified) presented high COD removal (94.7%) and methane production (6813.4 mL L_r^{-1}). *Methanosaeta* was the genus identified with the highest relative abundance in the second stage (60-62%).

Assay 6 (20% EDP + 80% SS acidified) was inhibited due to the accumulation of volatile fatty acids (11427.9 mg L^{-1}), mainly acetic acid (8455.6 mg L^{-1}). These results demonstrate that excess organic matter led to an imbalance between acidogenic and methanogenic microorganisms, resulting in kinetic inhibition of acetoclastic archaea.

The results of this study confirmed the feasibility of EDP utilization as a substrate in the two-stages anaerobic digestion. However, more strategies should be investigated to increase the concentration above 20%, to surpass the VFA accumulation in methanogenic reactors.

ACKNOWLEDGMENTS

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GENERAL CONCLUSIONS

This study proposed valorization and recovery strategies for expired dairy products (EDP) from anaerobic digestion. The main information obtained from the experimental studies of this thesis (Chapters II-V) is summarized below:

i. A new autochthonous mixed culture was obtained from expired dairy products. This autochthonous mixed culture was able to consume and grow from different substrates such as glucose, sucrose, lactose, maltose, fructose, xylose, arabinose, rhamnose, EDP, and wastewater from banana processing (BPW). In addition, the autochthonous mixed culture favored the pathway for producing lactic acid (LA). Good results of LA production yields were observed from using sugars as the only substrate, but mainly from waste such as BPW and EDP. The new autochthonous mixed culture proved to be a promising alternative in bioprocesses contributing to generating value-added products from different carbon sources.

ii. EDP co-digested with synthetic sewage (SS) was an efficient substrate in single-stage anaerobic digestion. Assays with a concentration of up to 20 g COD L^{-1} (mixture of 10% EDP + 90% SS) effectively removed the organic matter from substrates and presented high yields in methane production. Nevertheless, concentrations above 20 g COD L^{-1} caused an accumulation of volatile fatty acids in the reactors with inhibition of methanogenic microorganisms and consequent failure of the biological process. Therefore, the co-digestion of EDP + SS is a viable alternative for recovering these residues in biological processes. However, further studies need to be conducted so that a higher concentration of EDP can be used in the anaerobic digestion.

iii. Co-digestion of EDP with SS was effective in two-stage anaerobic digestion. In the first stage, high values of carbohydrate removal were obtained with good yields of hydrogen production for all evaluated concentrations (20 – 40 g COD L^{-1}). In the second stage, efficient organic matter removals with high methane production were observed only for tests up to 30 g COD L^{-1}. At concentrations above 30 g COD L^{-1}, there was the accumulation of volatile fatty acids, with high concentrations of acetic acid. The accumulation of acids caused kinetic

limitation of the acetoclastic microorganisms and the consequent failure in the biological process.

Therefore, the strategies tested in this study bring new prospects for EDP valorization from their application in biological processes, contributing to mitigating the environmental impacts caused by their improper disposal. In addition, EDP utilization can contribute to its valorization by providing bioproducts and biofuels from renewable sources.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. Apply the evaluated parameters in a continuous process and scale-up reactors;
2. Evaluate the manure as a co-substrate for the anaerobic digestion of expired dairy products. The manure has a natural buffer capacity, which can contribute to avoiding the reactors' rapid acidification;
3. Evaluate pre-treatment using enzymatic hydrolysis to minimize the lipid's adverse effects (lag phase increasing and accumulation of long-chain fatty acids) in anaerobic digestion process.

APPENDIX A

CHAPTER II

Autochthonous Mixed Culture from Expired Dairy Products Applied to Bioproducts Production

Table S.1- Temporal variation of microbial growth, glucose removal, LA production of autochthonous mixed culture in APT culture medium

Time (hours)	**Optical Density** (600 nm)	**Glucose** ($g\ L^{-1}$)	**Lactic Acid** ($g\ L^{-1}$)
0	0.44±0.04	9.73±0.04	0±0
3	0.52±0.04	9.22±0.13	0.5±0.01
6	0.86±0.07	4.71±0.01	2.5±0.01
9	1.36±0.05	0.92±0.01	5.20±0.01
12	1.61±0.05	0.82±0.05	7.93±0.02
26	1.61±0.08	0.80±0.05	8.20±0.01
33	1.61±0.07	0.71±0.02	8.30±0.04

APPENDIX B

CHAPTER III

Evaluation of Biomethane Production from Anaerobic Co-digestion of Expired Dairy Products with Synthetic Sewage

Table S.2- Cumulative methane production.

Time (days)	Assay 1 (NmL CH_4 L_r^{-1})	Assay 2 (NmL CH_4 L_r^{-1})	Assay 3 (NmL CH_4 L_r^{-1})	Assay 4 (NmL CH_4 L_r^{-1})	Assay 5 (NmL CH_4 L_r^{-1})
0	0±0	0±0	0±0	0±0	0±0
1	0±0	518.0±31.5	589.2±10.2	388.4±0.2	568.2±3.0
2	43.8±8.1	738.0±27.5	788.4±1.3	493.0±9.7	814.6±3.5
3	103.3±2.2	1051.5±14.9	1032.5±1.5	586.3±6.7	996.9±10.6
4	187.3±5.1	1128.5±3.4	1062.2±2.9	702.5±15.3	1137.4±26.9
5	282.8±6.1	1301.9±7.9	1148.7±21.0	823.3±23.0	1137.4±0
6	376.4±1.5	1382.6±1.4	1322.1±22.8	982.4±9.6	1137.4±0
7	458.8±2.2	1507.2±4.5	1430.4±16.8	1161.1±35.9	1137.4±0
8	526.3±6.6	1729.1±12.8	1505.7±16.8	1316.1±46.7	1137.4±0
9	578.9±24.2	2063.4±61.7	1664.9±53.0	1501.3±55.5	1137.4±0
10	618.4±15.3	2453.5±16.8	1792.7±62.9	1764.4±93.4	1137.4±0
11	647.3±14.9	2637.3±6.1	1943.0±67.9	2035.3±105.6	1137.4±0
12	668.1±23.3	2835.8±3.6	2228.2±90.8	2449.0±104.9	1137.4±0
13	683.0±27.5	3045.9±2.2	2476.0±67.6	3303.4±212.4	1137.4±0
14	692.3±15.2	3175.3±14.5	2825.0±48.2	4349.5±100.7	1137.4±0
15	701.0±12.8	3198.9±6.6	3368.0±15.9	5162.6±151.0	1137.4±0
17	708.6±25.3	3233.9±7.5	4046.2±66.6	5566.1±390.8	1137.4±0
18	712.0±16.8	3290.3±9.4	4477.5±130.2	5909.9±317.6	1137.4±0
19	713.8±7.5		4731.1±111.3	6084.2±58.2	1137.4±0
20	715.0±9.4		4901.5±59.4	6212.1±87.3	1137.4±0
21	715.8±0.1		4940.5±12.0	6280.0±39.9	1137.4±0
22	716.4±0		4962.7±15.7	6280.0±0	1137.4±0
23	716.8±0		5005.0±0.3	6280.0±0	1137.4±0
24	717.0±0		5005.0±0	6280.0±0	1137.4±0

APPENDIX C

CHAPTER IV

Reuse of Expired Dairy Products and Synthetic Sewage in the Production of Biogas by Anaerobic Digestion

Table S.3 - Cumulative methane production.

Assay I (Dairy waste I)		**Assay II (Dairy waste II)**	
Time (d)	**Cumulative CH_4 (mL)**	**Time (d)**	**Cumulative CH_4 (mL)**
0	0±0	0	0.0±0
1	304.4±8.6	1	194.2±0.1
2	500.2±6.5	1	246.5±5.4
3	616.8±2.1	2	293.1±3.8
4	713.6±14.5	3	351.2±8.6
5	788.1±0	4	411.7±12.9
6	855.6±22.6	5	491.2±5.4
7	910.7±11.3	6	580.5±20.2
8	935.6±1.4	7	658.0±26.3
9	963.2±4.2	8	750.7±31.2
10	987.2±12.7	9	882.2±52.6
11	1004.9±2.8	10	1017.6±59.4
12	1051.2±0	11	1224.5±59.0
13	1073.4±1.4	13	1651.7±119.5
14	1166.7±9.9	15	2174.7±56.6
15	1268.9±7.1	16	2581.3±85.0
16	1399.6±5.7	17	2783.1±219.9
17	1530.2±19.8	19	2955.0±178.7
18	1690.2±17.0	20	3042.1±32.8
19	1851.1±7.1	21	3106.1±49.1
20	1986.2±50.9	22	3140.0±22.5
21	2121.2±56.6	23	3140.0±0
22	2300.8±22.6	24	3140.0±0
23	2387.0±33.9	25	3140.0±0
24	2435.0±8.5		
25	2468.3±3.5		
27	2547.4±21.2		
28	2657.6±2.8		
29	2699.4±4.2		
31	2722.5±0		

APPENDIX D

CHAPTER V

Application of Two-stage Anaerobic Systems for Recovery of Expired Dairy Products and Biogas Production

Table S.4 - Cumulative hydrogen production.

Time (h)	Assay 1 (NmL H_2 L_r^{-1})	Assay 2 (NmL H_2 L_r^{-1})	Assay 3 (NmL H_2 L_r^{-1})
0	0±0	0±0	0±0
9	86.2±3.5	118.2±3.5	108.1±13.4
11	226.1±10.6	331.8±3.5	413.2±3.5
13	374.6±14.1	568.6±7.1	768.2±3.5
16	528.8±3.5	820.1±1.4	1107.2±11.3
19	602.6±4.2	941.4±8.5	1242.1±2.1
35	652.3±3.5	1022.9±3.5	1309.2±3.5
43	652.6±0	1023.3±0.7	1309.3±8.5
60	652.6±0	1023.4±1.4	1309.3±0
107	652.6±0	1023.4±0	1309.3±0

Table S.5 - Cumulative methane production.

Time (h)	Assay 4 (NmL CH_4 L_r^{-1})	Assay 5 (NmL CH_4 L_r^{-1})	Assay 6 (NmL CH_4 L_r^{-1})
0	0±0	0±0	0±0
10	283.1±1.4	332.5±9.9	409.2±4.2
33	390.6±5.7	467.0±5.7	529.3±12.7
57	511.4±0.7	619.9±1.4	663.4±15.6
98	719.5±17.0	884.9±2.8	899.1±35.4
146	937.2±11.3	1162.7±15.6	1161.9±5.7
198	1121.7±4.2	1397.0±25.5	1408.0±42.4
246	1244.±5.7	1551.4±9.9	1591.4±39.6
313	1356.7±14.1	1691.2±9.9	1780.4±15.6
342	1395.4±7.1	1751.9±7.1	1841.5±53.7
389	1492.4±26.9	2060.1±0.7	1919.4±22.6
416	1605.5±23.0	2474.1±22.6	1954.4±8.5
458	1927.7±120.2	3418.7±38.9	1997.5±7.1
482	2203.4±131.5	4014.4±7.1	2017.0±0
506	2539.3±67.2	4576.8±67.2	2033.5±0
529	2904.6±130.8	5052.0±63.6	2046.9±0
554	3330.7±35.4	5483.0±21.2	2059.2±0
585	3870.3±89.1	5895.9±11.3	2071.7±0
649	4896.6±36.8	6412.6±67.2	2090.0±0
674	5237.3±91.9	6529.±0.1	2095.2±0

698	5527.3±91.9	6611.3±1.4	2099.3±0
729	5848.4±238.3	6686.3±0.3	2103.7±0
745	5991.8±10.6	6714.7±1.9	2105.6±0
769	6180.8±7.1	6747.6±0.2	2108.0±0
817	6180.8±0	6789.1±2.3	2111.8±0
846	6180.8±0	6804.1±0	2113.5±0
872	6180.8±0	6813.4±0	2114.7±0