Pre-acidification of a Synthetic Dairy Wastewater in Continuously Stirred Tank Reactors

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Preface

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Summary

Two-stage anaerobic digestion is considered as a sustainable and efficient way to deal with dairy effluent, and is commonly applied. The pre-acidification happening during the first stage of this process could be used to buffer the complex conditions in the initial dairy effluent, as well as produce intermediary product such as volatile fatty acids (VFA), and it is essential to be controlled. Various types of reactors have been used studying this process, among which, continuously stirred tank reactor (CSTR) is common with its special benefits. During the pre-acidification process, the adjustment of hydraulic retention time (HRT) and pH is crucial to affect the performance of reactors. In order to compare the performance under different operation conditions, CSTRs were used to treat a synthetic dairy effluent in this work. This experiment was divided into two periods. One is under the pH of 4.9, another is under the pH of 4.0. The first condition was operated for 55 days. Then the pH in both reactors was adjusted at 4.0 for another 22 days. The volatile fatty acids (VFA) as the major fermentation product was analyzed. The results shows that at the pH of 4.9, the acidification degree in the CSTR with the HRT of 18h reached 80%, while the one with lower HRT could only reach 18%. Besides, results also indicate that HRT also has impact on the distribution of acids. As for the influence of pH, the results show that after the pH was adjusted down to 4.0, the total amount of VFA production decreased significantly especially for the one with the HRT of 18h (the acidification degree dropped down from 80% to 15%). Moreover, the decreased pH had a distinct impact on the VFA distribution. Before reducing the pH butyric acid was the most generated acid, followed by propionic acid and acetic acid. After the pH dropped, the mostly produced VFA was acetic acid, and at the same time, the proportion of butyric acid dropped significantly. The fermentation product consists not only of VFA which was mainly analyzed in this work, but also of other compounds such as formic acid and lactic acid. However, other fermentation compounds were not specifically analyzed in this work.

Key words: pre-acidification process; hydraulic retention time; soluble chemical oxygen demand transformation into fermentation product; volatile fatty acids and their distribution.

List of abbreviations

ABR	Anaerobic bioreactor
AD	Anaerobic digestion
AF	Anaerobic filter
AMBBR	Anaerobic moving bed biofilm reactor
AnMBR	Anaerobic membrane bioreactor
BOD	Biological oxygen demand
Ca	Calcium
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
DFAF	Down-flow anaerobic filter
EAOP	Electrochemical advanced oxidation processes
GC	Gas chromatographic
HRT	Hydraulic retention time
Mg	Magnesium
N	Nitrogen
NH_4-N	N in ammonium
OLR	Organic loading rate
P	Phosphorous
PO_4-P	P in phosphate
SBR	Sequential batch reactor
sCOD	Soluble chemical oxygen demand
sN	Soluble nitrogen
SO_4	Sulphate
STR	Stirred tank reactor
tCOD	Total chemical oxygen demand
TKN	Total Kjeldahl nitrogen
TN	Total nitrogen
TP	Total phosphorous
TS	Total solids
TSS	Total suspended solids
UASB	Upflow anaerobic sludge blanket reactor
UFAF	Up-flow anaerobic filter
UFFB	Up-flow anaerobic fluidized bed reactor
UHT	Ultra-high temperature
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
WW	Wastewater

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1 Introduction

1.1 Background

The demand of dairy product grows rapidly due to the increasing population and living standards, and the dairy industries are accompanied by large amounts of dairy effluent. Generally, these effluents are known as high-strength wastewater with significant chemical oxygen demand (COD) and biological oxygen demand (BOD), and various types of organic compounds, which mainly consist of lactose, protein and lipids (Hassan and Nelson 2012). Such effluents could severely threaten the environment without proper treatment. Various biological, electrochemical and physicochemical methods have been developed and applied to deal with dairy effluents. However, the anaerobic digestion process as one of the biological methods, has been more attractive than other technologies in recent decades (Nualsri, *et al.*, 2016). This is due to its double benefits of reducing sludge production and generating energy (i.e. utilizable methane), which also shows great potential of nutrient recovery (Demirel *et al.,* 2005).

There are complex reactions and production processes involved in the anaerobic digestion process. Each of those processes involves specific bioreactor arrangements and an optimum set point of process parameters (Khan *et al.,* 2016). Therefore, a two-stage process which consists of acidification and methanogenesis has been proposed to enhance energy recovery and COD removal. Fermentation product generated during acidification stage is required for the formation of methane in the follow-up methanogenesis stage.

Moreover, the acidification process could be further divided in to pre-acidification (including hydrolysis and acidogenesis) and acetogenesis. During the pre-acidification process, complex and undissolved polymers such as carbohydrates and fats are preliminarily hydrolyzed into smaller molecule compounds, then the acidogenic bacteria converts those compounds into intermediary products such as volatile fatty acids (VFA). However, some of the produced VFAs from pre-acidification cannot be converted directly by the methanogens, thus the acetogenesis is needed in the next stage.

There is generally a buffer tank installed before the anaerobic reactors, in order to adjust the hydraulic loading rate as well as the organic loading rate (OLR) (Sachs *et al.,* 2003). The buffer tanks are generally operated using continuously stirred tank reactors (CSTR), and some level of acidification may occur in these buffer tanks. Therefore, CSTR could not only be operated to buffer the complex conditions in the initial dairy effluent, but also be used to produce VFA.

The hydraulic retention time (HRT) and pH are regard as critical factors that might impact the pre-acidification process, with regard to the fermentation product (such as VFA), therefore influence the whole AD process. (Nualsri *et al.,* 2016). Because they could determine the types of microbial communities in the reactors, since different metabolic and microbial communities might be generated and accumulated with varying HRTs and pH values (Palomo-Briones *et al.,* 2017). For instance, according to Chartrain, Bhatnagar and Zeikus (1987), the lactose-

hydrolyzing population was not affected by HRT ranging from 25 to 100 h, while the acetate-degrading organisms decreased to insignificant levels at HRT below 12 h. The substrate absorption efficiency is therefore affected significantly by the retention time. Hence, it is possible to evaluate acidification reactors by analyzing the performance with different HRTs. Furthermore, HRTs also influence the effective volume of reactors, energy input and operational cost. Generally, longer hydraulic retention time leads to higher cost, but it could also result in the decrease of COD and substratum (Hassan *et al.,* 2012). Nevertheless, too short hydraulic retention time might lead to failure in reactors, since they might not function well within such limited time.

1.2 Problem and research questions

This thesis focuses on pre-acidification of a synthetic dairy effluent in CSTRs, which is as part of a project carried out at company AnoxKaldnes, Veolia, Lund. This project focuses on the analysis of pre-acidification process, especially at varying HRTs and pH values, in comparison with the conventional HRT (18h) and pH (4.9). Generally low HRT associates with financial benefits and low pH in this case could reduce the cost for alkalinity dosing, but risk in dysfunction in reactors. There are three research questions to be answered in order to know the possibility of lowering the HRT and the pH during acidification process.

1. How does hydraulic retention time influence the pre-acidification performance of CSTRs when treating this synthetic dairy effluent?

2. What impacts could occur to the pre-acidification performance with these two HRT (18h and 6h)?

3. What impacts could be found when operating CSTRs at the pH of 4.0, compared with at the pH of 4.9 during pre-acidification?

1.3 Hypothesis

Varying HRT could affect the generation, accumulation, and interactions of various fermentative bacteria, which results in differences in acids production, both the quantities and acids distribution. For instance, long HRT (18h) might result in larger amounts of VFAs generated, especially with higher concentration of some specific acids, while smaller amounts of total VFAs, with other specific acids concentrated more at the lower HRT (6h).

The pH affects the acidification process. Changing the operation pH from 4.9 down to 4.0 might result in dysfunction of some bacteria in the system. Thus the reactors might not produce as much VFA as with the pH of 4.9, this might also be found in the VFA production, and the acids distribution.

1.4 Objectives

1. To evaluate the performance of CSTRs for the pre-acidification of a synthetic dairy effluent as a pre-treatment on anaerobic digestion.

2. To compare the performance of two parallel pre-acidification CSTRs under different hydraulic retention times (18h and 6h), and different pH values (4.9 and 4.0).

The dynamic tendency of the fermentation product especially the VFA is of great importance with regard to the pre-acidification efficiency. Periodical analysis from the influent and the effluents was conducted on daily or weekly bases for comparative purpose.

2 Literature review

2.1 Characteristics of dairy effluents

The consumption of dairy product increases significantly with the increasing population and living standards. According to Grelet *et al*. (2015), International Dairy Federation (IDF, 2015), 80.8 billion kilograms of dairy products have been consumed in 2015. Besides, dairy industries are accompanied by large amounts of dairy effluent, since a large amount of water is utilized in production processes and equipment cleaning. As Strku-Sokolowaska and Rodziewicz (2017) reported, the annual global production of milk is approximately 630,000 million liters, with the total volume of 2,016,000 million liters generated dairy wastewater.

The major compositions of dairy effluent are lactose, soluble proteins, lipids, and mineral substances (Traversi *et al.* and Perna *et al.* 2013). While the levels of fat, lactose, and protein are in the range of 35-500, 250-930, and 210-560 mg/L, respectively as is shown in Lalman and Bagley's research (2000). Dairy wastewater is also known for its high level of biological oxygen demand (BOD), chemical oxygen demand (COD) and nutrient compounds (nitrogen and phosphorous) (Karadag *et al*, 2015).

Moreover, the contaminated wastewaters from dairy industries varies significantly depending on the production types (Cheese, ice cream, butter, milk powder, synthetic milk etc.), processing methods, and also cleanser selection (Baskaran, Palmowski and Watson, 2003), moreover, it is noteworthy that the flow rate of dairy wastewater has significant seasonal variations, which is typically high with regard to flow rates during summer and low in winter (Kolarski and Nyhuis, 1995). A summary of data obtained from literature for general properties of dairy waste effluents from full-scale operations is given in *Table 2.1*. The significant COD and organic compounds existing in dairy wastewater shown below indicates that, although effluent is biodegradable, its release into the nature could seriously affect the environment leading to severe problems. Additionally, nutrients, detergents and sanitizing agents also commonly occur in dairy effluent, which could lead to eutrophication of receiving water and affect the aquatic life (Montuelle *el al*., 1992).

Table 2.1 *Characteristics of various dairy waste effluents. (WW: Wastewater. N: Nitrogen. P: Phosphorus. NA: Value not available.)*

WW source	pH	COD (g/L)	Solids (g/L)	Volatile solids (g/L)	N (mg/L)	P (mg/L)	Ref.
Butter	12.08	8.93	5.1 (TSS)	NA	NA	NA	(Janczukowicz *et al.,* 2008)
Cheese	1-7.5	5.5-9.5	0.5-2.5 (TSS)	NA	NA	NA	(Monroy *et al.,*1995)
Creamery	8.0-11	2.0-6	0.4-1 (TSS)	0.3-0.9 (VS)	50-60 (TKN)	NA	(Kasapgil *et al.,* 1994)
Dairy	7.1	5	3.9 (TS)	1.35 (VS)	16.5 (TKN)	38.6	(Banu *et al.,* 2008)
Dairy	7.12	4.6	4.4 (TS)	2.1 (VS)	89 (TKN)	9.9	(Pretti *et al.,*2011)
Fluid milk	5.0-9.5	1.0-2.4	NA	NA	NA	NA	(Ozturk *et al.,* 1993)
Milk permeate	5.6-6.5	55.2-63.5	2.7-3.8 (TS)	NA	300-400 (TKN)	350-450	(Wang *et al.,* 2009)
Milk processing	4.0-7.0	5.0-10	3.0-7 (TS)	NA	20-150 (TKN)	50-70	(Bezerra *et al.,* 2007)
Mixed dairy	3.35	63.1	12.5 (TSS)	12.1 (VSS)	NA	NA	(Hwang *et al.,*1998)
Ice cream	5.2	5.2	3.9 (TS)	2.6 (VS)	60 (TKN)	14	(Borja *et al.,*1995)
Whey	4.9	68.6	1.35 (TS)	NA	1120 (TKN)	500	(Traversi *et al.,*2013)

2.2 Treatment methods of dairy effluent

Due to the expansion of dairy plants and the strict legislative requirements, it has been a critical issue for the dairy industry to find more advanced and cost-effective waste treatment or utilization methods. Various physicochemical, electrochemical, and biological methods have been adopted, and some of them have already been applied for treating dairy wastewater. For instance, physicochemical methods such as coagulation–flocculation, the optimum process

conditions were obtained by using 250 ppm salt and ferrous sulfate at the pH of 8.5. Under these conditions, chemical oxygen demand (COD) is reduced by 50% (Rivas *et al.,* 2010). Besides, electrochemical methods, such as electro-Fenton process, which according to Ghoneim *et al.,* (2011) is one of the most popular electrochemical advanced oxidation processes (EAOPs). In 2018, as Davarnejad, Nikseresht and Ajideh reported, the optimal chemical oxygen removal rate (93.24%) was obtained by applying electro-Fenton process, under the conditions of pH value of 7.58, at the reaction time of 87.1min. Moreover, the biological methods consist of various aerobic and anaerobic processes. Aerobic processes include aerated lagoons, activated sludge process, oxidation processes etc. For instance, Fang (1991) completed three stages of activated sludge experiment, and the results show that the residual BOD was lower than the direct discharge value allowed by law, and the COD removal rate of this aerobic digestion reached 89%. Furthermore, Frigon *et al.,* (2009) have further combined an anaerobic reactor followed by an aerobic system, according to this sequence results, the COD removal rate approached 99%.

Among those methods, anaerobic digestion is considered as a sustainable and efficient way, with its triple benefits of reducing sludge production, renewable energy generation and nutrient recovery (Karadag *et al.,* 2015 and Kataki, 2016). As Demirel *et al.,* (2005) reported, approximately 50% of the biodegradable organic compounds in wastewater can be stabilized by aerobic digestion, while up to 90% of them could be degraded by anaerobic digestion. The anaerobic process is also known for high level of household waste stabilization and low area demand (Hassan and Nelson 2012). Therefore, anaerobic treatment increasingly become a favorable biological method for treating dairy wastewater.

2.3 Anaerobic digestion

2.3.1 Mechanism of anaerobic digestion

Anaerobic digestion refers to the process, during which the organic compounds are degraded by fermentative bacteria under anoxic conditions, with the production of untillable biogas. There are four major phases involved in this process (shown in *Fig 2.1*): hydrolysis, acidogenesis, acetogenesis and methanogenesis; various fermentative bacteria take part in each of those phases. During hydrolysis, the complex and undissolved polymers such as carbohydrates, proteins and fats are converted into soluble amino acids, saccharides, fatty acids, and alcohols by hydrolysis enzymes (Khan *et al,.* 2016 and Henze *et al,* 2008); followed by the acidogenesis process, where the acidogenic bacteria converts the products from the initial hydrolysis stage into simpler compounds such as volatile fatty acids (VFAs) in the cells of fermentative bacteria, and secreted to the extracellular environment (Adekunle and Okolie, 2015; Liu *et al.,* 2012a,b); nevertheless, according to Wu *et al.,* (2016) some of the produced VFAs from acidogenesis cannot be converted directly by the methanogens, hence, the third stage (acetogenesis) is required for the conversion of VFAs (propionic, butyric acid, etc.) and alcohol into hydrogen gas and carbon dioxide. Finally during the last phase, acetic acid are converted to methane and carbon dioxide by acetotrophic microbes, while hydrogen and carbon dioxide are converted into methane by hydrogen trophic methanogens.

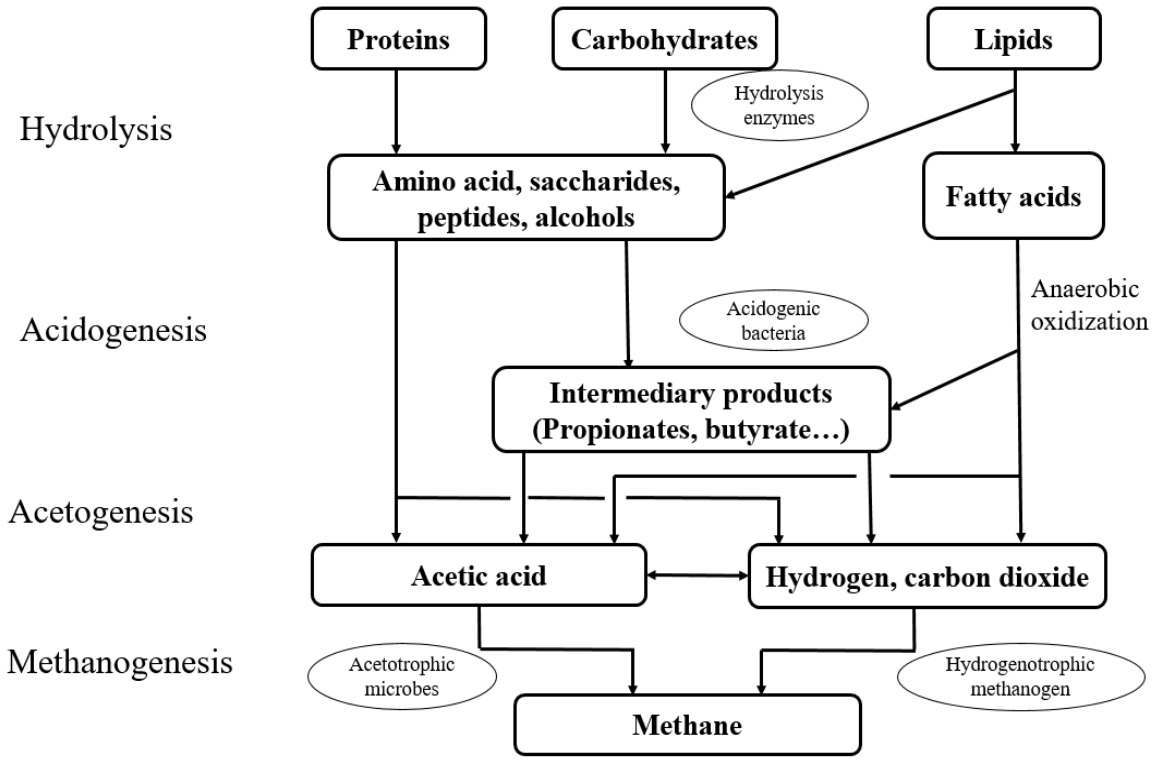

Figure 2.1 *The metabolic pathway of substrate degradation in anaerobic digestion.*

Hydrolysis

In this process, hydrolytic bacteria produce and release extracellular enzymes such as lipase, protease and cellulose, thus large polymers could be decomposed into simpler compounds by those enzymes with the produced hydrolysate of glucose molecules, amino acids, glycerol and long chain fatty acids (Khan *et al*,. 2016).

Acidogenesis

Acidogenesis refers to the following process, where the generated hydrolysate is further fermented into short-chain volatile fatty acids, and some acetate, carbon dioxide and hydrogen gas might also be generated during this process. (Henze *et al.,* 2008)

Acedogenesis

Some parts of the short-chain volatile fatty acids produced in acidogenesis cannot be transformed by methanogenic bacteria directly. The third stage is to further convert those short-chain volatile fatty acids (propionic acid, butyric acid) and alcohol into hydrogen and carbon dioxide. (Adekunle and Okolie, 2015).

Methanogenesis

This is the final step of anaerobic digestion. Methanogens produce methane by metabolizing acetic acids, hydrogen, and CO_2. Biogas is obtained from this process, and its composition depends on the type of organic matter to be degraded, which may be affected by temperature (Cirne *el al*., 2007). Methanogens grow slowly and are sensitive to environmental conditions among the different communities involved in anaerobic digestion, their optimum pH is between 7.5 and 8.5. According to Fenchel *et al.,* (2012) acetoclastic-bacteria methanogenesis is most active in anaerobic digesters, during this process acetate accounts for about two-thirds of the methane production.

2.3.2 Conventional digesters for dairy wastewater

In the studies both in full-scale and laboratory-scale, different digesters have been designed and applied for anaerobic digestion of dairy wastewater treatment. *Table 2.2.* Summarizes various anaerobic digesters.

Up-flow Anaerobic Sludge Blanket reactor (UASB) is regarded a common and suitable configuration for dairy industry wastewater treatment due to its ability to treat large volumes in a relatively short period of time. The studies by Ramasamy *et al.,* (2004) established the feasibility of UASB reactors in treating dairy wastewaters, and obtained over 90% reduction in COD (>40 g/l).

In order to improve the treatment efficiency of conventional reactors Najafpaur *et al.,* (2008) combined the characteristics of granular and biofilm system. A hybrid reactor was developed by modifying the traditional anaerobic reactor. In a two-compartment reactor, the particles and biofilm communities grow and operate simultaneously in different chambers. Karadag *et al.,* (2015) studied the performance of using anaerobic hybrid reactors to treat synthetic dairy (with the COD concentration of 1.2 g/L). It was reported that this system could reach a COD removal rate at 78% at the OLR up to 31 kg COD/m^3/day, and a HRT of 0.75-5 days.

Anaerobic Filter (AF) is generally suitable for biological treatment if specific dairy wastewater contains a low concentration of suspended solids. Its feasibility to treat both low and high strength dairy wastewater efficiently under short HRT and high organic loading rate (OLR) conditions have been reported. (Alves *et al.,* 1998; De-Haast J *et al,*. 1985). De-Haast *et al.,* (1985) has evaluated the performance of treating synthetic whey wastewater (with COD of 13 g/ L) using AF at down-flow mode (DFAF) and reported that it gave a COD removal rates ranging from 66-94 %. Alves*, et al*. in 1998 has applied the AF at up-flow mode (UFAF), treating synthetic dairy (with the COD rage of 3-12 g/L), and it reached the COD removal rates up to 99 %.

Anaerobic moving bed biofilm reactor (AMBBR) was developed to improve COD removal efficiency. According to Wang *et al.,* (2009) biofilm carriers provide a large surface area for biofilm formation, which enhances the stability by preventing cell loss in wastewater. Since the carriers are not attached to the reactor, they could move with flow. It ensures the internal mixing, therefore, it could avoid the over-acidification due to the undiluted raw milk wastewater. Wang *et al.,* (2009) studied the performance using AMBBR to treat a high strength milk permeate

wastewater, the average methane production coefficient YG/S obtained by mass balance was 0.3 CH4 L^{-1} tCOD removal. Stow-kincannon predicted a maximum substrate utilization Umax of 89 g TCOD L^{-1} d^{-1}, indicating a higher potential AMBBR capacity.

Continuously stirred tank reactor (CSTR) has been widely used in anaerobic fermentation process due to its simple design and easy monitoring of important parameters. Besides, it is suitable for some important fast-growing bacteria especially in the acidification process (Wang *et al*., 2013). In earlier studies Yang *et al.,* 2003 has reported that 95% COD reduction was obtained at a total HRT of 10 days in treating cheese whey wastewater (10g COD/ L) using CSTRs.

2.3.3 Two-stage anaerobic digestion for dairy wastewater

Anaerobic digestion involves a variety of symbiotic microorganisms, which can be divided into two major groups: acid-producing bacteria and methanogenic bacteria. Those two groups have great differences in physiology, kinetics and growth demand (Yang *et al.,* 2003). It is mainly optimized the pH in order to improve the growth rates for the microbial community. The microorganisms during acidification grow rapidly, with the pH range of 5.5-6.5 (Khanal *et al.,* 2004); while the suitable pH for methanogenic bacteria with slower growth rate is 6.8-7.2 (Ward *et al.,* 2008). In order to optimize the conditions of those microorganisms and improve the process efficiency, a two-stage process including pre-acidification and methanogenesis in series were developed.

Typically, a buffer tank is installed between each stages of these processes to adjust the pH before another process (Sachs *et al.,* 2003). However, as DiStefano and Palomar (2010) stated, the buffer tank increases the design complexity, therefore, they recommended that the two reactors should be directly integrated to reduce costs and complexity and achieve continuous operation. Besides, according to Wang *et al.,* (2013), CSTRs are suitable for some important fast-growing bacteria in acidification process, and it is convenient to monitor important parameters. Therefore, they are commonly used in the first stage for pre-acidification, in order to adjust the complex conditions in the initial dairy effluent such as the OLR and pH, prior to the fermentation process. Some examples of the performance of using CSTR-combined anaerobic digestion to treat dairy effluents are shown in *Table 2.3*.

Table 2.2 *Various anaerobic performance for dairy effluent among different digesters. (NA: Value not available.)*

Method	**Dairy effluent type**	**pH**	**HRT (d)**	**OLR (kg COD/m^3**	**COD (g/l)**	**COD removal (%)**	**CH4 yield (Nm^3kg^{-1}**	**Ref.**
UASB	Synthetic dairy	NA	0.13-0.5	2.4-13.5	1.4	37-96.3	NA	(Ramasamy *et al.,* 2004)

UASB	Cheese products	6.8-7.3	6	7.5	2.5	85-90	NA	(Gavala *et al.,* 1999)
HYBRID	Synthetic dairy	NA	0.75-5	2.2-31	1.2	78	0.27	(Kundu *et al.,* 2013)
UFAF	Synthetic dairy	NA	2	1.4–6.3	3–12	98	0.32–0.36	(De-Haast *et al.,* 1985)
DFAF	Synthetic whey	5.9–7.8	5	2.6	13	66-93.6	0.24-0.26	(Rodgers *et al.,* 2004)
AMBBR	Whey	7.0–7.8	0.6–17	1–21	6–17	70–97	0.33	(Wang *et al.,* 2009)
CSTR	Cheese whey	7	10	0.95	10	94.6	0.34	(Yang *et al.,* 2003)

Table 2.3 *Two-stage anaerobic treatment performance overview for dairy effluents. (ABR: anaerobic bioreactor. AnMBR: anaerobic membrane bioreactor. NA: Value not available)*

Method	Dairy effluent type	pH	HRT	OLR (kg COD/m^3 per day)	COD (g/l)	COD removal rate (%)	CH4 yield (Nm^3 kg^{-1} COD)	Ref.
CSTR+ UFAF	Wastewater from milk bottling plant	NA	2 days	2.4-13.5	1.4	90	NA	(Ramasamy *et al.,* 2004)
CSTR+ UFAF	Wastewater from milk and cream	7.0-7.5	1.5 days	7	2.0-6	95	NA	(O. Ince, 1998)
CSTR+ CSTR	Cheese whey	5.2;NA	21 days	30	60.5	95.3	NA	(Ven etsaneas *et al.,* 2009)
CSTR+ ABR	Dairy	6.8;NA	7.6h; 1.3h	1.3-4.5	1.2	82	0.26	(Jürgensen *et al.,* 2017)
CSTR+ UASB	Cheese whey	6.4	9.5h; 12-18h	20	9.5	90	0.37	Diamantis *et al.,* 2014)
CSTR+ AnMBR	Cheese whey	7.3-8.5	4 days	3-19.8	11.0-80	98.5	NA	Saddoud *et al.,* 2007)

2.4 Acidification of dairy effluent

Acidification includes pre-acidification (including hydrogenesis and acidogenesis) and acedogenesis, which occur in the first phase during two-stage fermentation process.

Nathao *et al.,* (2013) observed two-stage fermentation of food waste, and stated that the first stage of fermentation played an important role in the overall degradation efficiency and energy recovery of the substrate, especially the pre-acidification. The main production during pre-acidification is VFA, which contain a large amount of soluble organic acids, including acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-pentanoic acid, pentanoic acid and caproic acid. Among those products, acetic acid, butyric acid have been reported as the main precursors for methane production, according to Yu*et al. (*2016b), especially acetic acid, it accounts for 65-95% of the methane production.

2.4.1 Types of bioreactors for dairy wastewater acidification

There were various bioreactors used for acidification. For instance, a downflow–upflow hybrid anaerobic reactor was used to treat cheese whey (Malaspina *et al.,* 1995). According to the authors, it demonstrated COD removal rates of 98% at a considerably high organic loading rate, and reduced the investment costs due to its ability to obtain phase separation in the same reactor.

In 2000, Ramasamy and Abbasi attempted to upgrade stirred tank reactor, performance by adding a biofilm support system to an existing reactor, and significantly improvement on the efficiency of the STR was found after adding nylon reels (with diameter of 2cm and height of 1mm).

Furthermore, in the research conducted by Garrido *et al.,* (2001), an anaerobic filter and a sequential batch reactor were used to treat wastewater from an industrial milk analysis laboratory, they found that high degree of fractionation (50%-85%) of COD was carried out, and the remaining COD and most nitrogen were removed in SBR and then concluded that the combination of an AF and a SBR is a suitable alternative for treating dairy wastewaters.

However, among various reactors, continuous stirred tank reactor is regarded as the most commonly used bioreactor. According to Eddy (1991), suspension growth is one of the most commonly used technologies for VFAs production, and CSTR offers an ideal mixture of the effluent and microorganisms in the presence of suspended solids, and a complete mixture of the waste and biomass (Lee *et al.,* 2014).

2.4.2 Factors affecting acidification performance

HRT

Hydraulic residence time, also known as hydraulic residence time, is the average residence time of soluble compounds in the bioreactors. It has significant impact on substrate absorption efficiency metabolism and determine microbial community (Wang *et al.,* 2013). Thus adjusting HRT is regarded as a practical application due to its simplicity, which provides important information for engineering and/or design of microbial communities when optimizing acidification conditions.

The acidogens require a minimum HRT for hydrolysis and acidogenesis. Regular hydraulic retention time of a system depends on the type and composition of the substrate. For example, in anaerobic leaching bed reactors that digest high solid content substrates, 1.5 days of HRT was used for VFA production and profiling (Cysneiros *et al.,* 2012), while 1.9 days of HRT performed best in acid-producing anaerobic digestion (Aguilar *et al.,* 2013), more example related to HRT are shown in *Table 2-4.* It shows how different HRTs affects the production of VFAs, and distribution of different acids. It also illustrates that pre-acidification systems should be operated at different HRT with different substrate

pH

In the process of acidification, pH value plays an important role. The pH range within extremely acidic (less than 3) or extremely basic (more than 12) conditions is reported to be the acidogenic inhibition condition (Liu *et al.,* 2012a, b). However, the optimum pH for VFA production varies with the types of wastewater, for instance, VFA/ sCOD ratios are high (up to 75%) in pH 5.0-6.0 regardless of the type of inoculant used to produce VFA from food waste, while Bengtsson *et al.,* (2008) reported that the optimal pH for one wastewater is 5.3 to 6.0. Besides, although the composition of VFA produced mainly depends on the composition of substrates, however, differences in pH value could also control the types of VFAs produced during acidification (Lee *et al.,* 2014). Generally, in the low pH range (4-7.5), the dominant products should be butyrate and acetate, while in the high pH range (7.5-8), the yield of butyrate should be decreased due to the lack of the dominant presence of klebsiella, an enzyme related to butyrate production (Temudo *et al.,* 2008).

Other factors

Various microbes and complex reactions are involved in acidification process. Apart from HRT and pH, there are other factors could affect the fermentation performance, including the organic loading rate, temperature, hydrogen pressure, etc. For instance, Fang and Yu (2001) observed that lactose degradation increased from 20 to 55°C and decreased at 60°C during acidogenesis; With regards to hydrogen pressure, as Ryhiner *et al* (1993) stated that, hydrogen pressure plays an important role in the control of the anaerobic fermentation process, since the propionic acid and butyric acid are converted to acetic acid only under low hydrogen partial pressure. Besides, as is Thauer *et al.,* (1977) pointed out that, when if the hydrogen pressure is less than 10^{-4} atm, the oxidation of propionic acid in acetic acid is thermodynamically possible, also the accumulation of propionic acid is accompanied by a decrease in pH with an increase in dissolved hydrogen and acetic acid concentration.

Table 2.4 *Fermentation studies with varying HRT. Hac: acetic acid. Hpr: propionic acid, Hbu: butyric acid.*

WW type	**Reactor types**	**pH**	**HRT (h)**	**Total VFA**	**Main VFA Contribution**				**Ref.**
					Hac	Hpr	Hbu	Other	
Glucose	CSTR	5.5–6.5	8	1.5 (g COD/L)	47	2	26	24	(Hafez *et al.,* 2010)
			12	15 (g COD/L)	7	20	-	-	
Lactose	CSTR	5.9	12	32.1 (mmol/L)	37	0	30	33	(Palomo-Briones *et al.,* 2017)
			18	50.7 (mmol/L)	25	29	44	2	
			24	45.10 (mmol/L)	11	7	65	17	
Sugarcane stalks	CSTR	4.5-5.8	12	18.7gCOD/L	39	-	17	44	(Chatchawin *et al.,*2016)
			6	15.9gCOD/L	40	-	21	39	
			4	13.4gCOD/L	38	-	36	26	
			3	12.7gCOD/L	18	-	53	29	
PS and WAS	Batch glass bottles	10	5d	0.02 (g/g VS)	52	22	14	12	(Jankowska *et al.,* 2015)
			10d	NA	62	14	12	10	
Paper mill WW	CSTR	6	11	0.7(g COD/g COD)	39	14	39	0	(Bengtsson *et al.,* 2008)
			24	0.7(g COD/g COD)	42	40	11	0	
Whey	CSTR	6	8	0.8(g COD/g COD)	45	29	17	0	(Bengtsson *et al.,* 2008)
			50	0.9(g COD/g COD)	33	61	2	0	

2.5 Summary

Great amount of studies have been carried out to treat dairy wastewater. With regard to anaerobic digestion method, the two-stage process is more favorable due to its controllability and flexibility. Different combinations of digesters have been studied in laboratory-scale, and some of them have been applied according to the characteristics of various dairy effluents. Besides, when it comes to acidification process, researchers focus more on hydrogen and methanol production as well as BOD/COD removal efficiency.

Researchers show great interests in acidification stage according to this review, a great deal of reports have described how different factors (such as HRT, pH, OLR etc.) affect the performance of acidification. Besides, CSTR is commonly used for acidification process, although there are also other advanced digesters coming out to upgrade the existing system such as AMBBR, and integrated fixed activated sludge. However, when it comes to dairy effluent, more attention is put on whey production and hydrogen production. There are limited researches focusing on how various factors (HRT, OLR, pH etc.) affect the performance of the initial stage of acidification that is pre-acidification. Especially the pre-acidification process with regard to a specific milk production wastewater in a CSTR system.

3 Experimental approach

3.1 Substrate and inoculum source

The activated sludge from a full-scale municipality wastewater treatment plants (Klagshamn, Malmö, Sweden) was used as the original microorganisms' source, the reason to choose the activated sludge instead of digested sludge is to avoid selected bacteria in the system, in order to ensure that the different fermentation performance is only due to different operation conditions. This activated sludge was added into two continuous stirred tank reactors with the final concentration of 1320.0 g/L of volatile suspended solids (VSS).

For both reactors the synthetic dairy influent was used to simulate the wastewater from dairy industry. The influent consisted of homogenized pork blood powder (the Netherlands), Ultra-high temperature sterilized (UHT) milk with 3.5% fat (detailed information for this milk could be found in *Appendix A. Table A-1.*), granulated white sugar, and vithane + iron solution (2.5 mL/L of vithane stock and 10.2 mL/L of $FeCl_3$ stock). The vithane stock is one type of micro-nutrient solution, containing trace element, it is from the company Biothane, the Netherlands. It is usually used for anaerobic wastewater treatment. The recipe of this influent is shown in *Table 3.1* and the final influent contained 17 g/L of COD, 11 g/L of soluble COD (sCOD), 258 mgCOD-VFA /L of volatile fatty acids (VFA) and 2 g/L of VSS. The influent was prepared every day and stored in the fridge with the temperature under 8ºC to prevent its self-acidification. Besides, mixers were used for the influent to avoid precipitation in the influent on weekdays, but due to practical implementation conditions, the influent was not continuously mixed in the fridge on weekends.

Table 3.1 *The recipe for each liter of synthetic dairy influent.*

Component	**Quantity**	**Unit**	**Preparation**
Full fat milk (UHT sterilized)	48	mL	Added directly
Sugar (granulated white)	3.8	g	Added directly
Blood (freeze dried)	1.9	g	Homogenized with 0.2 L of water
Vithane + Iron Solution	1.65	mL	Added directly
Tap water	-	-	To make up to 1 liter

3.2 Operation conditions

The CSTRs with the HRT of 18h (CSTR 18h) and the HRT of 6h (CSTR 6h) had been operated for 77 days in total, working with the operation volume of 1380 ml and 1350 ml due to implementation conditions. To start with, activated sludge was used as original microorganisms' source. One magnetic stirrer in the bottom of each reactor was added. Besides, in order to prevent the organisms from the adhesion to the inner wall, 6 plastic pellets were also added into each reactor. The influent went inside the CSTRs through two pumps (Watson Marlow 520S) with the pumping speed of 0.4 rpm for CSTR 18h and 1.4 rpm for CSTR 6h. This gave the

organic loading rate around 3.0 g/ (d*L) and 6.9 g / (d*L) for each reactor respectively. The simplified experimental setting is shown in *Fig.*3.1.

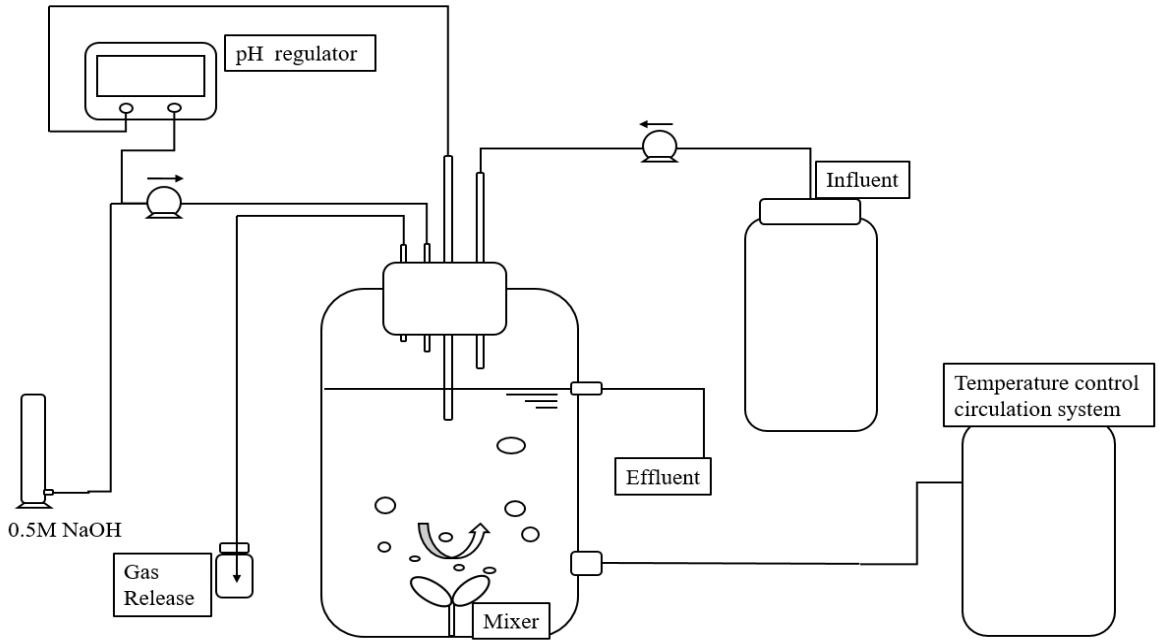

Figure 3.1 *Simplified experimental setting. (Fig. C-1 shows the practical operation setting could be find in Appendix C.)*

The temperature in both CSTRs were controlled as 19ºC by a water bath from a circulation system, the value could read from the circulation system. However, in order to ensure the reliability of this temperature control system, manual measurements of the temperature in the reactors were conducted directly using pH meter (Hanna H199100) every other week, and it gave the average result of 19.2 ºC$\pm$ 0ºC.

The pH in both reactors were measured by pH meter (SI Analytics H63) connected to the reactors. For the first 55 days, it was controlled at 4.9 ($\pm$0.05) by a pH regulator (Liquisys M, CPM 253) with the addition of 0.5M of NaOH as the buffer solution, then for the final 22 days, it was controlled at 4. The choice of 4.90 is because the microbes in the acidogenic phase are fast-growing bacteria at this pH (Khanal *et al.,* 2004). Similar with the temperature, the pH of the effluent are manually measured by the pH meter (Hanna H199100) three times a week to ensure the reliability of the pH regulator.

Additionally, a gas release system together with a water lock was added to each reactor, in order to prevent the contamination in NaOH solution due to the gas produced in reactors. The water lock is used to avoid air going to the reactors. Besides, to prevent the air going into the reactors when rinsing the inner wall and manually measuring the temperature, nitrogen gas was used.

3.3 Analysis method

3.3.1 Experimental measurements

The water quality for the influent and effluent was measured on a weekly base. The schedule is shown in *Table 3.2*. For COD and VFAs, analysis for both influent and effluent were conducted three times a week, on Monday, Wednesday and Friday; nitrogen, phosphorus, TS/VS, and TSS/VSS from influent were analyzed once per week on Wednesday, but those analyses for effluent were conducted twice a week, on Monday and Wednesday. Other parameter related to minerals such as alkalinity, SO_4, Ca and Mg were analyzed once a week on Wednesday for both influent and effluent.

Table 3.2 *Analysis schedule.*

	Analysis Method	**Monday**		**Wednesday**		**Friday**	
		Influent	Effluent	Influent	Effluent	Influent	Effluent
sCOD	LCK(914)	x	x	x	x	x	x
COD	LCK(914)	x	x	x	x	x	x
VFA (HACH)	LCK(365)	x	x	x	x	x	x
VFA (GC)	GC	x	x	x	x	x	x
NH_4-N	LCK(303)		x	x	x		
sN	LCK(303)		x	x	x		
TN	LCK(238)		x	x	x		
PO_4-P	LCK(350)		x	x	x		
TP	LCK(350)		x	x	x		
TS/VS	(Standard		x	x	x		
TSS/VSS	APHA,1995)		x	x	x		
Alkalinity	LCK(362)			x	x		
SO_4	LCK(153)			x	x		
Ca	LCK(327)			x	x		
Mg	LCK(327)			x	x		

There were two batches of samples being prepared before analysis. The first batch was prepared without any filtration, and was used for analyzing COD, TN, TP, TS/VS and TSS/VSS. Another batch of samples were first filtered by MGA-filter, followed by a syringe filter with the pore size of 0.45μm (Q-Max Syringe Filter Cat No.CA25050S). The filtered samples were used for the analysis of sCOD, VFA, NH_4-N, sN, PO_4-P and the mineral parameters. Those prepared samples were mostly stored in the fridge at the temperature of 4°C until testing, except for those

being analyzed by GC method for VFA value. Those samples were stored in the freezer, and were defreezed just before testing.

Hach kits were used in most of the analysis for both influent and effluent, and it includes: sCOD/tCOD (LCK 914), VFAs (LCK365), Alkalinity (LCK362), SO_4^{2-} (LCK 153), Ca^{2+} /Mg^{2+} (LCK 327), NH_4-N (LCK 303), soluble nitrogen (LCK238), TN (LCK238), PO_4-P (LCK 350), and TP (LCK350). Besides, the distilled water is used when the sample dilution is needed.

The total solids (TS), total suspended solids (TSS), volatile solids (VS) and volatile suspended solids (VSS) were measured using standard procedures for wastewater analysis (APHA,1995): for TS and TSS, the samples were dried in 105ºC for 2h; as for VS and VSS, the dried samples were further incinerated under 550ºC for 2h. Moreover, distilled water was used when sample dilutions were needed. Besides, microscope analysis for the sludge was also conducted.

In addition, the VFA analysis was done by two methods. One is using Hach kits, this method could merely give a general value for total VFAs. The results from that were usually used to monitor the general conditions of the system, because the change of the VFAs is usually faster than other factors such as pH and OLR. Another is the quantitative Gas Chromatographic (GC) method, which has been successfully used for the detection of both total VFAs, and the distribution of different acids (In this work, acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid, and caproic acid are analyzed) with more precise results. The chromatographic conditions used by these methods vary from temperature-programmed to thermostatic. In this work, column (from PerkinElmer ® Elite-FFAP Capillary Column) with the length of 30 m, and diameter of 0.25 mm was used. In general, samples need complex and time-consuming esterification pretreatment, or they could be directly analyzed after acidification. It is generally believed that the addition of acids has two benefits: an acid environment can inhibit the growth of microorganisms, which is conducive to the preservation of samples; secondly, the addition of acid could improve the sensitivity of sample detection.

3.3.2 Data analysis

HRT

HRT can be calculated, using the volume of CSTR (V) divided by the flowrate Q, as is shown in *Eq. 3.1*:

$$HRT = V/Q \qquad Eq.\ 3.1$$

Degree of acidification

The VFA results from the GC analysis could provide the concentration of each acids. In this case, the main acids including, acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid and caproic acid were chosen, thus the total concentration of the VFAs could be calculated by summing up all those chosen acids together.

The degree of acidification is a parameter used to evaluate the performance of the acid-phase digestion. It can be quantified using the percentage of the initial substrate concentration (the soluble COD) converted to VFA, using the *equation 3.2*. The initial substrate concentration could be measured in sCOD, and the quantity of VFA was converted into the theoretical equivalent of COD, using the COD equivalent of each VFA (Burak and Orhan, 2004). The COD equivalents of each VFA are given in *Table 3.2*.

$$Degree\ of\ acidification = VFA\_CODeq\ /\ (sCOD \text{ x } 1000) \text{ x}100 \qquad Eq.\ 3.2$$

Table 3.2 *The COD equivalent of each volatile acid, detailed calculation and example is shown in Appendix A.*

Volatile acid	**Acetic acid**	**Propionic acid**	**Iso-butyric acid**	**Butyric acid**	**Iso-valeric acid**	**Valeric acid**	**Caproic acid**
COD Equivalent	1.07	1.51	1.82	1.82	2.04	2.04	2.20

4 Results and discussion

4.1 Influent characteristics

The characteristics of the influent (synthetic dairy) is shown in *Table 4.1*.

Table 4.1 *The characteristics of influent.*

Parameter	Unit	Average concentration	
		In1	In2
pH		7	7
Total COD (COD)	g/L	17	17
sCOD / COD	-	0.7	0.7
Total VFA	mg COD-VFA /L	258	216
Total Solids (TS)	g/L	12	11
Total volatile solids (VS)	g/L	10	10
Total Suspended solids (TSS)	g/L	3	3
Volatile suspended solids (VSS)	g/L	2	2
VSS/TSS	-	0.7	0.7
sN	mg/L	258	313
NH_4-N	mg/L	1	1
Total nitrogen	mg/L	488	509
Total phosphorus	mg/L	51	52
PO_4-P	mg/L	19	20
Alkalinity	mgCaCO3/L	520	543
SO_4	mg/L	503	554
Ca	mg/L	67	66
Mg	mg/L	16	16

As is mentioned above, the influent was prepared every day, and was separated into two containers (In1 for CSTR 18h and In2 for CSTR 6h) in order to get it well-mixed. The average

pH of the influent is 7.2 in both containers, it contained 16 g/L of total COD, with the sCOD/ tCOD ratio of 0.8. The total solids were 12 g/L and 11 g/L in In1 and In2 respectively. The total suspended solids were 3 g/L, and 67 % of them were volatile suspended solids, in both influents. Additionally, the influents contained a total VFA of 258 and 216 mg COD-VFA /L in In1 and In2, respectively.

4.2 Operating conditions

The experiment lasted 77 days, and the operation conditions including the HRT, OLR, and pH are shown in Fig 4.1 and Fig 4.2. Within steady conditions, CSTR 18h was operated at the HRTs within the rage of 17.6-19.8 hours in an organic loading rate of 19 ± 2 g COD L^{-1} d^{-1}, while CSTR 6h was operated at the HRTs ranging from 5.4 to 6.8 hours with the OLR of 60 ± 8 g COD L^{-1} d^{-1}. The disturbances shown in the figures such as the high values appear in the OLR of CSTR 18h during day 8-11, and day 68-70 were due to the clogging of inlet tubes during these weekends. This is because the solids could accumulate inside the tubes, which results in the decrease of flow rate. As for the pH, during the first 55 days, the pH was controlled within 4.9-5.0 and then it was controlled at around 4.0 during the rest 22 days. The variations were mainly due to the clogging in the NaOH solution dosing tubes, which resulted in lack of pH buffering. There was gas produced in the reactors, and when it went into the tubes, precipitation of NaOH could happen insides those tubes. This problem was improved by adding a gas release system. The reactors were operated for about 25 days at each HRT in order to attain a steady-state conditions.

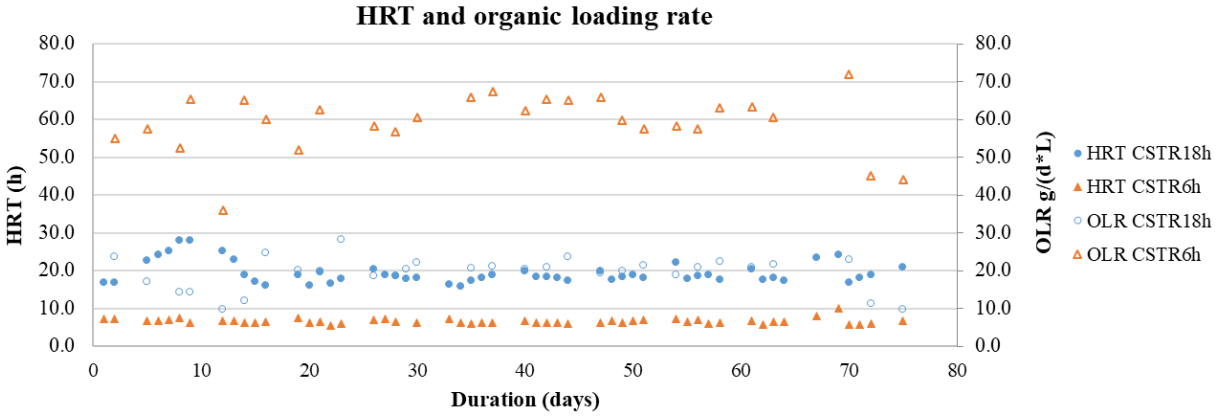

Figure 4.1 *The record of hydraulic retention times and organic loading rates.*

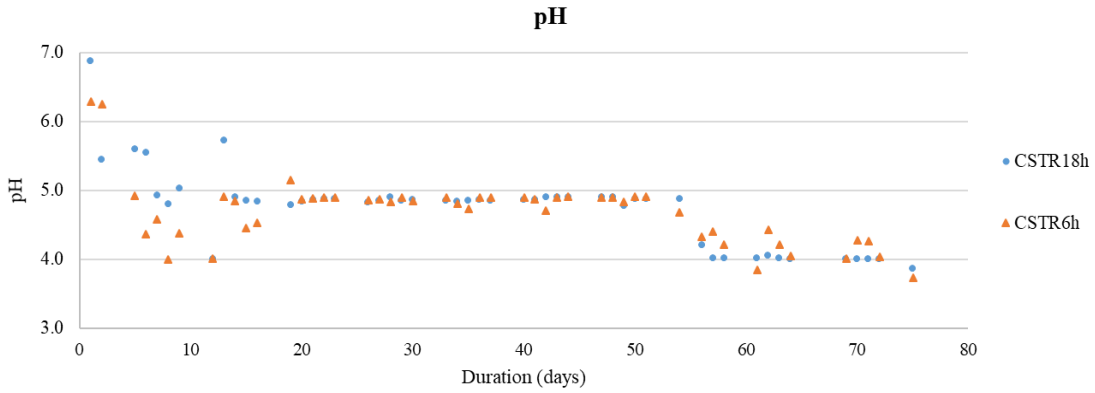

Figure 4.2 *The record of pH values.*

4.3 COD profiles

According to the results, for both pH conditions (pH at 4.9 and 4.0) after stabilization, the total COD in CSTR 18h and CSTR 6h were 15.7 ± 1.7 g/L (n =20). When comparing the total COD concentration in the reactors with the concentration in the influent (with the COD concentration of 16.7 ± 0.4 g/L, n =20), as is also shown in *Fig. 4.3*, there was no distinct differences. It indicates that there was no total COD removal occurring during the pre-acidification process. This is reasonable, since during the first-stage of fermentation, no COD is expected to be oxidized within the anaerobic environment, it is only transformed into fermentation products. Therefore, the condition of total COD concentration was also an important parameter to keep on track during the experiment, in order to ensure that the reactors were operated under a strictly anaerobic environment.

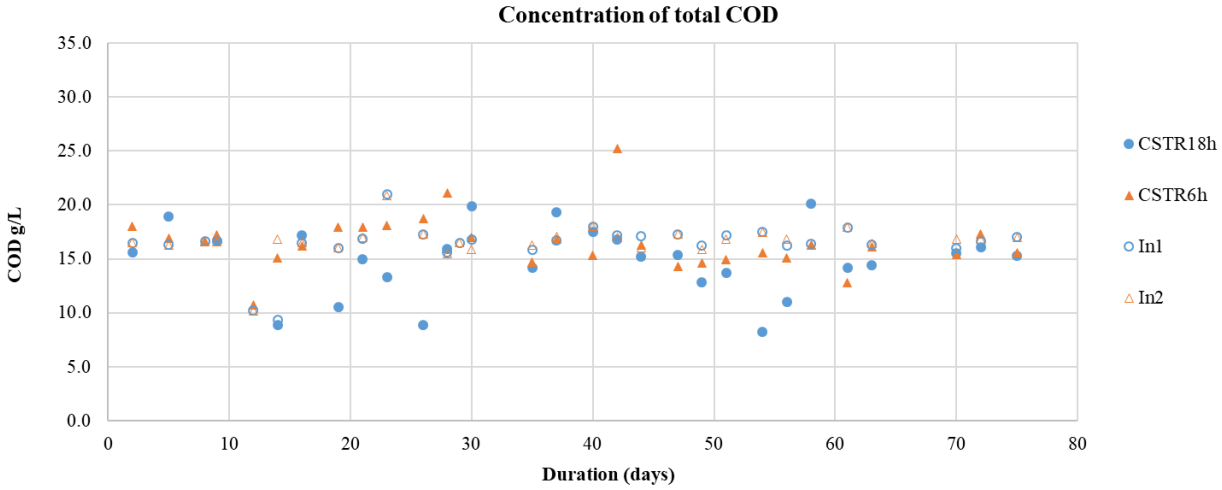

Figure *4.3 Total COD profile in both CSTRs and the influents. In1, In2 indicate influent for CSTR 18h and CSTR 6h, respectively.*

The total COD concentration in the CSTRs was not supposed to change, while the soluble COD was expected to transform into VFA. According to the results (shown in *Table 4.2*, Profile of the soluble COD among the entire duration could be found in *Appendix A. Fig.A-1*) at the pH

of 4.9, 50% of COD is soluble, and the average concentration of soluble COD in CSTR 18h and CSTR 6h is 6.6 and 7.5 mg/L. It means that the sCOD decreased by 34.8% and 30.4% respectively, compared with the influents. This is due to the agglomeration of the emulsion and casein micelle content in milk. In an acid environment, partial flocculation (which is the agglomeration of fat globules with no identity changes of the globules in the floc) could happen to these compounds, and it results in the decrease of soluble compounds (Birdi, K.S., 2009). However, they were not removed from the system, but existed in the form of particulate matter.

Table 4.2 *Average value of COD in CSTRs in different operation conditions. (CI: Confidence interval under the significance level 0f 0.05)*

Operation condition	sCOD (g/L)	COD (g/L)	Decrease of sCOD (%)	sCOD/ COD ratio
HRT 18h; pH 4.9	6.2 (CI 0.2)	14.8 (CI 1.6)	35 (CI 6)	0.5 (CI 0.1)
HRT 6h; pH 4.9	7.3 (CI 6.3)	17.0 (CI 1.6)	30 (CI 6)	0.5 (CI 0)
HRT 18h; pH 4.0	8.6 (CI 0.1)	15.2 (CI 1.5)	13 (CI 14)	0.6 (CI 0)
HRT 18h; pH 4.0	9.5 (CI 14.2)	15.5 (CI 1.0)	9 (18)	0.6 (CI 0)

4.4 COD transformation to VFA production

4.4.1 Quantity of total VFA production

The total VFA production profile from the Hach analysis is shown in *Fig. 4.4*. As is mentioned in Chapter 3, the results from Hach is not precise, since it not only detected the VFA but all the bio-acids, thus it is only used to monitor the condition of the reactors. The accurate results should be provided from the GC analysis shown in *Fig. 4.5.* According to the Hach analysis together with the operation conditions, it is reasonable to believe that the reactors were stabilized after day 25 until day 55 when the pH was changed into 4.0. However, according to the laboratory records, the sudden increase of VFA production in CSTR 6h on day 30 was due to the influent was not given into the reactor on day 29 for 16h by mistake, that means the HRT increased to 22h. Therefore, the fermentative bacteria stayed longer thus there were more VFA produced. On day 33, it is observed that the still water level increased in CSTR 6h, which means the volume increased. It also resulted in the increase of HRT. This situation was improved after 2 days, until it was found that the issue was due to the pressure in the outlet point in the container was higher than that in the reactors, it is also because of the biomass clogging in the outlet tubes.

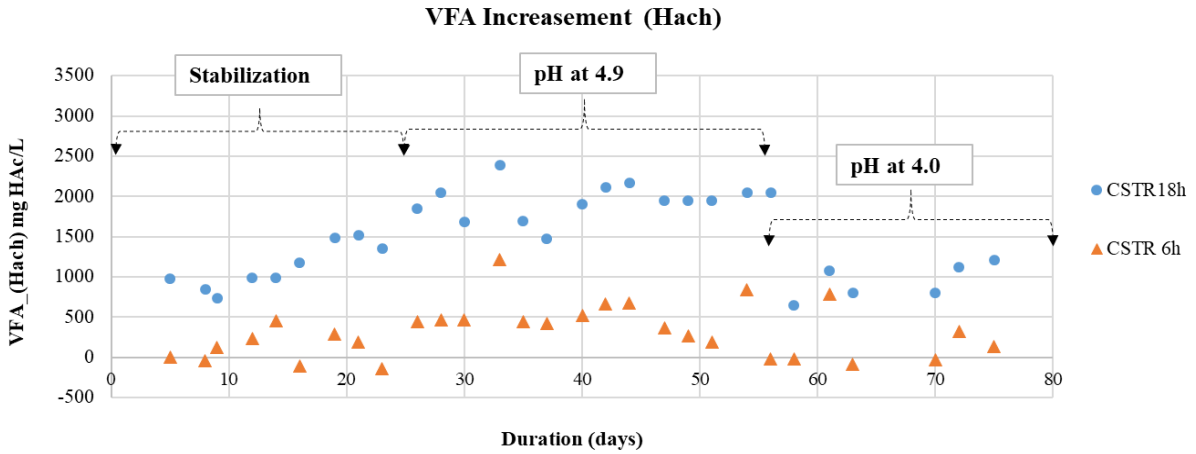

Figure 4.4 *The VFA increment. Results from Hach analysis.*

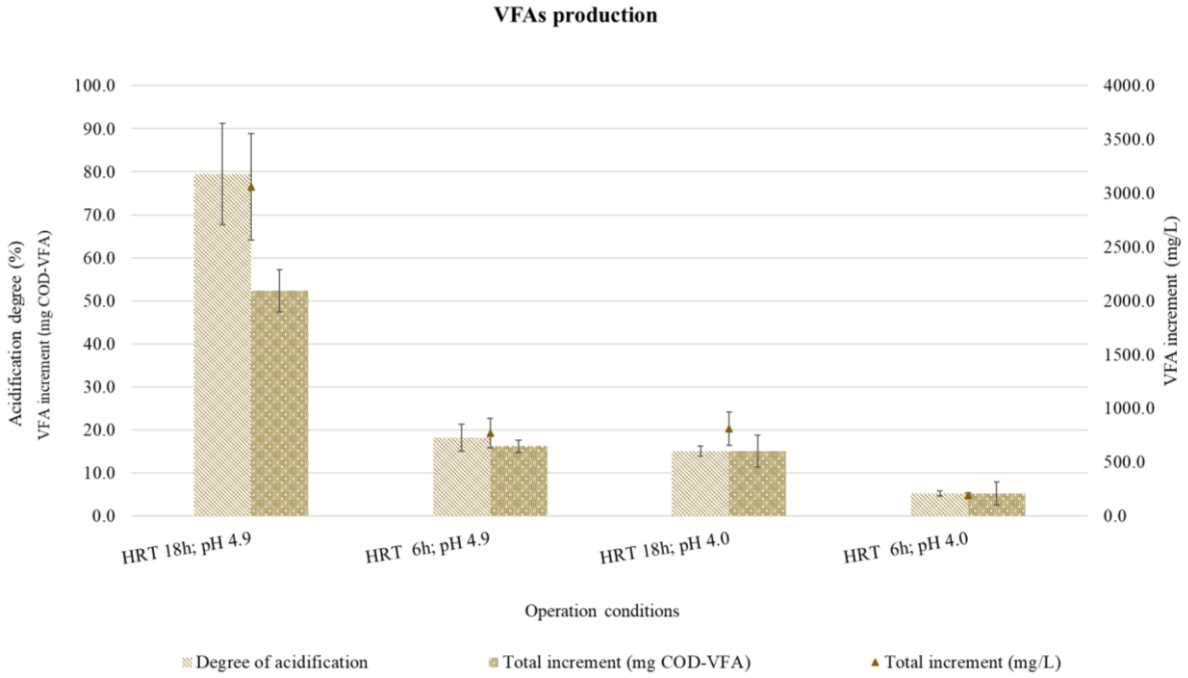

Figure 4.5 *The fermentation performance of CSTRs under four different operation conditions. (With regard to VFA production). The data for different acids was the average value during steady-state, for pH at 4.9 it was from day 25 to day 55. For pH at 4.0 it was from day 56 to day 76. (Confidence interval is under the significance level 0f 0.05.)*

The performance with regards to VFA under different conditions after stabilization are shown in *Fig. 4.5*. At the pH of 4.9, the acidification degree is around 80%, and the VFA produced in CSTR 18h in steady states are 3061 mg COD-VFA/L. Although there was still VFA produced in CSTR 6h, however the degree of acidification was only 18%, which yielded ¼ of the output, and that was 769 mg/L. It shows that the HRT has significant impact on VFA production. It is also obvious that with the HRT of 6 h, there was insufficient VFA produced, which means that the reactor could not function well within such a short retention time. Besides, the results also illustrates that after day 55, when the CSTRs were operated at the pH of 4.0, both reactors could

not function well when the pH dropped down to 4.0. The VFA yield decreased dramatically in CSTR 18h and CSTR 6h, with the significant reduction of acidification degree dropped down to 15% and 5% respectively.

The results provide strong evidences showing that the HRT has significant impact on VFA production. Although factors such as the composition of the growth medium and the source of inoculation could affect fermentation conditions, this scenario was consistent with previous studies on acidification of dairy wastewater where similar trends have been reported. For instance, Palomo-Briones *et al.* (2017) used lactose as substrate, and observed the fermentation performance in CSTRs, they confirm that with the HRT of 24 h, the lactose degradation efficiency could reach 82%, while it was only 35% with the HRT of 6 h. Also the results from (Jankowska *et al.,* 2015) describes the similar tendency of the VFA production increment. Horiuchi *et al.* (2002) has reported that soluble protein, carbohydrate and lipid can directly be fermented into acetate, propionate and butyrate, while iso-valerate and valerate are mainly generated from the degradation of protein. With a sufficient HRT, more soluble proteins and carbohydrates could be generated, thus the amount of VFA could accelerate.

As for the impacts from pH, virtual impacts of the pH could be seen from the significant change of VFA production after decreasing the pH value. According to Temudo *et al*. (2008), the pH in the substrate could determine the proportion of unionized acids that are able to permeate the cell membranes. Therefore, it could shape the microbial communities. As Rodriguez *et al.* (2006) also stated the fermentation rate is related to the inhibitory effect of non-ionized acids on the permeability of cell membranes. At lower pH values, (in this case at the pH of 4.0) the transport of undissociated acids requires more energy, than at higher pH values, since the freedom of the acids enhance the utilization of energy. With regard to the total VFA production, after decreasing the pH down to 4.0, the amount of VFA produced dropped in both reactors. Especially in CSTR 18h, a dramatic decline of the acidification degree (from 80% to 15%) was seen. This phenomenon was also observed in the studies from Jankowska *et al.* (2015), Infantes *et al*. (2011) and Yu and Fang (2002). For example in 2002, Yu and Fang used milk the substrate, and found that with increased pH from 4.0 to 5.5, the degradation amount of fat, protein and carbohydrate also rises.

4.4.2 Distribution of acids

The HRT and pH not only affect the amount of VFA produced, but also influence the distribution of different acids. The general distribution trend among the duration is shown in *Fig. 4.6*, and the average values after stabilization are shown in *Fig. 4.7and Fig. 4.8*. At the functional pH of 4.9, with the higher HRT of 18h, 38% of the total VFA was butyric acid. It was the most concentrated acid, followed by propionic acid and acetic acid, which accounted for 31% and 23% of the total VFA respectively. While at the HRT of 6 h, butyric acid was mostly generated, which was 46% of the total VFA. Besides, the proportion of acetic acid and propionic acid was 35% and 15% respectively. It elaborates that when the HRT was 6h, more acetic acid were produced than propionic acids. When the pH dropped down to 4.0, apart from the fact that the total amount of VFA dropped dramatically, it is noticeable that in both reactors

most part of the VFA generated was acetic acid. At the same time, the proportion of butyric acid dropped significantly.

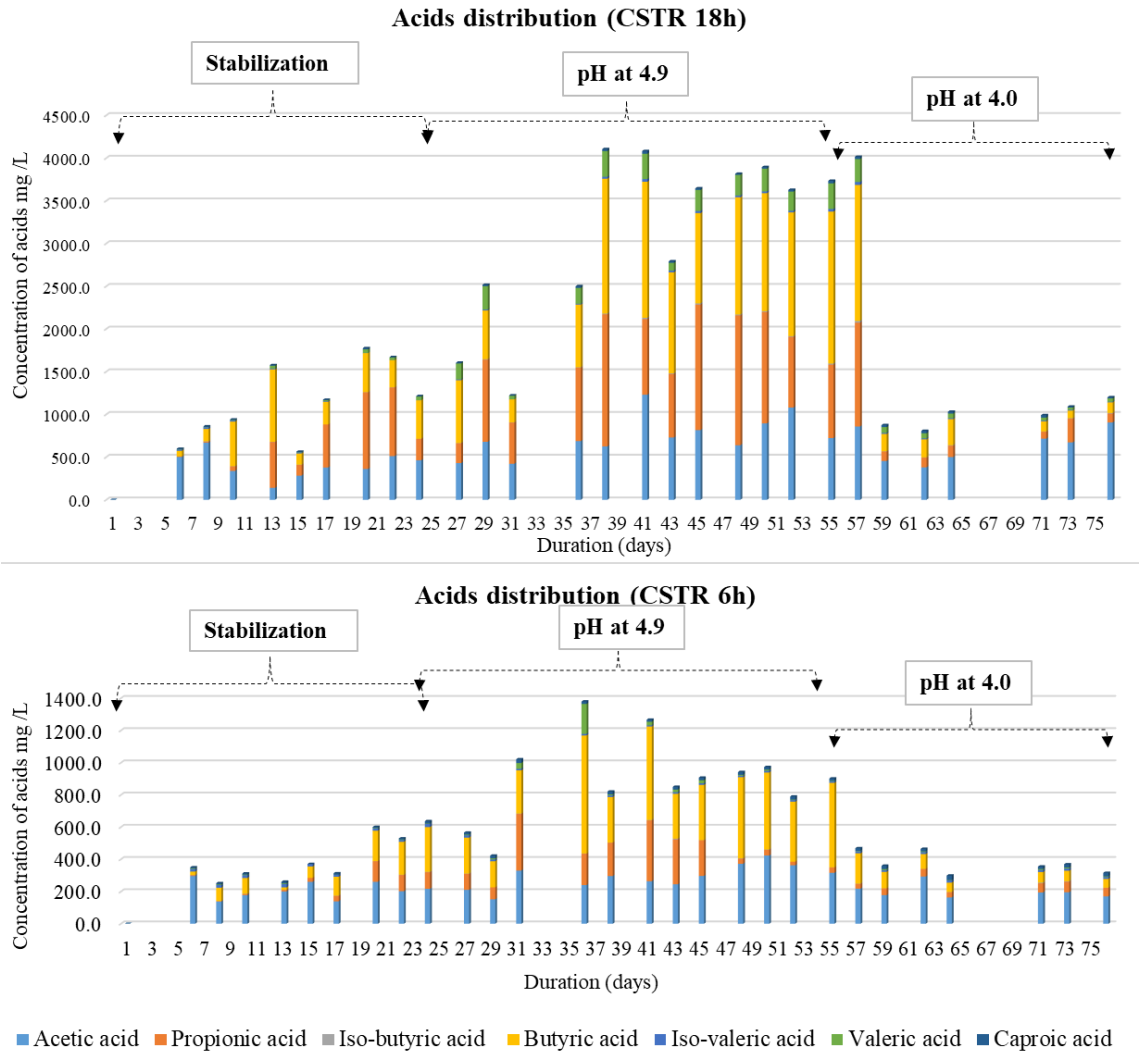

Figure 4.6 *Acids distribution among the experimental duration.*

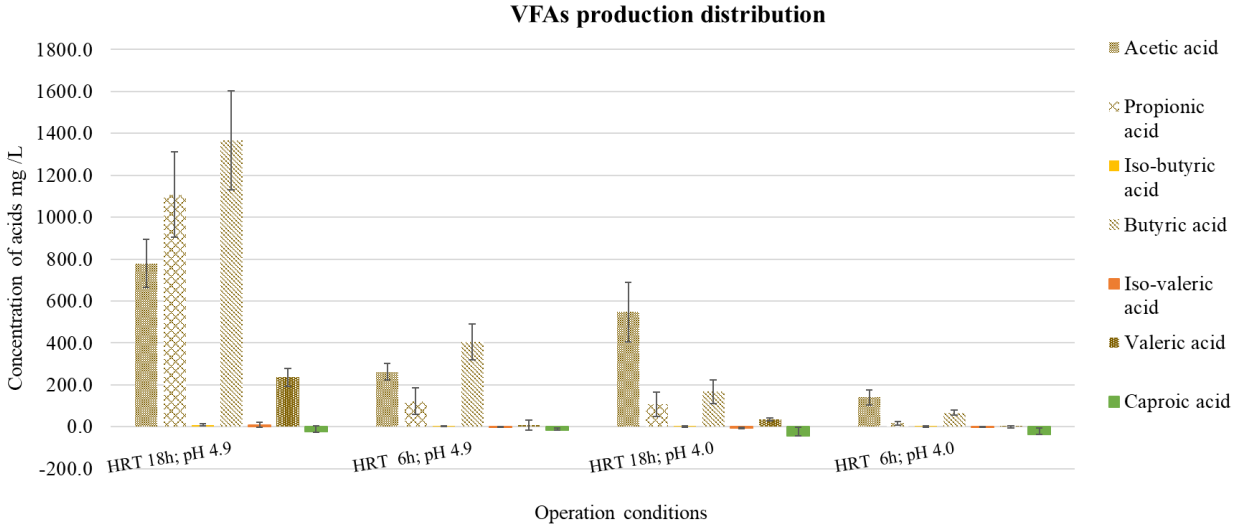

Figure 4.7 *Acids distribution with four operation conditions. Results from GC analysis, the average values after stabilization are chosen to present. (Confidence interval is under the significance level 0f 0.05.)*

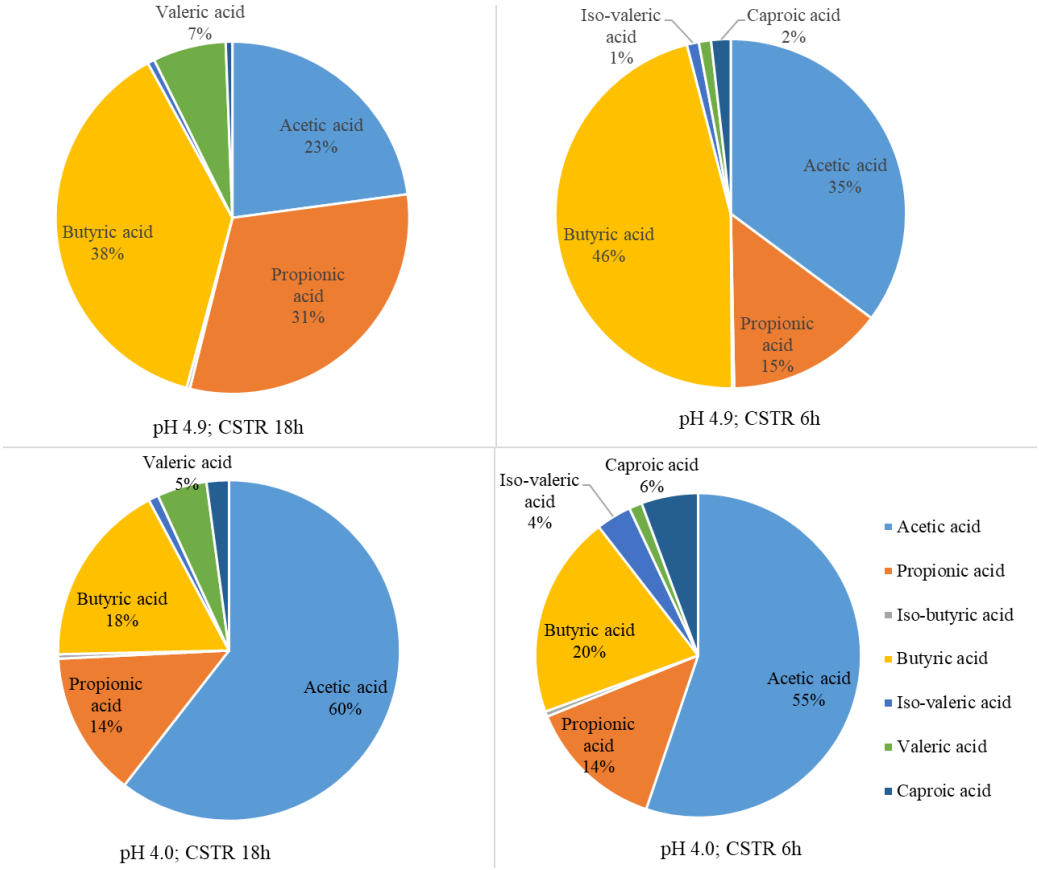

Figure 4.8 *The proportion of different acids of the total VFA, under four operation conditions.*

With regard to the influence from HRT. At the HRT of 18h, butyric acid, propionic acid acetic acids and valeric acid were the main acids produced in the reactors. With a reduced HRT to 6h, acetic acid, butyric acid were the main acids consist of the total VFA, while the proportion of propionic acid and valeric acid dropped dramatically. Another evidence is found in this work

in CSTR 6h, during day 30 to day 40, when the HRT increased up to 22h by operational mistake. Apart from the sudden rising of the total VFA production, the results from those days also shows a remarkable tendency of growing proportions of butyric acid, propionic acid and valeric acid. This scenario matched the result for CSTR 18h with the HRT of 18h, which indicates that the production of these three types of acids (butyric acid, propionic acid and valeric acid) was strongly correlated with the HRT. This can be confirmed from the report by Palomo-Briones *et al.* (2017), although they focused more on H_2 production. According to their reports, at a low HRT such as 12h and 6h, more soluble COD was transformed into H_2. It is consistent with relatively high yields of acetic acid and butyric acid, since those acids were metabolically linked to H_2 production. They also found that more lactate was produced at a HRT of 24 h. Lactate is fermented by lactic acid bacteria from lactose (Diamantis *et al.,* 2014). Therefore, more lactate was generated, which indicates there was more lactose produced at a higher HRT. This could further explain the high concentration and proportion of propionate in CSTR 18h in this work, since propionate is the main product of lactose fermentation, and should be further converted to acetate before methane production (Pakarinen *et al.,* 2011).

The decreased pH also resulted in significant changes of acids distribution. The acetic acid took the place of butyric acid, becoming the main component. At the same time, the population of propionic acids went down in CSTR 18h. This also matches the results from Jankowska *et al.* (2015), they found that at the pH of 4, acetic acid dominates the VFA, followed by propionate acid.

4.4.3 Other fermentation product

The COD transformation analysis in this work mainly focused on the observation of VFA product. However, other fermentation product such as formic acid and lactic acid could also be generated during acidification process. According to a single analysis for lactic acid in this work, there was noticeable lactic acid generated in CSTR 18h at the pH of 4.0. One evidence might be found in the Hach analysis for the VFA, it shows that the acids' production was stabilized after day 25 days, however, in the GC analysis, and there was more variation even after day 25. This is due to the fact that with the Hach analysis, all the organic acids were detected. While only VFA were analyzed with the GC method. It might indicate that apart from the VFA, there were other acids generated in the reactors. It is reasonable to assume that at the pH of 4.9 there was also a considerable amount of lactic acid produced.

4.4.4 Alkalinity consumption due to acids production

During the first 55 days the two reactors were operated at the pH of 4.9, 0.5M of NaOH solution was used to buffer the acids produced in both reactors. The consumption of NaOH was shown in Fig. 4.9, 36 mL NaOH per meq/L of the synthetic dairy wastewater was consumed at the HRT of 18h, while with the HRT of 6h, and only around 7 mL NaOH per meq/L of the influent was consumed. Furthermore, the NaOH consumption with regard to per mg of VFA is supposed to have approximately the same results under four conditions. However, results show that more NaOH was consumed at the pH of 4.9 than at the pH of 4.0 in both reactors, and more was consumed in the reactors with higher HRT than with the lower one. This might indicate that the

COD was not only transformed to those VFAs detected in this work, but also to other fermentation product, such as lactic acid. One evidence is that, a single analysis on lactic acid was conducted at the pH of 4.0, results shows that in CSTR 18h, there was 1200mg/L of lactic acid produced. Results shown in Appendix C.

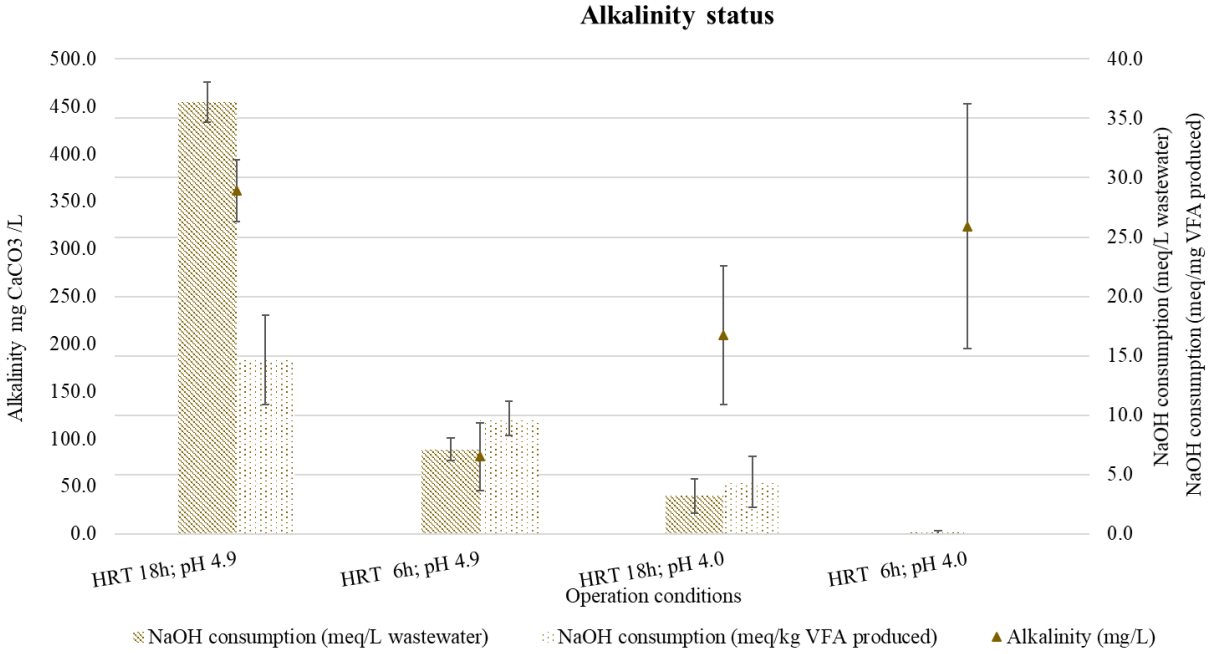

Figure 4.9 *Alkalinity status and NaOH consumption with regard to the wastewater and the VFA production. (Confidence interval is under the significance level of 0.05.)*

Besides, according to the results of alkalinity, when the reactors were operated at the pH at 4.9, the alkalinity level in reactors after acidification process dropped by 44% and 83% in CSTR 18h and CSTR 6h compared with the influent. The concentration of alkalinity in CSTR 18h (320.6 mg/L) was 4 times higher than that in CSTR 6h. This is because more acids were produced in CSTR 18h, which required more NaOH solution in order to keep the pH at 4.9, thus the alkalinity level was higher with the HRT of 18h. After the pH went down to 4.0, the alkalinity in CSTR 6h increased dramatically with an average value of 323.9 mg/L, while it decreased to 209 mg/L in CSTR 18h. This is because that with the influent going in the reactor, bringing around 580 mg/L of alkalinity (shown in *Table 4.1*), and the acidification degree (shown in *Fig. 4.6*) in CSTR 6h was only 5 %, which indicates that there was not enough acids produced to buffer the incoming alkalinity, therefore it increased significantly. The same reason for the scenario in CSTR 18h, but since the acidification degree is 15% there, there was still acids produced, there was NaOH going inside the CSTR 18h, therefore, the alkalinity decreased but not as much as that in CSTR 6h.

4.5 Further information during fermentation process

4.5.1 Biomass

The biomass condition in reactors could be analyzed by the solids. The results of solids contents (shown in *Fig.4.10* and *Table 4.3*) illustrate that, during the fermentation process, the total solids and total volatile solids in the reactors decreased after the pre-acidification process, while the suspended solids increased. The proportion of the volatile suspended solids in suspended solids could affect the efficiency of the fermentation process (Ewelina *et al.,* 2015). According to the results, the ratio of VSS to TSS in both reactors was high (ranging between 96.8 to 98.7%), meaning that most of the suspended solids consisted with biomass as well as compounds from the milk. Besides, from the microscope analysis (shown in *Fig. 4.11*), there were fermentative bacteria suspended and moving in the activated sludge in both reactors. The larger compounds between those bacteria in this case might due to the agglomeration fermentative microbes and the fat from the milk. Furthermore, when the pH was controlled as 4.9, more compound tended to agglomerate, this is because CSTR 6h had a higher OLR with the low HRT (see in *Fig. 4.1*). Similar scenario was also found in the study by Palomo-Briones *et al.* (2017), according to them an explanation could be that microorganisms tend to use the major fraction of the consumed substrate for biomass synthesis, in order to maintain themselves in the system under a short HRT (mainly 6 and 12 h), otherwise they will be washed out.

Figure 4.10 *Solid conditions with four operation conditions. In1 stands for CSTR 18h and In2 stands for CSTR 6h. (Confidence interval is under the significance level of 0.05.)*

Table 4.3 *The biomass condition with regard to the solids analysis. Percentage increase is the increase percentage of VSS in the reactors compared with the influents. (SD: Standard deviation. CI: Confidence interval under the significance level of 0.05).*

Experiment condition	**TS**	**VS**	**TSS**	**VSS**		**VSS/TSS**
	Content (g/ L)	Content (g/ L)	Content (g/ L)	Content (g/ L)	Percentage increase (%)	Ratio (%)
HRT 18h; pH 4.9	7.9 (SD 1.5)	5.5 (SD 1.8)	3.6 (SD 1.6)	3.4 (SD 1.6)	84 (CI 48)	97 (CI 2)
HRT 6h; pH 4.9	10.5 (SD 1.6)	9.5 (SD 2.0)	5 (SD 1.8)	5.2 (SD 1.6)	200 (CI 19)	98 (CI 0)
HRT 18h; pH 4.0	8.7 (SD 1.2)	7.8 (SD 1.2)	3.8 (SD 0.9)	3.8 (SD 1.6)	62 (CI 67)	98 (CI 1)
HRT 6h; pH 4.0	8.7 (SD 0.8)	8.1 (SD 0.8)	2 (SD 0.9)	1.9 (SD 1.6)	41 (28)	98 (CI 1)

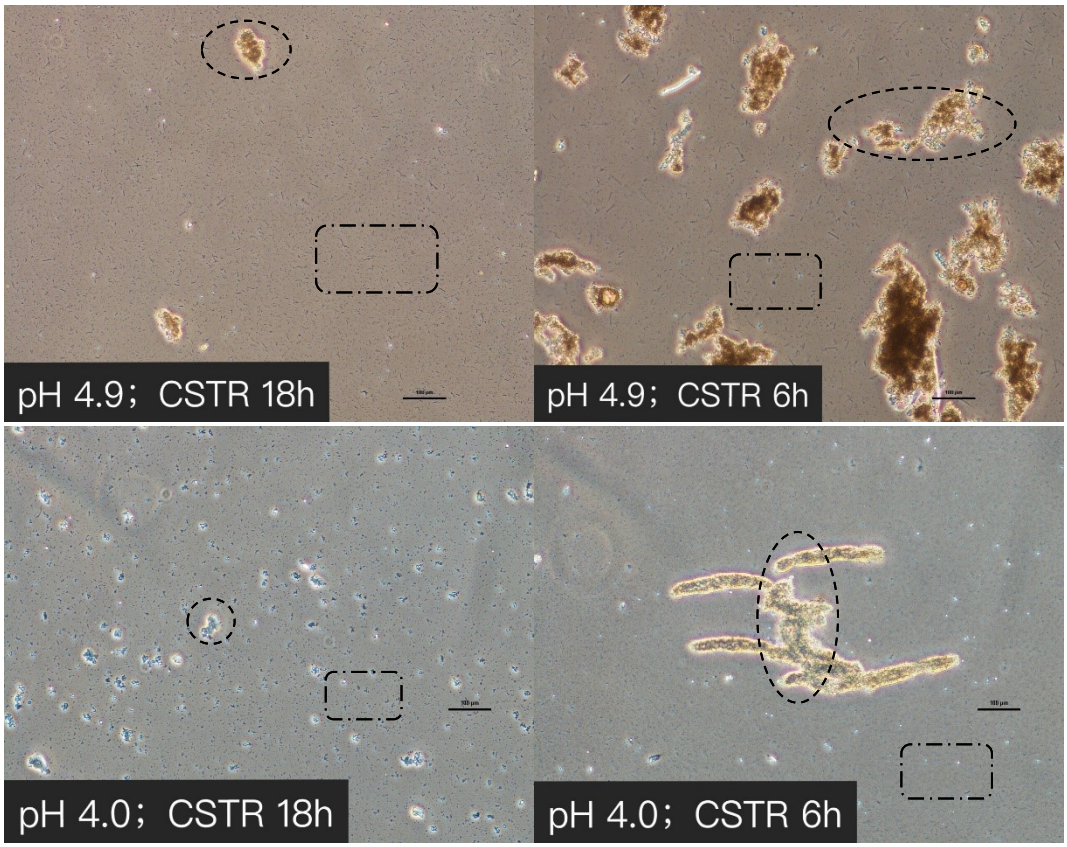

Figure 4.11 *the pictures of biomass under from microscope (with 10 times magnification). Those within the elliptical dotted line were examples of the flocculation, which might consist of bacteria or/ and fat from the milk. Those within the square chain dotted line were examples of the fermentative bacteria suspended in the water. Detailed pictures see Appendix B. Fig. B-1.*

With regard to the status of minerals during acidification, results (shown in *Fig. 4.11*, Detailed data for could be found in *Appendix B. Table B-2.*) shows that calcium and magnesium did not vary significantly with different conditions. However, the concentration of SO_4 in both reactors decreased after pre-acidification process. According to Chartrain *et al.* (1987), during acidification process, there is a common acid-producing bacterium, called vibrio desulfurization. With the presence of sulfates it could ferment lactic acid, ethanol into hydrogen, acetate, hydrogen sulfide and a small amount of ethanol. Therefore, the reduction of SO_4 might be due to the occurrence of vibrio desulfurization, which transformed the sulfate to sulfide. According to Yang *et al*. (2006), sulfuric acid reducing bacteria such as vibrio desulfurization could grow with a wide range of pH value (generally between 5.0 and 9.0), and Yang *et al.* also found that at the pH value of 5 is the best environmental conditions for their growth. While they could not survive at pH value less than 5 or more than 9. This could also explain that at the pH of 4.9, sulfate in both reactors decreased significantly compared with at the pH of 4.0.

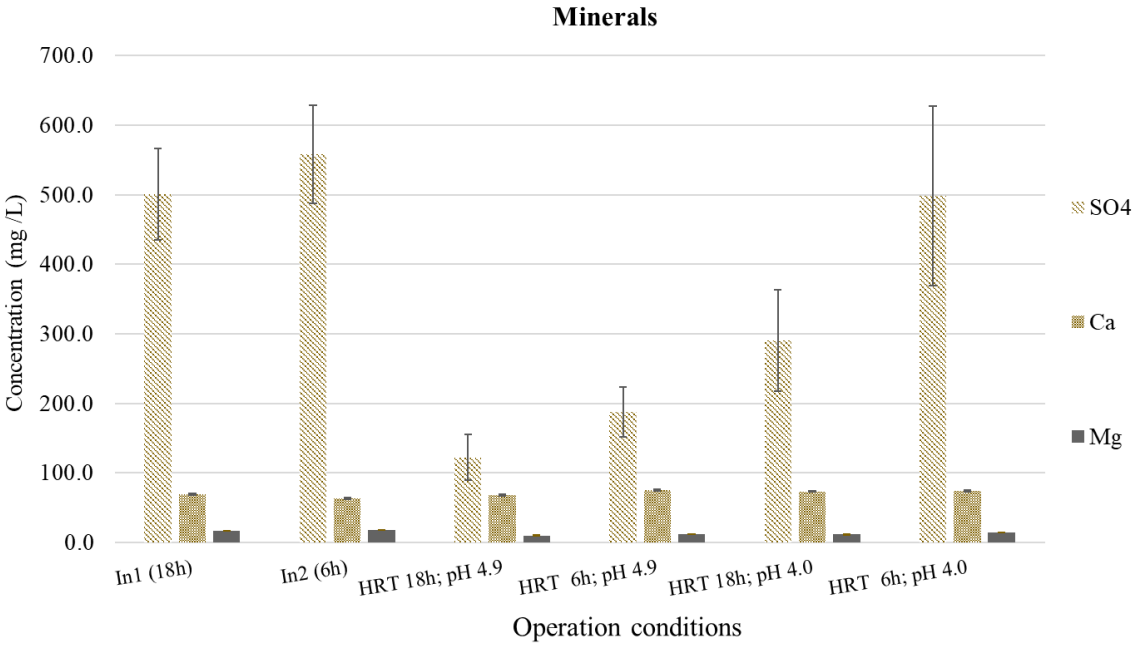

***Figure 4.12**. Status of SO4, Ca and Mg during pre-acidification. In1 stands for CSTR 18h and In2 stands for CSTR 6h. (Confidence interval is under the significance level 0f 0.05.)*

4.5.2 Nitrogen and phosphorus with regards to hydrolysis

Nitrogen and phosphorus in the dairy wastewater are mainly from the protein. Protein could be transformed into amino acids during hydrolysis, the total nitrogen and phosphorus existing in the system are supposed to stay constantly, while the ammonium nitrogen and phosphate are expected to increase due to the hydrolysis process (Yokota and Ikeda, 2017). As is shown in *Fig. 12* (detailed data for nitrogen and phosphorus could be found in *Appendix B. Table B-1)*, the concentration of the phosphate slightly increased in the reactors as expected, while ammonium nitrogen did not show a significant growth, it even decreased in CSTR 6h at the pH of 4.9. This result indicates that there is not much hydrolysis happening to the protein in this work. The total phosphorus increased in the reactors compared with the influent, one explanation could be that, phosphorus existed in the system not only with the form of protein but also with the form of bacterial cell. It is also noticeable that at the pH of 4.9, the soluble nitrogen decreased in both reactors, similar reason for this scenario as is for the decreased COD mentioned before, and this is due to the agglomeration of the protein particle at the acid environment.

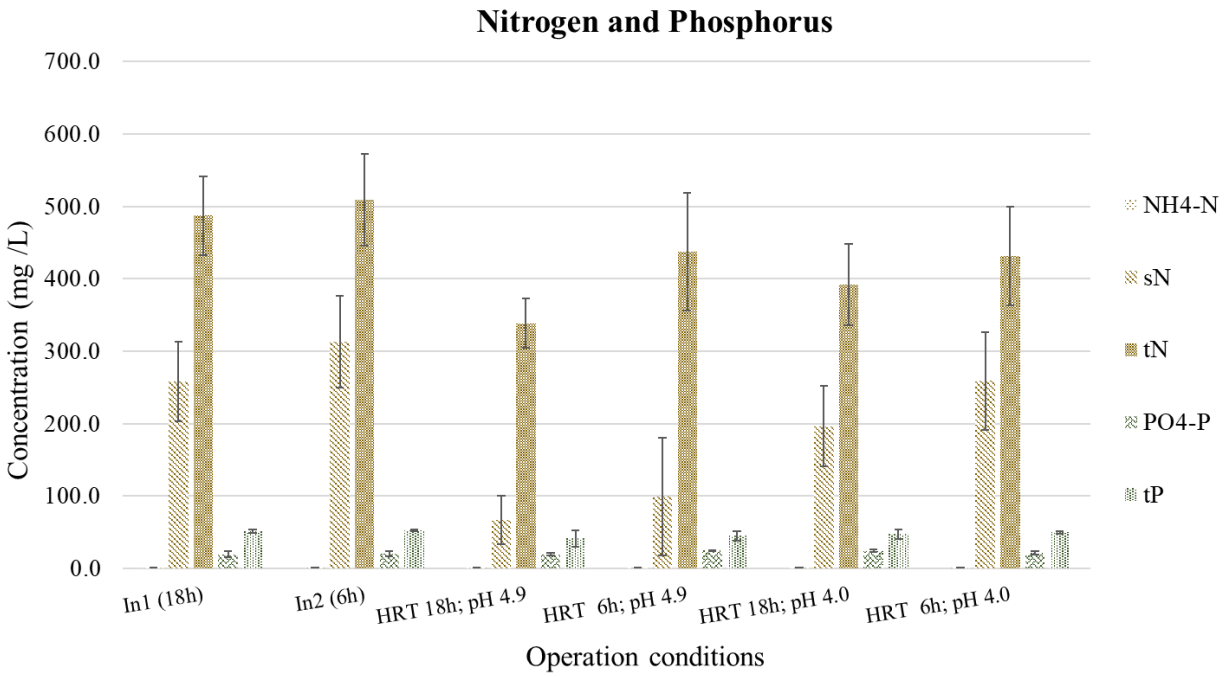

Figure 4.13 *Nitrogen and phosphorus status in CSTRs with four operation conditions. In1 stands for CSTR 18h and In2 stands for CSTR 6h. (Confidence interval is under the significance level of 0.05)*

5 Conclusions

In conclusion, both HRT and pH are crucial factors during the pre-acidification process in CSTRs. A sufficient HRT could enhance fermentation efficiency, with high degree of acidification and large amount of VFA production. In this case, the CSTR performed at an acidification degree up to 80% with the HRT of 18 h, it is reasonable to conclude that 18 h is suitable for the pre-acidification process, with this specific dairy wastewater. In comparison to 6 h, at the HRT of 18 h, butyric acid, propionic acid acetic acids and valeric acid were the main acids produced in the reactors. While at the HRT of 6 h, the CSTR produced less VFA with a lower acidification degree down to 15%, with acetic acid, butyric acid as the mostly generated acids. Moreover, the proportion of propionic acid and valeric acid dropped dramatically at the HRT of 6h.

The pH played a critical role for the VFA production and its distribution. A low pH at 4.0 remarkably inhibited the generation of VFA. Besides, the acetic acid took the place of butyric acid, becoming the main component, compared with at a pH of 4.9.

Furthermore, during the acidification process it is possible to make adjustments on HRT and/or pH in order to get specific types of acids.

6 Recommendations for future work

For the researchers, studies on more varying HRTs between 6 h and 18 h are recommended, in order to get an optimized HRT value for similar dairy wastewater. One suggestion for the influent is that, the pH buffer (such as NaOH solution) could be added directly to the influent instead of dosing it gradually into the reactors, since it might avoid additional experimental errors.

Furthermore, the lactic acid analysis is highly recommended in the future pre-acidification studies. There was a considerable amount of lactic acids detected in CSTR 18h at the pH of 4, the data at the pH of 4.9 is lacking in this work.

Additionally, appropriate reduction of the frequency on mineral analysis for Ca and Mg is recommended, since those two minerals did not vary visibly during this acidification process. Meanwhile the analysis of sulfite is recommended to add, since it might bring interesting facts and evidence to explain the reduction of sulfate in this work.

As for WWTPs, it is noteworthy that any changes in HRT and pH values could have impacts on the fermentation efficiency during the anaerobic digestion process. Besides, suitable HRTs and optimized pH values could help to determine and select different product.

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Appendix

Appendix A. COD transformation to fermentation product

1. Further information of COD conditions

Table A-1. COD conditions in the milk.

Milk (/100 mL)		**COD equivalent**		**g COD/L Milk**	**COD percentage**
Fat (g)	3.6	3	g COD/g of fat	108	51.8
Carbohydrates (g)	4.8	1.1	g COD/g of lactose	52.8	25.3
Proteins (g)	3.4	1.4	g COD/g of protein	47.6	22.8

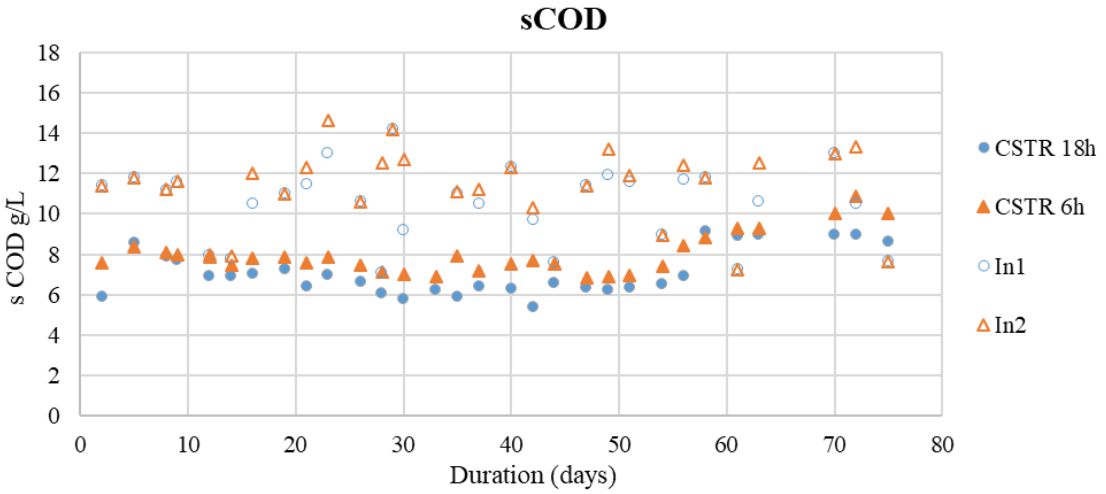


Figure A-1. The soluble COD concentration change in the influents and the reactors, among the entire duration. The soluble COD decreased in the reactors compared with the influents due to the agglomeration of the fat and protein compounds.

2. *Detailed information about the fermentation product with regard to VFA.*

Table A-2. Acids quantification.

Conversion Factors		**g/ mol**	**meq/ mmol**
C2（Acetic acid)	$C_2H_4O_2$	60.1	1
C3 (Propionic acid)	$C_3H_6O_2$	74.1	1
iC4 (Iso-butyric acid)	$C_4H_8O_2$	88.1	1
C4 (Butyric acid)	$C_4H_8O_2$	88.1	1
iC5 (Iso-valeric acid)	$C_5H_{10}O_2$	102.1	1
C5 (Valeric acid)	$C_5H_{10}O_2$	102.1	1
C6 (Caproic acid)	$C_6H_{12}O_2$	116.2	1

$$The\ total\ VFA\ concentration\ =\ Sum\ of\ all\ the\ acids'\ concentration$$

If the results from GC analysis are presented as the *Table A-3,* for example:

$$Total\ VFA\ = 1230 + 886 + 15 + 1593 + 30 + 297 + 32 = 4082\ (\frac{mg}{L})$$

$$COD_{eq} = 1230 * 1.07 + 886 * 1.51 + 15 * 1.51 + 1593 * 1.82 + 30 * 2.04 + 297 * 2.04 + 32 * 2.2 = 6316\ COD_{eq}\ mg/L$$

Table A-3. Result example from GC analysis.

Acetic acid	Propionic acid	Iso-butyric acid	Butyric acid	Iso-valeric acid	Valeric acid	Caproic acid)
mg/L						
1230	886	15	1593	30	297	32

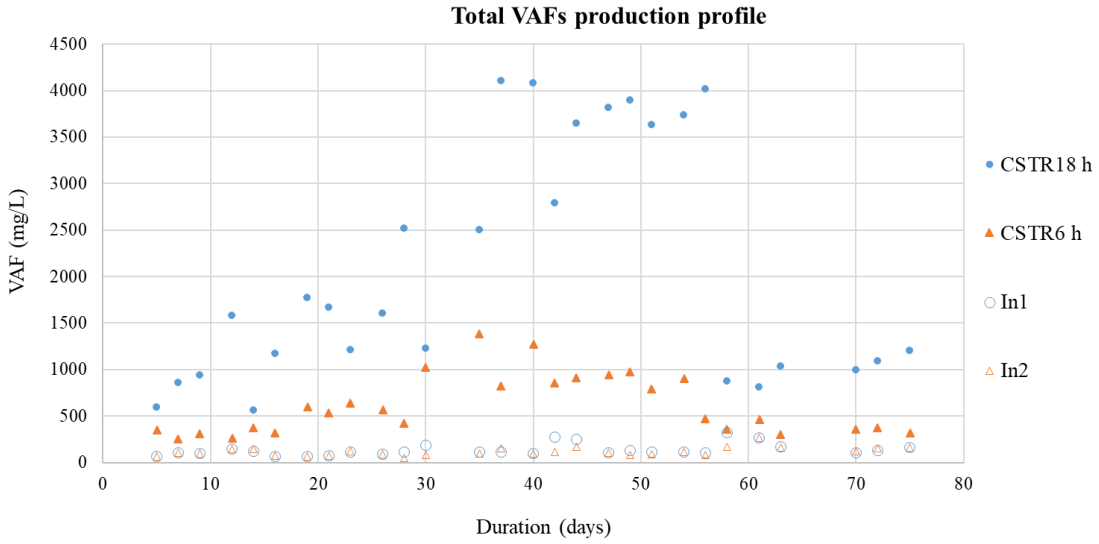


Figure A-2. VFAs production profile among the entire duration, showing that the VFA level in the influents was constantly low. (Results from GC analysis)

3. *Fermentation product - Lactic acid*

One single analysis for lactic acid on the last day (day 76) was conducted after the pH was adjusted down to 4.0. The results shows that there was 700 mg/L of lactic acid in the influent, and 1900 mg/L of it in the CSTR 18h. Therefore, there was 1284 $COD_{eq}\ mg/L$ of lactic acid produced in CSTR 18h on day 76, indicating that there could be significant amounts of lactic acid produced at the pH of 4.9 as well.

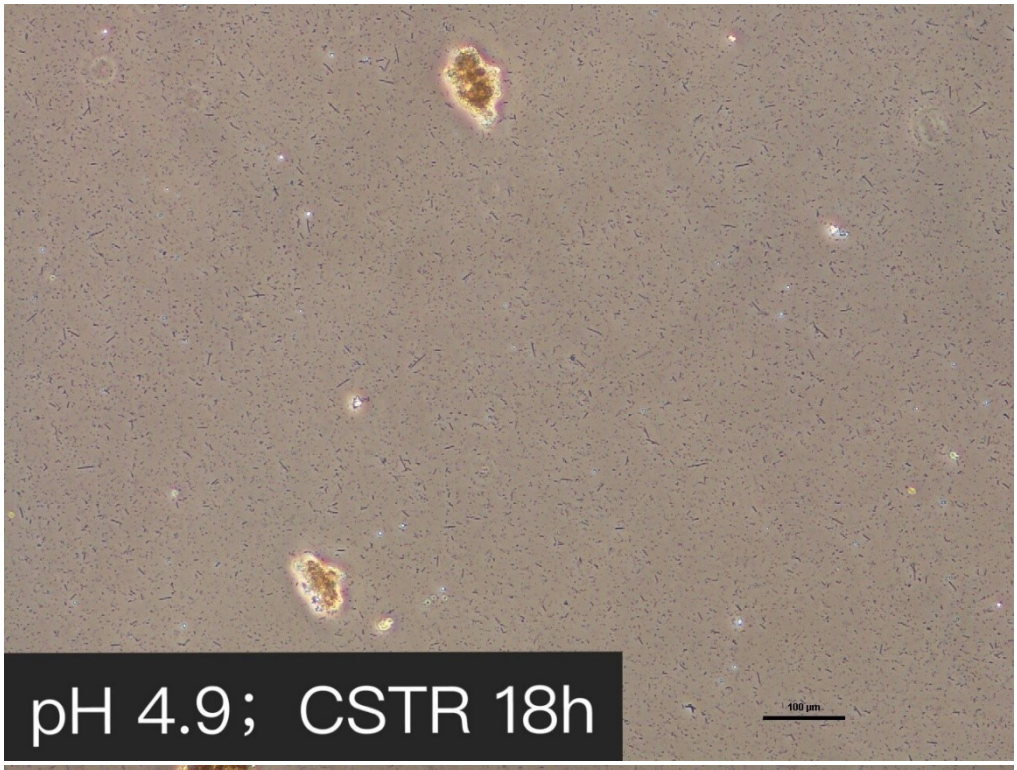
Calculation of lactic acid in the influent:

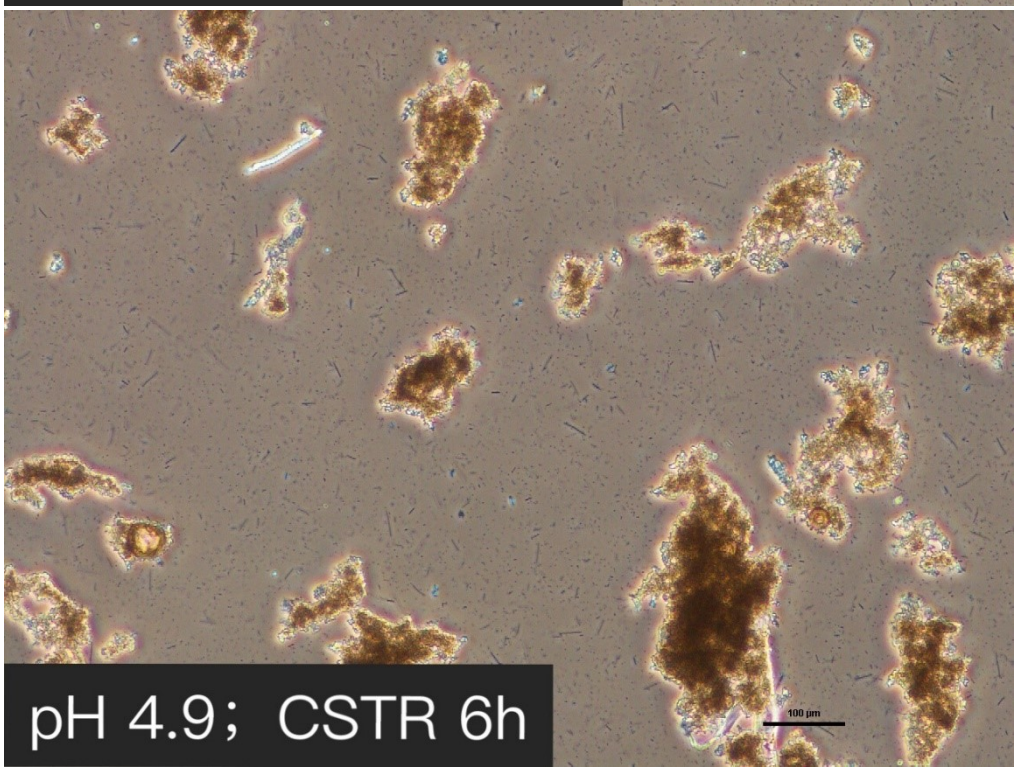
$$Lactic\ acid\ in\ the\ influent = 1.07 * 700 = 749\ COD_{eq}\ mg/L$$

Calculation of lactic acid in CSTR 18h:

$$Lactic\ acid\ in\ CSTR\ 18h = 1.07 * 1900 = 2033\ COD_{eq}\ mg/L$$

Appendix B. The specific data of compounds in the reactors.





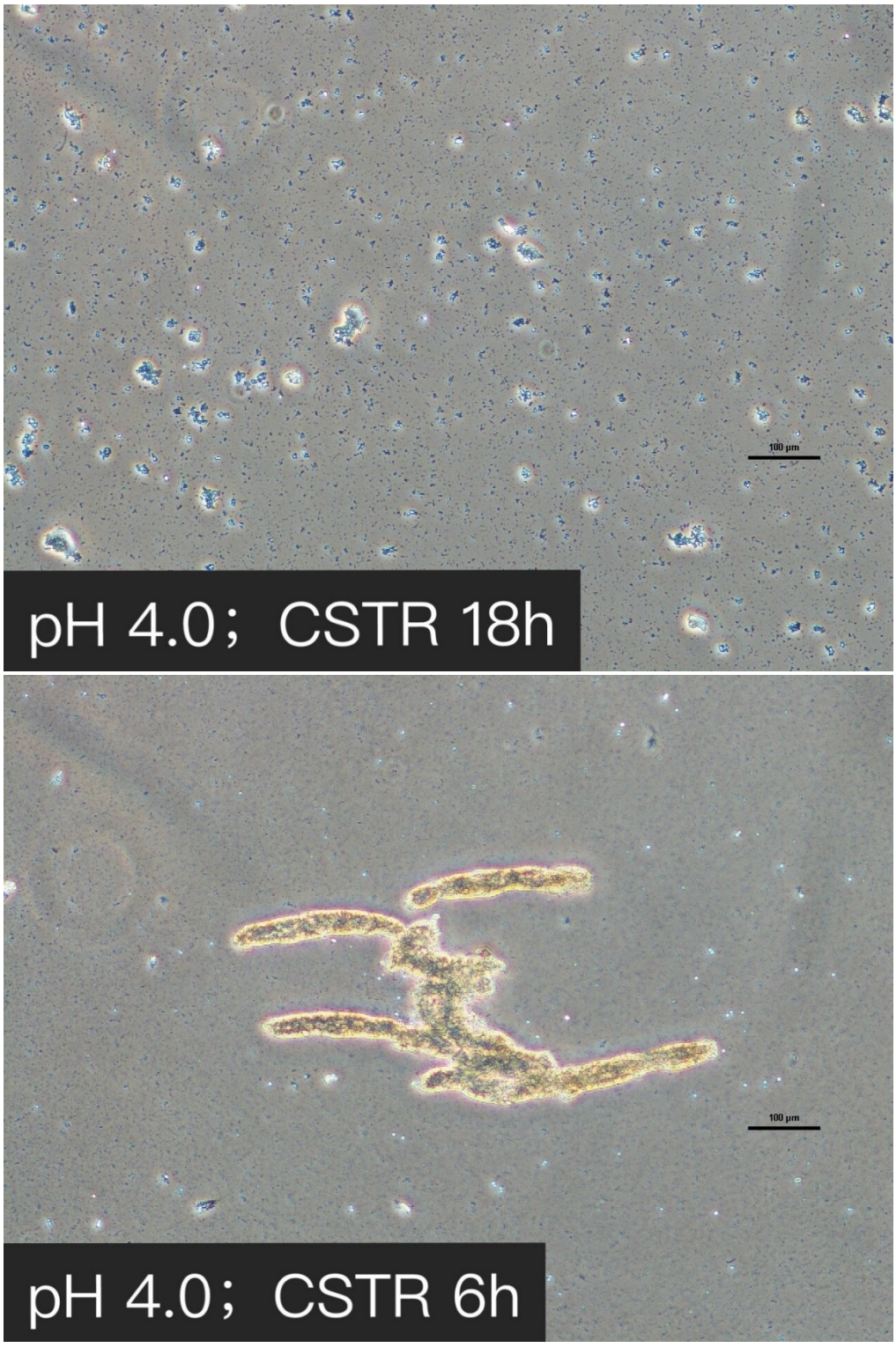


Figure *B-1. Detailed the pictures of biomass from microscope (with 10 times magnification).*

Table *B-1. The nitrogen and phosphorus status in CSTRs with four operation conditions. (+)/ (-) mean the concentration of that compound increased/ decreased respectively in the reactors compared with the influent.*

Experimental condition	Elements	Content (mg/L)	Average change (mg/L)
HRT 18h; pH 4.9	NH_4-N	1.2	(+) 0.2
	sN	66.9	(-) 191.1
	TN	338.6	(-) 148.6
	PO_4-P	19.6	(+) 0.2
	TP	41.6	(-) 9.8
HRT 6h; pH 4.9	NH_4-N	0.5	(-) 0.5
	sN	98.8	(-) 214.2
	TN	437.0	(-) 72.3
	PO_4-P	24.8	(+) 4.5
	TP	45.2	(-) 7.4
HRT 18h; pH 4.0	NH_4-N	0.8	(-) 0.2
	sN	196.3	(+) 61.7
	TN	392.0	(+) 95.2
	PO_4-P	24.1	(-) 4.7
	TP	47.2	(+) 4.2
HRT 6h; pH 4.0	NH_4-N	0.7	(+) 0.7
	sN	285.8	(+) 285.8
	TN	431.3	(+) 431.3
	PO_4-P	21.8	(+) 21.8
	TP	49.7	(+) 49.7

***Table** B-2. Status of SO_4 Ca and Mg in the influents and reactors.*

Experimental condition	SO_4 (mg/L)	Ca (mg/L)	Mg (mg/L)
In1 (18h)	500.4	68.9	16.6
In2 (6h)	558.5	63.3	17.6
HRT 18h; pH 4.9	122.3	68.1	9.7
HRT 6h; pH 4.9	187.4	75.2	11.9
HRT 18h; pH 4.0	290.0	73.4	11.3
HRT 18h; pH 4.0	473.3	73.6	14.1

Appendix C. Practical operation setting.

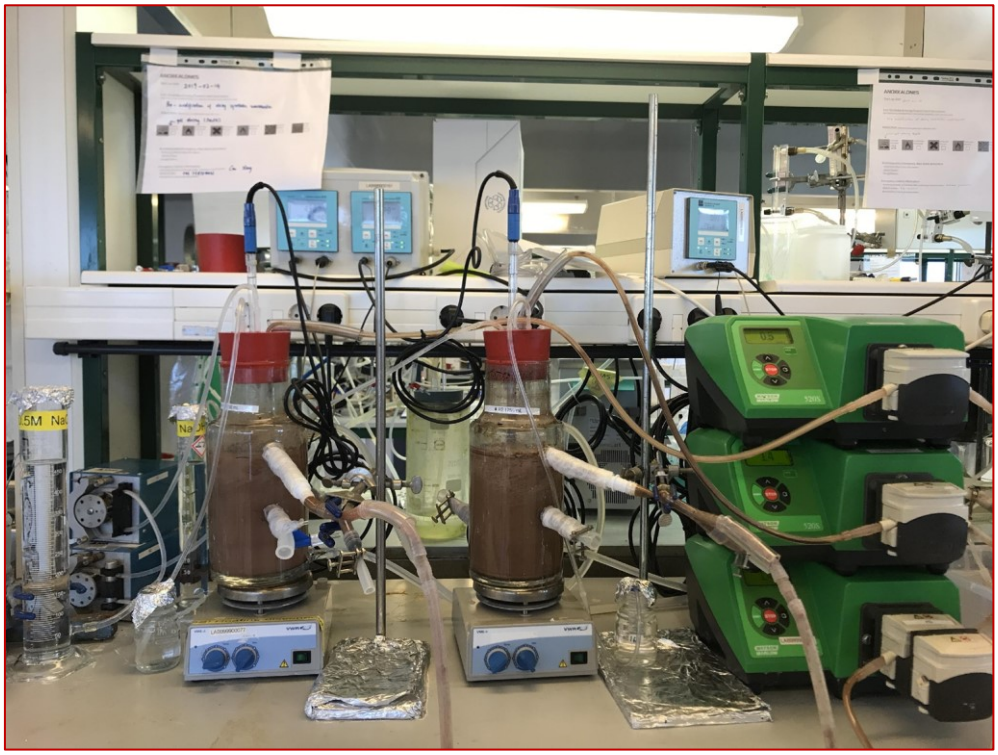


Figure C-1. Operational setting in the laboratory.

Popular science summary

Pre-acidification performances of a dairy wastewater under different hydraulic retention times and pH.

Pre-acidification is the initial process during anaerobic digestion treatment. This study evaluated different performances of pre-acidification with reduced hydraulic retention time (HRT) and pH value. Since these two factors not only affect the efficiency of pre-acidification, but also greatly related to financial issues during treatment processes.

The demand of dairy product grows rapidly, and dairy industries are accompanied by large amounts of dairy effluent. What are both efficient and cost-effective ways to treat dairy effluent? Anaerobic digestion (AD) as one of the biological methods is attractive because it could reduce sludge production, and produce utilized biogas. Pre-acidification is the initial process in AD with its main product of volatile fatty acids (VFA), this product is used to produce utilized biogas in further steps. What could be done during pre-acidification in order to save space and money? It is noteworthy that generally low HRT associates with financial benefits and low pH could reduce the cost for buffer solutions during pre-acidification, but it might also affect the performance of this process.

What would happen during pre-acidification if the HRT is decreased by 67% from the conventional HRT (18h), or if the pH value is decreased to 4.0, while the conventional pH is around 5? This project is part of a project carried out at company AnoxKaldnes, Veolia, Lund, focusing on the analysis of pre-acidification of a synthetic dairy effluent at reduced HRT (6h) and pH (4.0), in two parallel continuously stirred tank reactors (CSTR), in comparison with the longer HRT (18h) and higher pH (4.9) and it. Volatile fatty acids as the major product from pre-acidification were the most interesting parameters to observe and analyze.

According to the results, both HRT and pH are crucial factors impacting the pre-acidification process, with regard to VFA production. In this case, the longer HRT resulted in larger amounts of VFA generated. The CSTR performed at an acidification degree up to 80% with the HRT of 18 h, it is reasonable to state that 18 h is suitable for the pre-acidification process, with this specific dairy wastewater, while 6h is too short for the reactor to perform well. Studies on more varying HRTs between 6 h and 18 h could be more interesting in future work.

Besides, the results indicates that pH buffering is important during pre-acidification. A low pH at 4.0 remarkably inhibited the generation of VFA, compared with the pH of 4.9. As for the acids distribution, at a higher pH value, butyric acid and propionic acid were mostly generated in the reactors, while at the pH of 4.0, the acetic acid became the main component in the produced VFA. It indicates that it is possible to make adjustments on HRT and/or pH in order to get specific types of acids during the pre-acidification process.