MICROBIAL BIOTECHNOLOGIES FOR PRODUCTION OF BIOCHEMICALS INTERMEDIATES, BIOENERGY AND BIOMATERIALS

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Thesis

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TABLE OF CONTENTS

LIST OF TABLES

concentration of 0 g NaCl L^{-1}, 10 g NaCl L^{-1} and 20 g NaCl L^{-1}. The composition of the obtained polymer is also reported as HB:HV ratio (% w w^{-1}).

Table 7.3.2 PHA percentage and concentration at the end of each feeding pulse of the accumulation tests with MMCs acclimatized at salinity of 10 g NaCl L^{-1}, under salinity concentration of 0g NaCl L^{-1}, 10 g NaCl $L^{-1}$and 20 g NaCl L^{-1}. The composition of the obtained polymer is also reported as HB:HV ratio (% w w^{-1}).

Table 7.3.3 Organic acids concentration in the fermented cheese whey used as feeding substrate. The organic acids are divided in HB and HV precursors respectively in order to calculate an expected polymer composition based on the feed.

LIST OF FIGURES

inoculum.

Figure 4.1. Initial microbial characterization of the cheese whey, buttermilk and mixture (ratio 2:1).

Figure 4.2. H_2 (white symbols) and CH_4 (black symbols) produced during incubation time from test A (triangle) and test B (square).

Figure 4.3. Concentrations of lactose, galactose, lactic acid, acetic acid, ethanol and propionic acid in samples collected from test A (A) and test B (B).

Figure 4.4. Counts of the principal microbial groups in test A (A) and test B (B) and counts of methanogen and LAB (C) in both tests during incubation time.

Figure 4.5. Molecular analysis by the culture-independent method (PCR-DGGE) – bacterial community structure. The DGGE band patterns (A) and the cluster analysis (B) were representative of the samples collected from test A and test B at different days of the incubation time. Lanes: 1, animal manure inoculum (I); 2, test A at initial time (T0 A); 3, test A at day 7 (T7 A); 4, test A at day 14 (T14 A); 5, test A at day 21 (T21 A); 6, test A at day 28 (T28 A); 7, test B at initial time (T0 B); 8, test B at day 7 (T7 B); 9, test B at day 14 (T14 B); 10, test B at day 21 (T21 B); 11, test B at day 28 (T28 B).

Figure 4.6. Molecular analysis by the culture-independent method (PCR-DGGE) – archaeal community structure. The DGGE band patterns (A) and the cluster analysis (B) were representative of samples collected from test A and test B at different days of the incubation time. Lanes: 1, Marker: a: *Methanococcus voltae*; b: *Methanobrevibacter ruminantium*; c: *Methanobacterium congolense*; d: *Methanosarcina acetivorans*; e: *Methanosarcina barkeri*; f: *Methanosarcina mazei*; 2, animal manure inoculum (I); 3, test A at initial time (T0 A); 4, test A at day 7 (T7 A); 5, test A at day 14 (T14 A); 6, test A at day 21 (T21 A); 7, test A at day 28 (T28 A); 8, test B at initial time (T0 B); 9, test B at day 7 (T7 B); 10, test B at day 14 (T14 B); 11, test B at day 21 (T21 B); 12, test B at day 28 (T28 B).

Figure 5.1. Viable counts of main bacterial groups evaluated in samples of cheese whey, buttermilk and their mixture. The error bars represent the means ± SD of two replicates.

Figure 5.2. Cumulative specific production of H_2 and CH_4 monitored during 30 days of incubation. X-axis displays the day, and the Y-axis is the corresponding cumulative H_2 or CH_4 specific production per gram of the initial concentration of VS of the dairy waste mixture. The error bars represent the means ± SD of two replicates.

Figure 5.3. pH and concentration of lactose, lactic acid and ethanol (a), and concentration of acetic acid and propionic acid (b) in liquid samples collected from biodigesters at different times of incubation.

Figure 5.4. Abundance of bacterial families during the anaerobic process. Only OTUs with an incidence > 1% in at least one sample are shown. Abbreviations: Mix_T0, mixture of cheese whey and buttermilk at day 0 of incubation; MixI_T0, mixture of cheese whey and buttermilk inoculated at day 0 of incubation; B1_T7, sample collected from B1 after 7 days of incubation; B2_T7, sample collected from B2 after 7 days of incubation; B1_T14, sample collected from B1 after 14 days of incubation; B2_T14, sample collected from B2 after 14 days of incubation; B1_T30, sample collected from B1 after 30 days of incubation; B2_T30, sample collected from B2 after 30 days of incubation.

Figure 5.5. Neighbor-joining tree for the representative *Clostridiaceae* and *Tissierellaceae* OTUs (representatives with relative abundance > 0.1%). OTUs from this study were shown in bold reporting in brackets the total relative abundance. The sequence accession numbers of reference sequences from NCBI used for the phylogenetic analysis are shown in *parentheses* following the species name. Bootstrap values (> 50%, expressed as percentages of 1,000 replications) are given at the nodes. The scale bar estimates the number of substitutions per site.

Figure 5.6. Principal Coordinates Analysis of weighted UniFrac distances for bacterial (panel a) and archaeal (panel b) 16S rRNA gene sequence data of samples during the incubation process. Colour label. red: mixture of cheese whey and buttermilk at day 0 of incubation; green: samples collected after 7 days of incubation; blue: samples collected after 14 days of incubation; orange: samples collected after 30 days of incubation.

Figure 5.7. Abundance of archaeal genera during the anaerobic process. Only OTUs with an incidence > 1% in at least one sample are shown. Abbreviations: Mix_T0, mixture of cheese whey and buttermilk at day 0 of incubation; MixI_T0, mixture of cheese whey and buttermilk inoculated at day 0 of incubation; B1_T7, sample collected from B1 after 7 days of incubation; B2_T7, sample collected from B2 after 7 days of incubation; B1_T14, sample collected from B1 after 14 days of incubation; B2_T14, sample collected from B2 after 14 days of incubation; B1_T30, sample collected from B1 after 30 days of incubation; B2_T30, sample collected from B2 after 30 days of incubation.

Figure 5.8. Neighbor-joining tree for the representative *Methanoculleus* OTUs (representatives with relative abundance > 0.1%). OTUs from this study were shown in bold reporting in brackets the total relative abundance. The sequence accession numbers of reference sequences from NCBI used for the phylogenetic analysis are shown in *parentheses* following

the species name. Bootstrap values (> 50%, expressed as percentages of 1,000 replications) are given at the nodes. The scale bar estimates the number of substitutions per site.

Figure 6.3.1 Microbial growth of different strains of *C. necator* (a,b) *Pseudomonas* spp. (c,d) and *Azotobacter* spp. (e,f) on specific optimal medium without (a,c,e) and with (b,d,f) the synthetic acids addition.

Figure 6.3.2 Microbial growth of different of *C. necator* 13513, 531 and 428 on the optimal medium (LB) with the addition of $VFA_{extracted}$.

Figure 6.3.3 Microbial growth of different strains of *P. oleovorans* DSM 21016, *P. fluorescens* DSM 50415, *P. resinovorans* SS233, SA38 and SA39 on the optimal medium (TSB) with the addition of $VFA_{extracted}$.

Figure 6.3.4 *C. necator* DSM 13513 growth in the batch tests with LB as optimal medium (a) and with the VFA (b) addition in terms of microbial count (log CFU mL^{-1}) and PHB production (%) during 48 h of incubation at 30 °C.

Figure 6.3.5 Observations with fluorescence microscope (Nikon Eclipse E400) of *C. necator* DSM 13513 growth after 9 (a), 24 (b), 30 (c) and 48 h (d) in the batch tests with the synthetic VFA addition.

Figure 6.3.6 *P. resinovorans* SA38 growth in the batch tests with TSB as optimal medium (a) and with the VFA (b) addition in terms of microbial count (log CFU mL^{-1}) and PHB production (%) during 48 h of incubation at 28 °C.

Figure 6.3.7 Observations with fluorescence microscope (Nikon Eclipse E400) of *P. resinovorans* SA38 growth after 9 (a), 24 (b), 30 (c) and 48 h (d) in the batch tests with the synthetic VFA addition.

Figure 6.3.8 *P. resinovorans* SA39 growth in the batch tests with TSB as optimal medium (a) and with the VFA (b) addition in terms of microbial count (log CFU mL^{-1}) and PHB production (%) during 48 h of incubation at 28 °C.

Figure 6.3.9 Observations with fluorescence microscope of *P. resinovorans* SA39 growth after 9 (a), 24 (b), 30 (c) and 48 h (d) in the batch tests with the synthetic VFA addition.

Figure 6.3.10 *C. necator* DSM13513 growth in the batch tests with TSB and with the $VFA_{extracted}$ addition in terms of microbial count (log CFU mL^{-1}) and PHB production (%) during 48 h of incubation at 30 °C.

Figure 6.3.11 Observations under optical and fluorescence microscope (Axiovert 200M, Zeiss) of *C.necator* DSM 13513 growth after 9 (a, e), 24 (b, f), 30 (c, g) and 48 h (d, h) in the batch tests with the $VFA_{extracted}$ addition.

Figure 6.3.12 *P. resinovorans* SA39 growth in the batch tests with TSB and with the $VFA_{extracted}$ addition in terms of microbial count (log CFU mL^{-1}) and PHB production (%) during 48 h of incubation at 28 °C.

Figure 6.3.13 Observations under optical and fluorescence microscope (Axiovert 200M, Zeiss) of *P. resinovorans* SA39 growth after 9 (a, e), 24 (b, f), 30 (c, g) and 48 h (d, h) in the batch tests with the $VFA_{extracted}$ addition.

Figure 7.3.1 Trend of DO concentration (mg L^{-1}) from the tests with MMCs acclimatized at salinity of 20 g NaCl L^{-1}, under salinity concentration of 0g NaCl L^{-1} (a), 10 g NaCl L^{-1} (b) and 20 g NaCl L^{-1} (c). The black dashed lines indicate the time of fermented cheese whey addition in pulses.

Figure 7.3.2 Trend of DO concentration (mg L^{-1}) from tests with MMCs acclimatized at salinity of 10 g g NaCl L^{-1}, under salinity concentration of 0g g NaCl L^{-1} (a), 10 g g NaCl L^{-1} (b) and 20 g NaCl L^{-1} (c). The black dashed lines indicate the time of fermented cheese whey addition in pulses.

Figure 7.3.3 Observations under optical (a,b,c) and fluorescence microscope (d,e,f) before each feeding pulse (I; a, d; II; b, e; III; c, f) in the experiment with MMCs adapted at 20 g NaCl L^{-1} under salinity condition of 10 g NaCl L^{-1}.

Figure 7.3.4 Observations under optical (a,b,c) and fluorescence microscope (d,e,f) before each feeding pulse (I; a, d; II; b, e; III; c, f) in the experiment with MMCs adapted at 10 g NaCl L^{-1} under salinity condition of 10 g NaCl L^{-1}.

Figure 7.3.5 Biopolymers recovered from the pellet obtained at the end of the accumulation tests. a) d) and b) are examples of the bioplastic disk obtained; c) Bioplastic disks detached from the Petri dishes; e) demonstration of the elastomeric properties of the bioplastics obtained.

LIST OF ABBREVIATIONS

3HV: 3-hydroxyvalerate;

4HB: 4- hydroxybutyrate;

AO: alginate oligosaccharides;

BMP: bio- methane potential test;

C/N: carbon to nitrogen ratio;

CDW: cell dry weight;

COD: chemical oxygen demand;

CWs: cellulose wastes;

EPS: exopolysaccharides;

GHG: green house gas;

HRT: hydraulic retention time;

LCA: life cycle assessment;

MCL: medium chain- length;

MMC: mixed microbial cultures.

OFMSW: organic fraction of municipal solid waste;

OLR: organic loading rate;

P(3HB): poly(3-hydroxybutyrate);

P(3HB-3HV): poly(3-hydroxybutyrateco- 3-hydroxyvalerate);

PCR-DGGE: polymerase chain reaction denaturing gradient gel electrophoresis;

PHAs: polyhydroxyalkanoates;

PHB: polyhydroxybutyrate;

PHBV: poly (hydroxybutyrate-co-hydroxyvalerate);

S/I: substrate/inoculums ratio;

SCL: short-chain-length;

SRT: solid retention time;

TS: total solids;

TTA: total titratable acidity;

UASB: upflow anaerobic sludge blanket;

VFAs: volatile fatty acids;

VS: volatile solids;

WBM: Mixture of cheese whey and buttermilk;

CHAPTER 1

Brief thesis presentation

1.1 Short overview

Recently, issues concerning the sustainable and harmless disposal of organic solid waste have generated interest in microbial biotechnologies aimed at converting waste materials into bioenergy and biomaterials, thus contributing to a reduction in economic dependence on fossil fuels.

In particular, the development of high-performance microbial strains to bioenergy and biomaterials production using organic by-products and waste, could reasonably make their production costs comparable to those required by fossil fuels and petrochemical-derived plastics, thus promoting their use.

For this purpose, promising food processing by-products are dairy wastes, such as cheese whey and buttermilk, because they can be conveniently processed and valorized in a biorefinery value chain since they are abundant, zero-cost and all year round available. For this reasons, in the recent past, many studies have been focused on the use of dairy waste for the production of either bioenergy or biopolymers separately. On the contrary, only few studies have been focused on a new integrated system based on a series of anaerobic and aerobic stages aimed at yielding biogas and/or hydrogen coupled with polyhydroxyalkanoates (PHA) production. In fact, in the same processing chain, the volatile fatty acids (VFAs) resulting from the acidogenic and acetogenic steps of the anaerobic digestion are used as substrates for aerobically producing PHA. In this context, a comprehensive knowledge of the microbial species involved in producing biological gases (biohydrogen and biogas) and valuable intermediates (VFAs) from dairy wastes is necessary to further improve the performance of the integrated system. Therefore, critical aspects, designing as well as managing criteria and future perspectives of this integrated system are handled and discussed as topics in this PhD thesis.

1.2 Aims and outline

The objective of this research was achieved by developing the experimental work in two main stages, respectively focused on the following intermediate aims: i) enhance the current knowledge on the microbial communities involved in the anaerobic digestion of dairy wastes; ii) address the valuable chemical intermediates (VFAs) resulting from the anaerobic phase to polyhydroxyalkanoates (PHAs) synthesis. Therefore in the first stage of the research, it was

investigated the role of microbial groups in the anaerobic processes and the correlation between the intermediates or end-products (e.g., H_2, CH_4 and VFAs) with the bacterial groups during the anaerobic process, whereas through the second stage it was examined the PHAs formation from the organic acids resulting from the acidogenic and acetogenic phases (anaerobic process) using pure cultures as well as mixed. This research led to intermediate results that are reported in the different chapters that compose this thesis according to the following structure:

In ***Chapter 2***, an overview of the suitable substrates and microbial strains used in low-cost polyhydroxyalkanoates as well as biohydrogen and biogas production is given with the possibility of creating a unique integrated system. The results of a preliminary study on the anaerobic digestion of cheese whey and buttermilk collected from a buffalo mozzarella cheese factory located in Casoria in the Campania region (Italy) under different operating conditions (inoculum percentage and pH) are reported in ***Chapter 3***: the inoculum concentrations was set between 1-5% (*w/v*), natural acid conditions were set and different microbial groups related to biogas production were selected. In order to limit the use of the inoculum and make the process performance independent on its availability, in ***Chapter 4*** it was investigated the effect of inoculum ranging between 1-3% (*w/v*) on the anaerobic digestion of dairy waste. This effect was evaluated by monitoring the microbial growth and communities' structure with culture-dependent and independent methods: many archaeal species, mostly involved in the production of CH_4, were identified by sequencing denaturing gradient gel electrophoresis (DGGE) bands. Since the DGGE analysis, although is highly efficient, analyze a limited number of aspects if compared with the emerging metagenomic approaches based on high-throughput sequencing (HTS), in ***Chapter 5***, it was examined the use of a polyphasic approach including HTS in lab-scale batch tests addressed to follow the microbiota dynamic in different stages of the anaerobic process fed with cheese whey and buttermilk. The fermented cheese whey obtained in these last experiments with high concentrations of organic acids, were characterized and used in further assays aimed to PHAs accumulation. Actually, in ***Chapter 6*** pure culture of PHAs accumulating bacteria were screened for their ability to grow and accumulate PHAs by using the fermented cheese whey (organic acids resulting from the previous experiments) and comparing their performance with synthetic pure acids. In order to promote low cost processes for PHAs production, in ***Chapter 7***,the use of mixed microbial cultures (MMC) in producing PHAs was tested: bacteria were selected from the activated sludge of a wastewater treatment plant (Mutela, Portugal) using as carbon source a fermented cheese whey at different level of salinity. The last chapter, ***Chapter 8***, presents a critical synthesis of the main findings from the research and conclusions based on knowledge generated.

CHAPTER 2

Literature Review

This chapter has been published as:

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2.1 Introduction

Over the past few decades, the need to reduce pollutant emissions produced by conventional systems of organic waste disposal has promoted the development of technologies that convert organic waste into bioenergy and biomaterials. In the near future, this new approach in waste management, in addition to being eco-friendly, can reasonably replace fossil fuels with biomass (organic waste or energy crops) as a source of both energy and materials (e.g., plastics) and therefore make two contributions toward reducing greenhouse gas (GHG) emissions into the atmosphere (Bauen et al., 2009).

Petrochemical-derived materials can be replaced with biodegradable materials and biochemicals derived from renewable sources. In fact, organic waste materials are interesting renewable resources that can be converted into different value-added products, such as bioethanol or biochemicals obtained by sugar fermentation (Mezule et al., 2015; Liguori et al., 2016). Recent technological developments have explored the value of biochemical products as precursors to biopolymers, e.g., succinic acid (Ventorino et al., 2016a; Ventorino et al., 2017) and 2, 3-butanediol (Saratale et al., 2016) derived from lignocellulosic biomass. Some biopolymers can be produced by microorganisms from the accumulation of extracellular materials, such as exopolysaccharides (EPS) (Pepe et al., 2013), and used in the food, chemical, cosmetic, and packaging industries as adhesives, absorbents, lubricants, and cosmetics. Furthermore, several biopolymers, such as polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, and polysaccharides (Lee 1996), have already been successfully tested as bioplastics (Steinbüchel et al., 1998) because their physical and chemical properties perform just as well as conventional synthetic plastics. Among them, PHAs have gained much attention thanks to their complete biodegradability under various conditions within a period of one year (Cavalheiro et al., 2009). Different bacteria (e.g., *Alcaligenes* spp., *Azotobacter* spp., methylotrophs, *Pseudomonas* spp., *Bacillus* spp., and recombinant *Escherichia coli*) have been used in PHA production from different low-cost substrates. In fact, to replace conventional petrochemical-derived plastics, useful substrates for PHA production include organic waste and by-products. In fact, to commercialize PHAs, substantial effort has been devoted to reducing the production cost through the development of bacterial strains and more efficient fermentation/recovery processes because the price of the substrate has the largest influence on the production cost of PHA (Salehizadeh et al., 2004).

To make PHA production more feasible for industrial application, future prospects are mainly focused on promoting less expensive substrates, improved microorganism cultivation strategies,

and easier downstream processing methods, which are required for reducing production costs (Ahn et al., 2001). For this reason, different inexpensive substrates, such as molasses and sucrose, starch-based materials, cellulosic and hemicellulosic materials, sugars, whey, oils, fatty acids and glycerol, and organic matter from waste and wastewater (Castilho et al., 2009), have been tested to produce biopolymers, and the results are promising.

Furthermore, it is important to highlight that the same substrates used to produce biopolymers represent a source of renewable energy (biomethane and biohydrogen) obtainable through an anaerobic digestion process. Therefore, such substrates can be simultaneously used to produce bioenergy and biopolymers, thus achieving a maximum valorization when they are used as organic waste.

The anaerobic digestion process is characterized by biochemical reactions in series carried out by different consortia of bacteria that convert organic compounds into methane, carbon dioxide, water, and ammonia. In the first step, complex and not negligibly sized biomolecules of organic materials are disintegrated and subsequently hydrolyzed into soluble, biodegradable organics by extracellular enzymes (Panico et al., 2014). Then, acidogenic microorganisms metabolize products by hydrolysis into volatile fatty acids (VFAs) (Acidogenic phase) (Sans et al., 1995). Acidogenic products are first converted into acetic acid, hydrogen, and carbon dioxide (Acetogenic phase) and, finally, into methane by methane-producing Archaea (Methanogenic phase) (Chynoweth et al., 2001). The same substrates of methanogenic metabolism are the precursors that form PHAs (Patel et al., 2011). Thus, this review gives insights into the current methodology for producing PHAs and biogas, with a focus on the use of organic waste and by-products as raw materials to keep production costs low. Moreover, this review examines the potential of several biological processes that can occur in the development of an innovative unique integrated system able to simultaneously produce bioenergy and biopolymers.

2.2 Bio-based and biodegradable polymers: PHAs production and classification

Polyhydroxyalkanoates (PHAs) represent a group of bio-based and biodegradable polymers, considered similar to petroleum-based polymers (Carvalho Morais, 2013).

Many bacteria, such as *Cupriavidus* (*C.*) *necator* (Koutinas et al., 2007; Xu et al., 2010; Haas et al., 2008; Yu et al., 2008; Cavalheiro et al., 2009; Fukui and Doi, 1998; Kahar et al., 2004; Füchtenbusch et al., 2000; Yu, 2001; Wang and Yu, 2007) different *Pseudomonas* (*P.*) species *(P. fluorescens, P. hydrogenovora, P. oleovorans, P. resinovorans, P. aeruginosa, P. mendocina)* (Jiang et al., 2007a; Koller et al., 2008; Füchtenbusch et al., 2000; Cromwick et

al., 1996; Hori et al., 2002; Guo et al., 2011), strains belonging to *Azotobacter* (*A.*) species (*A. vinelandii*, *A. chroococcum*, *A. beijerinckii*) (Page et al., 1992; Chou et al., 1997; Kim 2000; Quagliano et al., 1999; Pal et al., 1999, *Bacillus* (*B.*) spp. (Halami 2008; Yilmaz and Beyatli 2005; Law et al., 2003), recombinant *Escherichia (E.) coli* (Lee 1996; Liu et al., 1998; Lee et al., 1997; Kim 2000; Nikel et al., 2006; Ahn et al., 2001; Park et al., 2002) and *Burkholderia* (*Bk.*) spp. (Nonato et al., 2001; Silva et al., 2004), synthesize PHAs as intracellular carbon and energy storage, accumulating these polyesters of hydroxyalkanoates as granules in the cytoplasm of cells (Reddy et al., 2003). Polyhydroxyalkanoic acids produced by bacteria are the building blocks of biodegradable thermoplastics and elastomers currently in use, or candidates to be used, in the medical and pharmaceutical industries as well as in agriculture (Suriyamongkol et al., 2007). The production of PHAs occurs mainly when cells are cultivated in the presence of a carbon source in excess, and their growth is limited by the lack of another nutrient, such as nitrogen, phosphorus, sulfur, or oxygen (Anderson et al., 1990). When the supply of the limiting nutrient is restored, PHAs are degraded by an intracellular depolymerase and subsequently metabolized as a carbon and energy source (Taidi et al., 1994) and the number of bacteria rapidly increases.

PHAs can be divided into two groups depending on the number of carbon atoms in the monomer units: short-chain-length (SCL) PHAs, which consist of 3-5 carbon atoms, and medium chain-length MCL-PHAs, which consist of 6-14 carbon atoms (Anderson et al., 1990). The length of the side chain and functional group has great importance for the physical properties. The SCL-PHAs are crystalline, brittle, and stiff polymers, with a high melting point and a low glass transition temperature. In contrast, MCL-PHAs show low crystallinity and tensile strength and lower melting points.

PHAs have the general formula shown in Figure 2.2 (Castilho et al., 2009), where “n” is equal to 1, and “R” is a methyl group. The most abundant PHA family member is poly(3-hydroxybutyrate) (P(3HB)). Using different substrates in a co-feeding system, copolymers of PHB (polyhydroxybutyrate) can be formed, such as polymers containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers. 3HV can be incorporated into the PHB molecule, forming poly(3-hydroxybutyrateco-3-hydroxyvalerate) [P(3HB-3HV)], resulting in a more brittle compound than P(3HB) (Reddy et al., 2003).

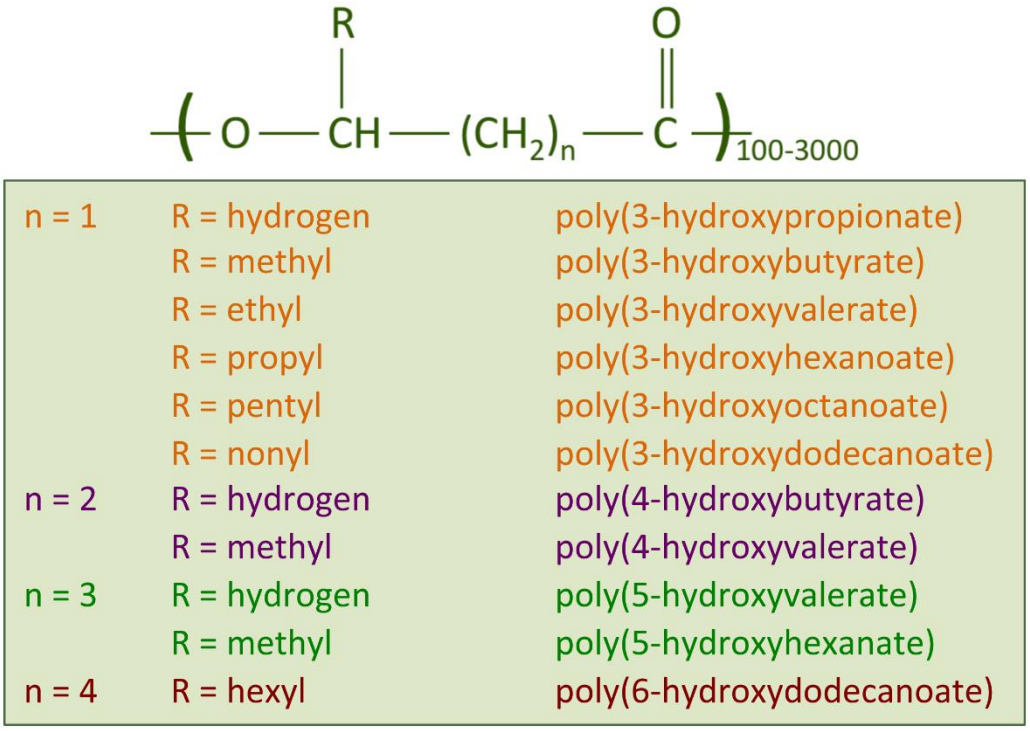

Figure 2.2. General structure of polyhydroxyalkanoates (PHAs). The most studied PHA type is the homopolymer P(3HB), for which n is equal to 1 and R is a methyl group (Castilho et al., 2009).

Thus, to reduce the environmental footprint by producing and using petrochemical-derived products, they can be replaced partially and even completely by polyesters derived from biological processes (i.e., PHAs) that have the significant advantage of being completely biocompatible (Reddy et al., 2003). Biocompatibility is the property shown by certain materials that generates non-toxic compounds when they are disposed of after use as well having the same physical property of the artificial material derived from petrol that they would replace (Castilho et al., 2009). Unlike petroleum-derived plastics that take several decades to degrade, PHAs can be completely bio-degraded within a year by a variety of microorganisms, mainly bacteria and fungi (Suriyamongkol et al., 2007). In particular, several aerobic and anaerobic PHA-degrading bacteria, such as *Comamonas* sp. (Jendrossek et al., 1993), *P. lemoignei* (Delafield et al., 1965) from soil, *Alcaligenes faecalis* (Tanio et al., 1982) and *P.fluorescens* from activated sludge (Mergaert et al., 1994) and *P. stutzeri* from lake water (Mukai et al., 1994), and fungi, such as *Aspergillus fumigatus* (Mergaert et al., 1994), have been isolated from various environments. These microorganisms excrete extracellular PHA depolymerases to degrade PHAs into water-soluble monomers and oligomers, using them as a carbon source (or methane under anaerobic conditions) (Lee, 1996).

Thus, life cycle assessment (LCA) conducted on the use of PHAs has been proven as the main advantage to avoid the accumulation of plastics in the environment (Patel et al., 2003). Therefore, PHAs are better than petrochemical analogues, such as polyethylene and polypropylene (Akiyama et al, 2003; Harding et al., 2007; Pietrini et al., 2007), in terms of sustainability and environmental protection (Atlić et al., 2011), but the realization and more widespread use of these environmentally friendly processes are related to the cost of the final product. The current PHA price also depends on monomer composition, and it is usually higher for copolymers; overall, it ranges from 2.2-5.0 € Kg^{-1} (Gholami et al., 2016; Chanprateep, 2010; Castilho et al., 2009), which is less than the typical range of 10-12 € Kg^{-1} reported at the beginning of the past decade (Gholami et al., 2016). Notwithstanding the burden of costs and the environmental impacts of plastic trash, the current PHA prices are not deemed to be commercially competitive with respect to conventional petroleum-based polymers, which typically cost less than 1.0 € Kg^{-1} (Gholami et al., 2016; Chanprateep, 2010; Valentino et al., 2017). Although the price of PHAs is high, several companies are producing PHA products worldwide to meet the demand of the market, including in the UK, Japan, US, Germany, Brazil, Italy, and China (De Marco, 2005; Tian et al., 2009).

2.3 Suitable substrates and bacterial strains for PHA production

The synthesis of PHAs occurs in many microorganisms under well-defined operating conditions and when they are supplemented with specific substrates, better known as PHA precursors. These compounds are incorporated into PHAs and used as the sole carbon source by microorganisms (or coupled with others as cosubstrates) if the cells are cultivated in the presence of an excess carbon source. Moreover, PHAs are also formed when growth is impaired or restricted by the lack of another nutrient, such as nitrogen, phosphorus, or oxygen (Lee, 1996). Thus, different PHAs could be synthesized from the combination of different substrates and microorganisms under different growth conditions (aerobic or anaerobic, temperature, and pH).

Relevant substrates for the production of PHAs are as follows: carbon dioxide (Tsuge, 2002) or fossil resources, such as low rank coal (Füchtenbusch et al., 1999) renewable resources (e.g., starch (Yu, 2001; Halami, 2008; Koutinas et al., 2007; Xu et al., 2010; Vandamme and Coenye, 2004), cellulose (Lee, 1998), sucrose (Jiang et al., 2007a; Page et al., Koutinas et al., 2007)), waste materials (e.g., molasses (Page et al., 1992; Yilmaz and Beyatli, 2005; Liu et al., 1998), whey (Ahn et al., 2001; Koller et al., 2008; Lee at al., 1997; Nikel et al., 2006; Park et al.,

2002), glycerol (Cavalheiro et al., 2009), and chemicals (e.g., propionic acid (Kalia et al., 2000)). To avoid the use of fossil resources due to environmental issues and to limit PHA production costs, renewable resource and waste materials are reasonably considered suitable and promising substrates.

In the following paragraphs, an overview of different works categorized on the basis of the different substrates used is given. The results are presented in terms of the PHA content (%PHAs, %) and concentration ([PHAs], g l^{-1}) calculated by the following equations 1 and 2, respectively, where mPHAs is the amount of PHAs [mg], mcells [mg] is the amount of freeze-dried biomass in samples, and CDW is the cell dry weight [g l^{-1}]:

$$\%PHAs = \frac{mPHAs}{mcells} \times 100 \quad (1)$$

$$[PHAs] = \frac{\%PHAs}{100} \times CDW \quad (2)$$

2.3.1 Starch-based material as a source of PHAs

Starch is a renewable carbon source available in large amounts. Prior to fermentation, starch is hydrolyzed to glucose by a two-step process, liquefaction and saccharification, because PHA-producing bacteria cannot synthetize amylase enzymes for starch degradation. Commercial hydrolyzing enzymes are often used, but they contribute to an increase in the cost of the glucose production process (Kim, 2000). Kim (2000) used soluble starch to produce P(3HB), obtained after 70 h of incubation with 25 g l^{-1} of PHB (content of 46% in cell dry weight), in fed-batch cultures of *A. chroococcum* strain 23 under oxygen-limiting conditions. Halami (2008) reported the ability of the isolated strain *Bacillus cereus* CFR06 to accumulate PHAs in a starch medium composed of soluble starch, yeast extract, and salts. The genus *Bacillus* was identified as one of the first Gram-positive bacteria suitable to produce PHAs and was cultivated under nitrogen limitation in Luria–Bertani (LB) broth for 24 h at 37°C on a rotary shaker at 100–150 rpm/min. The results obtained were less promising than those found by Kim (2000) because, after 72 h of incubation, a P(3HB) concentration of 0.48 g l^{-1} with a content of 48% was achieved.

Koutinas et al. (2007) proved the potential of *Cupriavidus necator* (synonym *Wautersia eutropha* and formerly classified as *Alcaligenes eutrophus,* formerly classified as *Ralstonia (R.) eutropha* (Vandamme et al., 2004)) in PHB production from a specific substrate derived from wheat. The authors conducted fed-batch tests using a 500 ml shake flask on a 250 rpm rotary shaker at 30°C and a pH range of 6.5–6.8. The results showed a PHB concentration of 51.1 g l^{-1} using a culture medium with free amino nitrogen as substrate at a concentration of 1.2 g l^{-1}. Under the same operating conditions (working volume, rpm, temperature, and pH range), Xu et al. (2010) compared the batch and fed-batch modes using *C. necator* NCIMB 11599 grown on wheat-derived media. They demonstrated that more PHB was accumulated in cells operating in fed-batch mode. In fact, the use of fed-batch mode allowed for an increase in PHB concentration to 130.2 g l^{-1} (PHB cells content ~80%) compared with batch fermentation that showed a production of 41.5 g PHB l^{-1} (PHB cells content ~66%). Haas et al. (2008) used saccharified waste potato starch as a carbon source for PHB production by *C. necator* NCIMB 11599, obtaining a PHB concentration of 94 g l^{-1}, with a specific yield from starch of 0.22 PHB g starch g^{-1} under phosphate-limiting conditions. Poomipuk et al. (2014) isolated and selected the strain *Cupriavidus* sp. KKU38, which was able to accumulate PHAs up to 65.27% (PHAs concentration of 2.8 g l^{-1}) from cassava starch hydrolysate as a sole carbon source in a 250 ml flask (Table 2.3.1).

However, to overcome the high costs of the hydrolysis of starch into glucose by a two-step process (liquefaction and saccharification), making this feedstock less economically viable, Bhatia et al. (2015) constructed the recombinant *E. coli* strain SKB99 harboring plasmids containing genes for starch hydrolysis (from *Paenibacillus* sp.) and PHB synthesis (from *R. eutropha*). This engineered strain utilized starch as the sole carbon source, with a maximum PHB production of 1.24 g l^{-1} (PHB content 40%) for 72 h with 2% (w/v) starch (Table 2.3.1). In addition, the accumulation of PHB started with the growth of the strain *E. coli* SKB99 and remained consistent until it attained the stationary phase, highlighting that PHB production in this engineered strain is not regulated by the stress response, unlike in *R. eutropha* and other microorganisms.

Therefore, starch-based materials are suitable substrates for PHA accumulation and, in particular, for P(3HB) accumulation. However, PHA accumulation strictly depends on the bacterial species and strains that exhibit different biotechnological performances depending on the carbon source and the culture conditions. The best results were obtained using *C. necator* NCIMB 11599 cultured on wheat and hydrolyzed waste potatoes under nutrient (nitrogen or phosphorus) limiting conditions, operating in batch and fed-batch mode, respectively.

Table 2.3.1 Overview of studies reporting PHAs production from starch-based materials

Strain	Type of PHA	Operation mode	Time to PHA_{max} [h]	PHAs concentration [g l^{-1}]	PHAs content [%]	Reference
***Azotobacter chroococcum* 23**	P(3HB)	Fed-batch	70	25	46	(Kim, 2000)
***Bacillus cereus* CFR06**	P(3HB)	Batch	72	0.48	48	(Halami et al., 2008)
***Cupriavidus necator* NCIMB 11599**	P(3HB)	Fed-batch	168	51.1	70	(Koutinas et al., 2007)
***Cupriavidus necator* NCIMB 11599**	P(3HB)	Batch Fed-batch	69 171	41.5 130.2	66 80	(Xu et al., 2010)
***Cupriavidus necator* NCIMB 11599**	P(3HB)	Batch	72	94	22	(Haas et al., 2008)
***Cupriavidus sp.* KKU38**	PHA	Batch	96	2.8	65.3	(Poomipuk et al., 2014)
***Recombinant E. coli* SKB99**	P(3HB)	Batch	72	1.2	40	(Bhatia et al., 2015)

2.3.2 Molasses and sucrose as sources for PHAs

Molasses is a common industrial by-product of sugar production, is much cheaper than glucose, and is extensively used as a carbon source for PHA production from biological processes (Table 2.3.2). Liu et al. (1998) demonstrated that recombinant *Escherichia coli* (HMS174/pTZ18u-PHB) can efficiently utilize molasses as the sole carbon source to produce PHB. A fed-batch feeding strategy was developed to improve cell growth and PHB production. The final PHA concentration was 31.6 g l^{-1}, and 80% of PHAs was accumulated. Jiang et al. (2007a) isolated a strain of (PHB)-accumulating bacteria from the soil in Alaska (USA), identified as *P. fluorescens* A2a5. This microorganism is capable of accumulating a large amount of granules in its cells when grown in sugarcane liquor medium. Batch cultivation was carried out at 25°C in a 5 l bioreactor inoculated with 1% inoculum (v/v) at pH 7.0. In this way, a maximum cell dry weight (CDW) of 32 g l^{-1} with a PHB concentration of 22.4 g l^{-1} was obtained, and the PHB content was approximately 70%. *C. necator* was aerobically grown in a well-balanced medium consisting of sugarcane and inorganic nutrients to reach a high cell density (Nonato et al., 2001). Then, cell growth was shifted to PHB synthesis by limiting nutrients other than the carbon source. The fed-batch fermentation process was carried out by continually feeding (45-50 h) a high concentration of sugar syrup to achieve a biomass of nearly 65-70% PHB, with a concentration ranging from 80 to 100 g l^{-1}.

The effect of different molasses concentrations (1-5 g molasses/100 ml water) on PHB production by *B. cereus* M5 was investigated by Yilmaz and Beyatli (2005). They observed that PHB productivity by this strain decreased with increasing molasses concentration. In fact, the highest P(3HB) concentration produced by this strain was 0.1 g l^{-1} (polymer content of 73.8%) with 1% molasses concentration.

A. vinelandii UWD was investigated by Page et al. (1992) using molasses as the sole carbon

Table 2.3.2. Overview of studies reporting PHAs production from molasses and sucrose

Strain	Type of PHA	Operation mode	Time to PHA $_{max}$ [h]	PHAs concentration [g l^{-1}]	PHAs content [%]	Reference
Recombinant *E. coli* (*HMS174/pTZ18u-PHB*) (*C. necator* genes)	P(3HB)	Fed-batch	31.5	31.6	80	(Liu et al., 1998)
***Pseudomonas fluorescens* A2a5**	P(3HB)	Batch	96	22.4	70	(Jiang et al., 2007a)
Cupriavidus necator	P(3HB)	Fed-batch	45-50	80-100	65-70	(Nonato et al., 2001)
***Bacillus cereus* M5**	P(3HB)	Batch	72	0.13	73.8	(Yilmaz and Beyatli, 2005)
***Azotobacter vinelandii* UWD**	P(3HB)	Fed-batch	35	23	66	(Page et al., 1992)
***Bacillus megaterium* BA-019**	P(3HB)	Fed-batch	24	72.7	42	(Kulpreecha et al., 2009)

source. Fed-batch bioreactors were operated with 5% (w/v) molasses at pH 7.2 and inoculated with 4%(v/v) of the pre-grown strain. In the beet molasses medium, NH_4 was depleted by 10 to 12 h to establish NH_4-limiting conditions and fix nitrogen during the PHA production phase of growth. After 35 h, a P(3HB) concentration of 23 g l^{-1} and a polymer content of 66% were achieved.

Kulpreecha et al. (2009) tested *B. megaterium* BA-019 on sugarcane molasses (20 g l^{-1}) as a carbon source and urea or ammonium sulfate at 0.8 g l^{-1} as the investigated nitrogen sources. In the experiments, a cell dry mass concentration of 72.7 g l^{-1} in 24 h, with a PHB content of 42% (w/w), was achieved under nitrogen-limiting conditions operating in fed-batch mode.

In addition, with sugarcane, *C. necator* showed the best PHA concentration among the bacterial strains (recombinant *E. coli, A. vinelandii* UWD, and *B. megaterium*) operating in fed-batch mode with molasses as a carbon source. In fact, *C. necator* is able to accumulate approximately 100 g l^{-1} synthesizing glucose (from starch) and sucrose (from sugarcane).

2.3.3 Lignocellulosic material as a source for PHAs

To produce fuels and other valuable bioproducts, lignocellulosic biomass from dedicated crops and agricultural and forestry waste are promising renewable sources (Di Pasqua et al., 2014; Ventorino et al., 2015; Ventorino et al., 2016a,b).

Lignocellulosic materials, consisting of lignin (complex polyphenolic structure), cellulosic (b-D-1,4-glucan), and hemicellulosic (D-arabinose, D-xylose, D-mannose, D-glucose, D-galactose, and sugar alcohols) fibers, constitute the most abundant renewable resources on our planet (Castilho et al., 2009).

The composition of lignocellulosic biomass differs in terms of lignin (10–25%), cellulose (30–60%), and hemicellulose (25–35%) content (Kumar et al., 2010).

Silva et al. (2004) studied the potential of two bacterial strains, *Bk.. cepacia* IPT 048 and *Bk. sacchari* IPT 101A, in producing P(3HB), comparing biosynthesis from xylose and glucose with bagasse hydrolysate. In high-cell-density cultures using a mixture of xylose with glucose under P limitation, both strains reached a maximum P(3HB) concentration of 60 g l^{-1} dry biomass, containing 60% biopolymer. Higher polymer content and yield were observed under P limitation than under N limitation for *Bk. sacchari* IPT 101A, whereas *Bk. cepacia* IPT 048 showed a similar performance in the presence of both growth-limiting nutrient conditions. Using bagasse hydrolysate as the carbon source, polymer contents reached 62% and 53% for

B. sacchari IPT 101A and *B. cepacia* IPT 048, respectively, with a CDW of 4.4 g l^{-1} for both strains under N limitation (Table 2.3.3). Yu and Stahl (2008) also studied the performance of *C. necator* with the same substrate. In their experiment, the cultures were shaken in flasks at 200 rpm and 30°C for 48 h with pH adjusted to approximately 7.5. They demonstrated that P(3HB) was the predominant biopolyester formed from the hydrolysis of sugarcane bagasse, with a concentration of 3.9 g l^{-1}, corresponding to a P(3HB) accumulation of 65% of the CDW, achieved with a high carbon to nitrogen ratio (C/N = 20 or above). Since a minimum nitrogen level should be maintained during cultivation, this high C/N ratio implies a high concentration of residual organic carbon or a high amount of hydrolysates. A moderate C/N ratio (7–10) may be used to yield a low concentration (less than 1 g l^{-1}) of residual carbons and a moderate level of PHA content in the cells (45–50% w/w). Lee et al. (1998) investigated P(3HB) production from xylose and hydrolyzed cellulose by growing recombinant *E. coli* strains with *C. necator* PHA biosynthesis genes, testing the effects of supplementing a complex nitrogen source on cell growth and PHB production. The cells were cultivated for 60 h in a 250 ml flask containing 50 ml of medium in a shaking incubator at 250 rpm. When the strain TG1 (pSYL107) was grown on 20 g l^{-1} xylose, it was capable of accumulating 1.7 g l^{-1} of P(3HB) with 35.8% of polymer content. A higher P(3HB) concentration, equal to 4.4 g l^{-1}, and a polymer content of 73.9% were reached when the previous culture medium was supplemented with 10 g l^{-1} of soybean hydrolysate. To evaluate the effects of the nitrogen source, tryptone and peptone were also tested, achieving 47.7% and 10.3% of PHB content, respectively.

The ability of *R. eutropha* ATCC 17699 (*C. necator*) to produce PHB in the presence of different waste biomass hydrolysates (rice paddy straw, soybean husk, sunflower husk, and wood straw) was evaluated by Saratale and Oh (2015). The most suitable substrate for PHB accumulation by this strain was the rice paddy straw hydrolysate, which was selected by the authors for optimization of the process, obtaining the maximal PHA accumulation (75.45%) and PHB production (11.42 g l^{-1}) within 48 h of fermentation. Moreover, lignin and its derivatives are also used for PHA production. Tomizawa et al. (2014) tested PHA-accumulating strains on mineral salt media containing each of the 18 lignin derivatives and hydroxybenzoic acids, including intermediates derived from the metabolism of lignin derivatives in bacteria. Most of the strains grew poorly in media containing lignin derivatives, such as p-coumaric acid, caffeic acid, ferulic acid, and sinapinic acid. On the contrary, *R. eutropha* PHB-4 accumulated P(3HB) from 3-hydroxybenzoic acid and 4-hydroxybenzoic acid as the sole carbon sources, with a PHA content of 65 and 63 wt % and a dry cell weight of 1.6 and 0.69 g l^{-1}, respectively.

Although *C. necator* species seems to be the best bacterial candidate for PHB production using lignocellulosic hydrolysate, the accumulation is lower than that obtained with sucrose- and starch-based materials as carbon sources. The lowest PHA accumulation could be due to the presence of specific toxic compounds (e.g., furfural, HMF, p-hydroxybenzoic aldehyde, and vaniline) that are usually released during the pretreatment of lignocellulosic biomass, which are known to have an inhibitory effect on microbial growth and metabolism.

Table 2.3.3. Overview of studies reporting PHAs production from lignocellulosic materials.

Strain	Type of PHA	Operation mode	Time to PHA_{max} [h]	PHAs concentration [g l^{-1}]	PHA content [%]	Reference
***Burkholderia sacchari* IPT 101**	P(3HB)	Batch	25	2.73	62	(Silva et al., 2004)
***Burkholderia cepacia* IPT 048**	P(3HB)	Batch	25	2.33	53	(Silva et al., 2004)
Cupriavidus necator	PHA	Batch	48	3.9	65	(Yu and Stahl, 2008)
Recombinant *E. coli* (*C. necator* genes)	P(3HB)	Batch	60 60	1.7 4.4	35.8 73.9	(Lee et al., 1998)
***R. eutropha* ATCC 17699 (*C. necator*)**	P(3HB)	Batch	48	11.4	75.5	(Saratale and Oh, 2015)

2.3.4 Whey-based culture media as a source for PHAs

Whey is the major by-product of cheese factories, representing 80–90% of the volume of transformed milk (Lee et al., 1997). It contains approximately 4.5% (w/v) lactose, 0.8% (w/v) protein, 1% (w/v) salts, and 0.1–0.8% (w/v) lactic acid, and its high biological oxygen demand (40 g l^{-1}) makes it difficult to dispose. The discharge of large amounts of cheese whey into the environment can damage the chemical and physical structure of soil and pollute groundwater and can also affect the air (Zhong et al., 2015). This by-product represents an attractive low-cost substrate for producing PHAs (Table 2.3.4).

As seen in the previous sections, *C. necator* is one of the best-known bacteria among PHA-producing microorganisms, but it is unable to hydrolyze lactose or metabolize galactose (Gomez et al., 2012). In fact, *C. necator* was able to use lactose only after the expression of genes encoding β-galactosidase and galactokinase, although at a very slow rate (Pries et al., 1990). Therefore, recombinant *E. coli* containing the *C. necator* PHA biosynthesis genes for the production of PHB from glucose is considered a good candidate for PHB production from whey (Lee et al., 1997). Lee et al. (1997) cultivated recombinant *E. coli* strains in a defined medium supplemented with varying concentrations of whey solution and obtained 5.2 g l^{-1} of PHB, corresponding to 81.3% (w/w) of PHB, with a concentration of 30 g l^{-1} of whey solution. Kim (2000) also studied recombinant *E. coli* strains as PHB-accumulating microorganisms under O_2 limitation compared with conditions without O_2 limitation. The highest PHB accumulation (80%) was observed under O_2-limiting conditions, with a PHB concentration of 25 g l^{-1}. Instead, without O_2 limitation, 57% of PHB was achieved with a concentration of 32 g l^{-1}. A recombinant *E. coli* strain containing the PHA biosynthetic genes from *Azotobacter* spp., specially designed for the production of PHB from milk whey, was studied by Nikel et al. (2006). Fed-batch cultures were carried out at 37°C in a 5.6-liter fermentor with a starting volume of 2.0 liters and a controlled pH of 7.20. The feeding solution used for fed-batch cultures was a concentrated and deproteinated whey solution containing 25% (w/v) lactose. They reported that after 24 h, the cells accumulated PHB up to 72.9% of their cell dry weight, reaching a PHA concentration of 51.1 g l^{-1}. Physical analysis of PHB collected from the recombinants showed that its molecular weight was similar to PHB produced by an *Azotobacter* spp. strain.

A new fermentation strategy using a cell recycle membrane system was developed by Ahn et al. (2001) for the efficient production of P(3HB) from whey by a recombinant *E. coli* strain

harboring the *Alcaligenes latus* PHA biosynthesis genes.

Cell fed-batch cultures of recombinant *E. coli* CGSC 4401 (pJC4) were carried out to overcome the volumetric limitation of a fermentor (2.7 l) fed with a solution with low lactose solubility to increase PHB productivity. A whey solution containing 210 g lactose l^{-1} was used as a feeding solution. The final cell concentration, PHB concentration, and PHB content obtained in 39 h were 150 g l^{-1}, 100 g l^{-1}, and 67%, respectively. In another experiment, a whey solution containing 280 g lactose l^{-1} was used as a feeding solution. After 36.5 h, a PHB concentration and a PHB content of 96.2 g l^{-1} and 80.5%, respectively, were obtained using a whey solution concentrated to contain 280 g lactose l^{-1} as a feeding medium. No inhibitory effects of the by-products or nutrients on cell growth and PHB production were found during fermentation by the authors.

The production of P(3HB) from whey by fed-batch cultures of recombinant *E. coli* harboring a plasmid containing the *Alcaligenes latus* PHA biosynthesis genes was examined by Park et al. (2002). Fed-batch cultures of recombinant *Escherichia coli* SGSC 4401 (pJC4) were carried out at 30°C in 30 l (working volume of 10 l) and 300 l (working volume of 150 l) fermenters supplying only air. The culture pH was controlled at 6.9. With lactose below 2 g l^{-1}, the cells grew to 12 g l^{-1} with 9% (w/w) P(3HB) content in a 30 l fermenter. The accumulation of P(3HB) could be triggered by increasing lactose to 20 g l^{-1}. Using this strategy, 35.5 g l^{-1} was obtained with a 70% (w/w) P(3HB) content after 26 h. The same fermentation strategy was used in a 300 l fermenter, and a 20 g l^{-1} with 67% (w/w) P(3HB) content was obtained in 20 h by Park et al. (2002). Koller et al. (2008) compared the production of PHB under nitrogen-limiting conditions obtained with *P. hydrogenovora* using the following two substrates: hydrolyzed whey permeate and glucose/galactose medium. Shake flasks (1 l) containing 250 ml of hydrolyzed whey permeate or synthetic medium supplemented with glucose and galactose (each 2.5 g l^{-1}) were both inoculated with 5% (v/v) *P. hydrogenovora*. The flasks were shaken at 30°C for 48 h. Furthermore, the study investigated the influence of the 3HV precursor sodium valerate on the bacterial growth of *P. hydrogenovora.* Thanks to its advanced properties compared with those of highly crystalline pure PHB (Koller et al. 2008), the ability of the strain to biosynthesize P(3HB-co-3HV) in media supplemented with hydrolyzed whey permeate and sodium valerate was evaluated. In these two different experiments, PHA content was confirmed at 12% for both types of PHAs, but the PHA concentration was higher when sodium valerate was added to P(3HB-co-3HV) production.

A recombinant strain of *E. coli* was generally used to obtain the PHA concentration (more than 90 g l^{-1}) from whey-based culture media because *C. necator* is unable to hydrolyze lactose. In

fact, several studies tested different lactose concentrations to correlate this parameter to PHA accumulation. Fed-batch experiments supplemented with a high amount of lactose (hydrolyzed from chees whey) were performed to obtain a higher PHA concentration. Otherwise, when increasing the lactose concentration to 280 g l^{-1}, a relevant increase in PHA concentration was not observed.

In addition, it is interesting to note that with whey-based culture media, the oxygen-limiting conditions enhance PHB biosynthesis from recombinant *E. coli* but decrease PHA concentration in the cells.

Table 2.3.4. Overview of studies reporting PHAs production from whey-based culture media.

Strain	Type of PHA	Operation mode	Time to PHA $_{max}$ [h]	PHAs concentration [g l^{-1}]	PHA content [%]	Reference
Recombinant *E. coli* (*C. necator* genes)	P(3HB)	Batch	49	5.2	81.3	(Lee et al., 1997)
Recombinant *E. coli* (*C. necator* genes) GCSC 6576	P(3HB)	Fed-batch with oxygen limitation	52	25	80	(Kim, 2000)
		Fed-batch without oxygen limitation	35	32	57	
***Pseudomonas hydrogenovora* DSM 1749**	P(3HB)	Fed-batch	41	1.27	12	(Koller et al., 2008)
	P(3HB-co-3HV)		31	1.44	12	
Recombinant *E. coli* K24K *(Azotobacter* spp. genes*)*	P(3HB)	Fed-batch	24	51.1	72.9	(Nikel et al., 2006)
Recombinant *E. coli* CGSC 4401	P(3HB)	Fed-batch	36.5	96.2	80.5	(Ahn et al., 2001)
Recombinant *E. coli* CGSC 4401 (*A. latus* genes)	P(3HB)	Fed batch 30 l bioreactor	26	35.5	70	(Park et al., 2002)
		Fed batch 300 l bioreactor	20	20	67	

2.3.5 Fatty acid and glycerol culture media as source for PHAs

Pure glycerol is an important industrial feedstock, with applications in the food, drug, cosmetic, and tobacco industries, while crude glycerol is the main by-product of biodiesel production, with low value due to the presence of impurities (such as methanol, salts, and fatty acids). Thus, crude glycerol represents a waste product with an associated disposal cost (Cavalheiro et al., 2009). For this reason, it can be used as an attractive substrate for PHA production (Table 2.3.5). *C. necator* DSM 545 was used by Cavalheiro et al. (2009) to accumulate P(3HB) from waste glycerol and from commercial glycerol as a control substrate. For *C. necator* cultivated on basal medium supplemented with pure glycerol and nitrogen depletion, a maximum of 51.2 g l^{-1} of P(3HB) at 33.5 h was reached, with a PHB content of 62%. On the contrary, using waste glycerol as a carbon source, productivity was lower because only 38.5 g l^{-1} was achieved with a PHB content of 50% in 34.5 h.

Production of PHAs from various plant oils or oleic acid by *C. necator* H16 was studied by Fukui and Doi (1998). The strain was tested on olive oil, corn oil, and palm oil and in all these plant oils. The strain was cultivated in a 100 ml nitrogen-limited mineral salt medium containing 1% plant oil at 30°C for 72 h. The wild-type strain produced P(3HB) at a high polymer content (79-82%) but at low concentrations (2.9-3.4 g l^{-1}).

Kahar et al. (2004) produced a copolymer of 3HB with 5 mol% (R)-3-hydroxyhexanoate, P(3HB-co-3HHx), from soybean oil as a sole carbon source with a recombinant strain of *C. necator*. The medium for PHA production in the fermentor was a mineral salt medium, and the initial concentration of NH_4Cl was set at 4 g l-1. Additional NH_4Cl was intermittently fed into the culture broth to avoid nitrogen source depletion. Soybean oil was added to the fermentor for an initial concentration of 20 g l-1. A high content of P(3HB) (85-95 g l^{-1}) and a high PHA content of 71–74% (w/w) were achieved during 96 h.

Table 2.3.5. Overview of studies reporting PHAs production from oil, fatty acid and glycerol culture media.

Strain	Type of PHA	Operation mode	Time to PHA $_{max}$ [h]	PHAs concentration [g l^{-1}]	PHA content [%]	Reference
C. necator **DSM 545**	P(3HB)	Fed-Batch	33.5	51.2	62	(Cavalheiro et al., 2009)
C. necator **H16 (ATCC 17699)**	P(3HB)	Batch	72	2.9-3.4	79-82	(Fukui and Doi, 1998)
C. necator **H16 (pJRDEE32d13)**	P(3HB)	Fed-Batch	96	85-95	72-76	(Kahar et al., 2004)
C. necator **H16**	P(3HB)	Batch	96	1.24	19.7	(Füchtenbusch et al., 2000)
Pseudomonas resinovorans	PHA	Batch	48	0.14	15.2	(Cromwick et al., 1996)

Füchtenbusch et al. (2000) studied *R. eutropha* and *P. oleovorans* cultivated in a mineral salt medium with the oil from rhamnose production as the sole carbon source under aerobic conditions at 30°C in nutrient broth or in mineral salt medium.

The concentration of ammonium was limited to 0.05% (mass/vol) to promote the accumulation of PHAs. The cultivation of *P. oleovorans* and *R. eutropha* was performed in 300 ml at 28°C. *C. necator* accumulated only P(3HB) at 6.3 g l^{-1}, with a polyester content of 19.7% during the first 96 h. The same authors tested *P. oleovorans* under the same operating conditions using the same carbon source. After 96 h, this strain accumulated 5 g l^{-1}, with a P(3HB-co-3HHx) content of 17.3%.

Different *Pseudomonas* species (*P. oleovorans*, *P. resinovorans*, *P. putida*, and *P. citronellolis*) were tested by Cromwick et al. (1996) in 2 l shake flasks. The bacteria were evaluated for their ability to grow and produce PHAs using tallow free fatty acids and tallow triglyceride as carbon substrates; however, only *P. resinovorans* was able to grow and produce PHAs. The PHA concentration in this case was 0.12–0.15 g l^{-1}, with a 15.2% polymer content, using unhydrolyzed tallow as the substrate.

The different fatty acids and glycerol waste materials used as substrates for PHA accumulation highlighted that *C. necator* was the best candidate operating under nitrogen source depletion, although PHA accumulation depended on the strain and operating mode. In fact, performing the experiments in fed-batch mode, more PHB was accumulated in the cells than in operating in batch mode.

2.3.6 Solid agro-industrial by-products and waste as a source of PHAs

Law et al. (2003) showed that recombinant *B. subtilis* could utilize malt waste in the medium as a carbon source better than glucose and thus could substantially lower the cost of PHA production (Table 2.3.6). In the paper by Law and co-authors, the *pha* genes (involved into PHAs accumulation) from *B. megaterium* were cloned into *B. subtilis*. The recombinant strain was cultivated by acid hydrolyzed malt waste, and a 1% inoculum was used in a fermentation flask incubated at 37°C at 280 rpm for 16 h. Their results showed PHA accumulation in a malt waste medium of 2.53% with a PHB concentration of 0.06 g l^{-1} in 12 h.

A. vinelandii UWD strains were tested by Cho et al. (1997) with most poly-3-hydroxybutyrate-co-valerate (PHBV) production from swine waste liquor. Strain UWD was cultured in a shake flask with 4% inoculum at 200 rpm, incubated at 30°C for 18–52 h.

Using undiluted swine waste liquor medium without glucose supplementation, cell growth was limited to 1.2 g l^{-1} with 37% in 48 h. Cell growth and PHBV production increased when swine waste liquor was diluted two-fold and supplemented with 30 g glucose l^{-1} (5.48 g l^{-1} and PHBV content 58%).

Industrial fruit and vegetable waste were successfully used as sole carbon sources by Ganzeveld et al. (1999) to produce PHBV by *R. eutrophus* under oxygen-limiting conditions. The fermentor was a 1 l standard fermentor with a working volume of 750 ml. The temperature was controlled at 30°C. The stirrer speed was adjusted manually to maintain the dissolved oxygen pressure above 30% of the saturation concentration. A concentration of 1.1 g PHBV l^{-1}, or 40% (w/w) of the cell dry weight, was obtained.

Starchy wastewater was used by Yu (2001). The waste was first digested in a thermophilic upflow anaerobic sludge blanket (UASB) reactor to form acetic, propionic, and butyric acids. PHA formation from individual acids was further investigated under nitrogen-limiting conditions by active biomass of *R. eutropha*. PHA formation from acid effluent in 48 h was 1.2 g l^{-1}, with a PHA content of 34.1%.

Another suitable substrate for PHA production is food scraps, a complex form of organic solid waste that is unusable by PHA-producing microbes, such as *R. eutropha*. Hydrolysis and acidogenesis are the main processes used to convert biodegradable solids into short-chain volatile fatty acids, such as acetic, propionic, and butyric acids, which are utilized by PHA-producing bacteria. This approach was used by Du et al. (2004) by coupling organic acid production with anaerobic acetogenesis to produce PHAs. The PHA-synthesis reactor (2 l air-bubbling bioreactor) was maintained at 30°C via a water jacket and pH 7.5. The dissolved oxygen concentration was maintained at 20% of air saturation or above. The PHA content and concentration reached their maximal values of 72.6% and 16.5 g l^{-1}, respectively, in 73 h.

Other studies were conducted on the use of excess activated sludge from a wastewater treatment plant fed with industrial waste streams as a substrate for PHB accumulation (Kumar et al., 2004). Wastewater from food processing (producing mainly potato chips, wafers, and sweets) and starch rich grain-based alcohol industries (rice grain-based and jowar grain-based distillery spent wash) was used as a substrate for PHB production by Khardenavis et al. (2007). In their work, different types of wastewater were tested in 250 ml conical flasks and incubated on a rotary shaker at 150 rpm at 30°C: wastewater derived directly from industry, filtered wastewater, and deproteinized wastewater, each in the absence and presence of an external nitrogen source; the highest biomass concentration of 6.6 g l^{-1} (dry weight) was produced in 96 h in a raw rice grain-based distillery spent wash with the addition of di-ammonium hydrogen

phosphate, accumulating 2.7 g l^{-1} PHB with a content of 67%; a deproteinized jowar grain-based distillery spent wash and filtered food processing wastewater yielded lower PHB and biomass accumulation.

The studies carried out using solid agro-industrial by-products and waste demonstrated that the accumulation of PHAs was lower than that obtained with the other complex starting matrices, which was also observed when lignocellulosic hydrolysates were used as carbon sources. In addition, with this organic biomass, the highest accumulation was achieved using *C. necator* species, although the operating mode strongly influenced the process. Interestingly, activated sludge from a wastewater treatment plant was used as mixed cultures for PHA production from industrial waste streams. In particular, the PHA concentration was similar to that observed with pure cultures, overcoming the high costs derived from the production of pure cultures and the disposal of waste activated sludge.

Table 2.3.6. Overview of studies reporting PHAs production from solid agro-industrial by-products.

Strain	Type of PHA	Operation mode	Time to PHA $_{max}$ [h]	PHAs concentration [g l^{-1}]	PHA content [%]	Reference
Recombinant *Bacillus subtilis* 1A304 (105 MU331)	P(3HB)	Batch	12	0.06	2.5	(Law et al., 2003)
***Azotobacter vinelandii* UWD (ATCC 53799)**	P(3HB-co-3HV)	Batch	48	0.43	37	(Cho et al., 1997)
			18	5.48	58.3	
		Batch	45	1.13	40.8	(Ganzeveld et al., 1999)
***C. necator* (*R. eutropha*)**	P(3HB-co-3HV)	Batch	48	1.2	34.1	(Yu, 2001)
		Fed-batch	73	16.5	72.6	(Du et al., 2004)
Activated sludge	P(3HB)	Batch	96	2.7	67	(Khardenavis et al., 2007)

2.4. Integrated systems to simultaneous produce intracellular (PHAs) and extracellular by-products (biosurfactants)

Bacterial strains actively involved in PHA accumulation can be used at the industrial scale to reduce the production costs of biopolymers due to their ability to convert waste materials into valuable intracellular and extracellular bi-products (e.g., PHAs and exopolysaccharides (EPS), respectively) that are useful for biochemical production. PHAs represent intracellular carbon and energy storage, while EPS and biosurfactants are produced as extracellular substances to protect the cells from desiccation and predation or are a carbon source. These substances are of industrial interest for washing powders and fabric softener production (Cameotra and Makkar, 1998). They are used also in the food, chemical, cosmetic, and packaging industries as adhesives, absorbents, lubricants, and cosmetics (Ding et al., 2015; Palomba et al., 2012; Torrieri et al., 2014). Biosurfactants are amphipathic molecules with polar and nonpolar heads produced by different bacterial genera (e.g., *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Rhodococcus*, and *Enterobacter* (Liang et al., 2014). Biosurfactants present as a wide variety of structures because their synthesis is influenced by the carbon source (Lin, 1996). In fact, they can be produced on different substrates, such as sugars, lipids, alkanes, and waste materials (Liang et al., 2014). The main property of biosurfactants is the ability to reduce surface and interfacial tension, forming microemulsions (Desai and Banat, 1997). Among biosurfactants, rhamnolipids are the most studied thanks to the simultaneous production of PHAs and rhamnolipids by *P. aeruginosa* IFO3924 (Hori et al., 2002). In their work, batch cultivation was conducted at 30°C in 3 l fermentors equipped with an agitator using 7 g l^{-1} of decanoate as a carbon source. In this experiment, basal salt medium was used to increase the concentration of the nitrogen source. After a 3-day cultivation, considerable PHA content (23% of CDW corresponding to a concentration of 2.2 g l^{-1}) and rhamnolipid amounts (298 mg l^{-1}) were produced. Another type of extracellular polymeric substance is EPS, a mixture of high molecular polymers, which supplies carbon units when substrate is limited. Wang and Yu (2007) studied the simultaneous biosynthesis of EPS (an extracellular product) and PHB (an intracellular product) by *R. eutropha*. They observed that EPS production was closely coupled with cell growth, while PHB was synthesized only under nitrogen-limiting conditions and cell growth-limiting conditions. In fact, the experiments were conducted at different concentrations of glucose and NH_4-N to evaluate their influence on EPS and PHB production. Furthermore, the previous authors observed that the PHB content in dry cells decreased with increasing nitrogen concentration, while the EPS concentration increased. While keeping the nitrogen

concentration constant, further experiments were conducted at varying glucose concentrations, and the results showed that an increase in glucose concentration promoted biomass growth and PHB production. The relevant production (shown in Table 2.4) of both polymers was observed when glucose and nitrogen were supplied at concentrations of 40 g l^{-1} and 3 g l^{-1}, respectively. Among EPS, alginates are of great commercial interest for their use in a wide range of applications in the food industry, such as in frozen custards, restructured foods, cream and cake mixtures, and beer production. They are composed of variable amounts of β-D-mannuronic acid and C5-epimer α-L-guluronic acid linked via β-1, 4-glycosidic bonds. When extracting alginates from harvested material, the uronic acids are converted into the salt forms mannuronate and guluronate through a neutralization step. The proportion, distribution, and length of these blocks determine the chemical and physical properties of the alginate molecules. Commercial alginates are currently extracted from marine algae, such as *Laminaria* and *Macrocystis*, but can also be obtained from bacterial species, such as *A. vinelandii*, *P. aeruginosa*, and *P. mendocina* (Hori et al., 2002). The co-production of alginates and PHAs by *P. mendocina* using glucose as a carbon source was studied by Guo et al. (2011). The simultaneous production of MCL-PHA and alginate oligosaccharide (AO) cultivation was performed in 200 l fermenters with 120 l mineral salt medium containing 20 g l^{-1} glucose at 30°C and 200 rpm of impeller speed for 48 h. The authors reported that 0.316 g l^{-1} PHA_{MCL} and 0.57 g l^{-1} AO were obtained at the end of the fermentation process. The MCL-PHA production reached a maximum of 0.360 g l^{-1} at 36 h when the carbon source was almost exhausted. At 48 h, the utilization of intracellular stored MCL-PHA took place, corresponding to a decrease in PHA content to 0.316 g l^{-1}. Moreover, the production of PHB and EPS by *Azotobacter beijerinckii* was investigated by Pal et al. (1999) under nitrogen-free conditions with an excess of carbon. This strain was maintained by growth on nitrogen-free glucose medium at 30°C for 48 h and was then stored at 4°C. Nitrogen-free liquid medium was inoculated with 4% (v/v) inoculum, and the flasks were incubated at 30°C on a rotary shaker. The highest production of PHB (2.73 g l^{-1}) was reached when glucose was supplemented at 3% (w/v), observing an EPS amount of 1.2 g l^{-1}. Quagliano and Miyazaki (1999) studied the simultaneous production of PHB and EPS by *A. chroococcum*, evaluating the influence of ammonium addition with glucose, fructose, and sucrose. The organism was grown aerobically in 250 and 500-ml flasks at a one-third volume of the culture medium with the carbon sources alone or supplemented with 0.1 g l^{-1} of $(NH_4)_2SO_4$. The flasks were incubated in a rotatory shaker at 220 rpm at 30°C for 72 h. The highest PHB content was obtained with sucrose (1.1 g l^{-1}), but EPS production was almost unobservable. Instead, the experiments conducted with

glucose showed a maximum EPS concentration (2.1 g l^{-1}), with PHB production of 0.74 g l^{-1}. Thus, some microorganisms, such as *P. aeruginosa, R. eutropha, A. beijerinckii, A. chroococcum*, and *P. mendocina*, are able to concurrently produce PHAs and biosurfactants using the same type of organic substrate. However, the bacterial technological performance during the coupled process of PHA and biosurfactant production leads to a lower accumulation of PHAs. In particular, the optimal operating conditions for PHA and biosurfactant production are different. In fact, Wang and Yu (2007)observed that without nitrogen-limiting conditions, the PHB content in dry cells decreased, whereas the EPS concentration increased, demonstrating that nutrient-limiting conditions promote only PHA accumulation.

Table 2.4. Overview of studies reporting PHAs production coupled to metabolites used in industry.

Strain	Type of PHA	Operation mode	Time to PHA $_{max}$ [h]	PHAs concentration [g l^{-1}]	PHA content [%]	Produced metabolites [g l-1]	Reference
***Pseudomonas aeruginosa* IFO3924**	PHA	Batch	72	0.5	23	Rhamnolipids 0.3	(Hori et al., 2002).
***Ralstonia eutropha* ATCC 17699**	PHB	Batch	60	12.7	62	EPS 0.18	(Wang and Yu, 2007)
***Azotobacter beijerinckii* WDN-01**	PHB	Batch	40	2.73	54.6	EPS 1.2	(Pal et al., 1999)
***Azotobacter chroococcum* 6B**	PHB	Batch	48	0.74	28	EPS 2.1	(Quagliano and Miyazaki, 1999)
***Pseudomonas mendocina* NK-01**	PHA_{MCL}	Batch	48	0.316	25.3	Alginate oligosaccharides 0.57	(Guo et al., 2011)

2.5 Bioenergy production from industrial and agricultural waste

2.5.1 Anaerobic digestion and biogas production

Anaerobic digestion is a consolidated biological treatment, mainly used for reducing organic content in the sludge produced from municipal waste water treatment plants, thus achieving its stabilization (Appels et al., 2008). In the past few decades, the need to drastically reduce the use of landfills for the disposal of organic waste and producing energy from renewable resources has promoted the use of anaerobic digestion for treating a wide range of organic solids, e.g., organic waste and energy crops (Lema and Omil, 2001; Lettinga, 2001). To calculate bioenergy production potential based on anaerobic digestion for biomethane, official data for food waste generation and management were collected by Dung et al. (2014) from 21 countries, evaluating a methane potential equal to 379.769 KWh $year^{-1}$.

Treatment systems based on the anaerobic digestion process are flexible because they can have different configurations according to the number of stages (one or two stages); can operate at different temperatures, mostly at 35°C (mesophilic) or 55°C (thermophilic); can be fed in batch, semi-batch, or continuous; can take place in completely stirred or plug flow reactors; and can work with a content of solids lower than 10% in mass (wet system) or higher than 20% (dry system), preceded by several innovative pretreatments to increase waste solubilization (Mancuso et al., 2016). Treating organic waste through anaerobic digestion results in economic and environmental advantages (Lettinga, 2001; Dung et al., 2014; Mancuso et al., 2016; Barton et al., 2008); after treatment, the waste material is reduced in quantity, and it is more stable and less harmful for the environment because it is a source of a renewable energy, e.g., biogas, that does not alter the balance of CO_2 in the atmosphere and therefore does not contribute to global warming (Abbasi et al., 2012). Additionally, biogas refined to biomethane is also used to feed gas networks (Bekkering et al., 2010) as a surrogate to natural gas, and, finally, the by-product of anaerobic digestion, named digestate, can be reused in agriculture as fertilizer (Tambone et al., 2009; Rehl and Müller et al., 2011) thanks to its relevant content of nutrients. The performance and results of anaerobic digestion are strictly dependent on the environmental conditions (Mata-Alvarez et al., 2000; Kerroum et al., 2014; Ariunbaatar et al., 2015; Kim et al., 2002), such as temperature, pH, nutrients content, presence of inhibitors (Ariunbaatar et al., 2015), substrate composition and particle size, micronutrient availability, and the microbial strains used as the inoculum. Anaerobic digestion is driven by a complex microbiome containing both bacteria and Archaea. Each trophic group in the microbiome contains different

microorganisms involved in different metabolic tasks (Kundu et al., 2017). A strong syntrophic relationship exists between different consortia of microorganisms, since biochemical reactions in series are carried out (Figure 2.5.1).

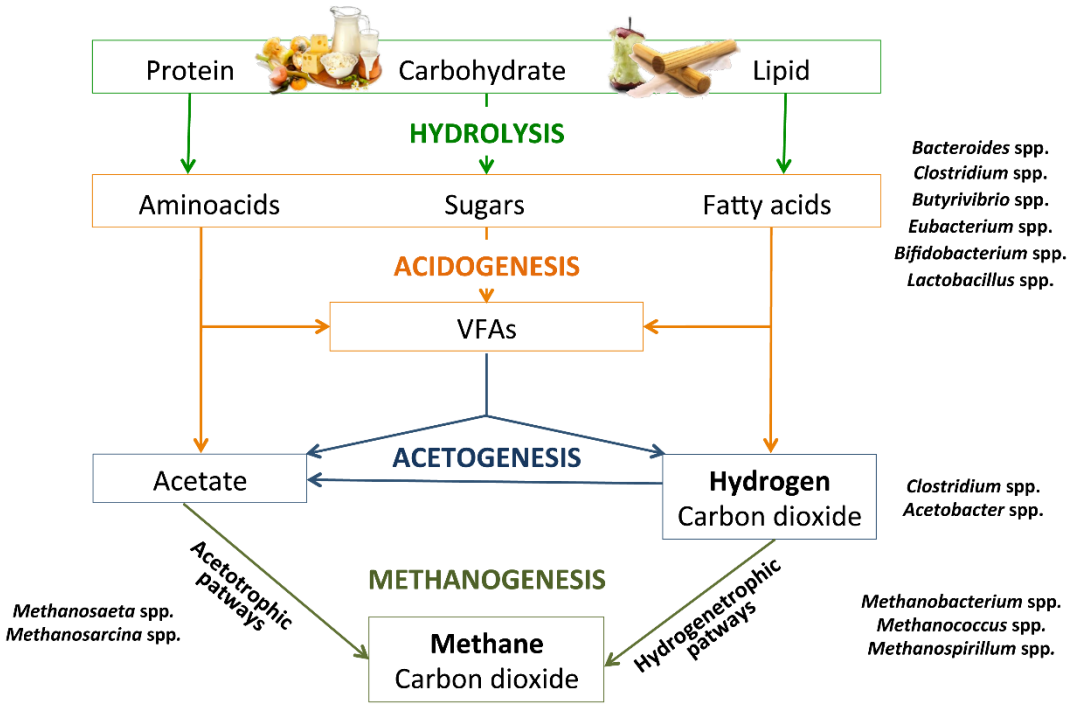

Figure 2.5.1. Phases of biological production of methane with the occurrence of VFAs, acetate, hydrogen and carbon dioxide. Anaerobic bacteria involved are positioned according to their probable role in the process.

Bacteria are crucial in the hydrolyzation and acidogenic step of the anaerobic digestion process. Novaes (1986) reported that the anaerobic species belonging to the families *Streptococcaceae* and *Enterobacteriaceae* as well as the genera *Bacteroides*, *Clostridium*, *Butyrivibrio*, *Eubacterium*, *Bifidobacterium*, and *Lactobacillus* are most commonly involved in the anaerobic digestion process. Furthermore, during the process, bacteria, such as *Clostridia*, fermented the hydrolyzed products of proteins to VFAs, CO_2, and hydrogen (H_2).

In addition, Archaea are important in the methanogenic phase of anaerobic digestion. Methanogenic Archaea are strictly anaerobic and are able to transform fermentation products into CH_4 (Gonzalez-Martinez et al., 2016). Some of these bacteria synthesize CH_4 using acetic acid, including the *Methanosaeta*, *Methanosarcina*, and *Methanothrix* genera. These are

acetoclastic or acetotrophic methanogens. Additionally, other groups of methanogens synthesize CH_4 by utilization of H_2 and CO_2 or methyl compounds, such as *Methanobacterium*, *Methanococcus*, *Methanospirillum*, or *Methanomassiliicoccus* (Gonzalez-Martinez et al., 2016). These bacteria are potentially able to use all types of biomass suitable for producing biogas: sewage sludge from aerobic wastewater treatment, animal manure, harvest residues, organic waste from agriculture and food processing factories, dairy waste, organic fraction of municipal solid waste (OFMSW), fruit and vegetable waste, and energy crops, which are substrates commonly used for feeding anaerobic digesters (Raposo et al., 2012).

The amount of biogas obtainable from a specific substrate depends on the operating conditions and its content of carbohydrates, proteins, and lipids. Lipids require a longer time than carbohydrates and proteins to be converted into biogas but have a more efficient conversion rate in terms of biogas produced per gram of substrate thanks to a high number of C and H atoms in their molecules (Cirne et al., 2007). Lipids are commonly present in food waste and in several wastewater types from factories, such as those that process meat, produce dairy, or refine fat (Li and Fang, 2007). Lipids can often be the cause of inconveniences, such as the inhibition of methanogenic microorganisms or their flotation and subsequent washout (Neves et al., 2006).Organic waste from agriculture, food waste, and OFMSW is mainly composed of carbohydrates. Such wastes are easily degraded; if their feeding is not accurately controlled, volatile fatty acids (VFAs) produced by the acidification step of the anaerobic digestion tend to accumulate, causing a sharp drop in the pH value, which inhibits the activity of methanogenic Archaea (Siegert and Banks, 2005) and leads to underperformance of the process. Wastes rich in proteins are commonly produced by meat and fish processing factories, slaughterhouses, and farms (animal slurry and manure). These wastes are characterized by a low C/N ratio (Callaghan et al., 2002; Cuetos et al., 2010; Edström et al., 2003) that can hamper and even inhibit the activities of microorganisms (Chen et al., 2008). Furthermore, proteins undergoing anaerobic digestion are converted into ammonia as an end product, which is rather toxic to microorganisms (Nielsen and Angelidaki, 2008) and should be considered when looking for cost-effective ammonia removal techniques (Limoli et al., 2016).

Wastes rich in cellulose (CWs) are produced by paper and cardboard as well as textile factories. CWs are also found, in large amounts, in unsorted municipal solid wastes (MSWs) and therefore are not useful for recycling. The C/N ratio in CWs is usually high, ranging from 173/1 up to values higher than 1000/1 (Zhang et al., 2008), while the optimum C/N ratio ranges from 20/1 to 30/1 (Hawkes, 1980).

Microalgae can be an alternative substrate for renewable energy recovery. The co-digestion of

microalgae with different types of wastes, such as pig/dairy manure (Astals et al., 2015), lipid waste (fat, oil, and grease) (Park et al., 2012), waste activated sludge (Wang and Park et al., 2015), and corn straw (Zhong et al., 2013), has been extensively evaluated for biomethane production. Zhen et al. (2016) examined the technical potential of methane production from microalgae through co-digesting with food waste. The results showed that supplementing food waste significantly improved microalgae digestion performance compared to the digestion of a single food waste, with the highest methane yield of 639.8 ± 1.3 ml/g VS_{added}.

In fact, an estimation of the amount of methane that can be produced from a specific substrate is commonly obtained through a specific test called the biomethane potential test (BMP). The BMP can be used as an index of the anaerobic biodegradation potential, as it is the experimental value of the ultimate specific biomethane production for the indefinite degradation time (Angelidaki and Sanders, 2004). However, in practice, BMP is estimated at a well-defined degradation time that can be a specific day, e.g., the 30th (Browne et al., 2011; Owens and Chynoweth, 1993) or 50th (Hansen et al., 2004) of incubation or the day when biomethane production is approximately zero (Xie et al., 2011) or less than 5 mld^{-1} (Browne et al., 2011). BMP can be expressed specifically as a volume of methane per amount of waste (dm^3-CH_4 kg^{-1}-waste), volume of waste (dm^3CH_4 dm^{-3} waste), per mass volatile solids added (dm^3 CH_4 kg^{-1} VS), or COD (chemical oxygen demand) added (dm^3 CH_4 kg^{-1} COD). The volume is usually expressed at standard conditions in terms of pressure (1 atm) and temperature (0°C). Other units for expressing methane potential are also used (Angelidaki et al., 2009).

For the same substrate, the BMP results can be variable because it is affected by the operating conditions in terms of temperature, mixing intensity, pH adjustment, substrate/inoculum (S/I) ratio, substrate particle size, liquid/volume ratio, nutrient content, inoculum, and if the substrate has been previously pretreated (e.g., mechanically, thermally, chemically) or mixed with one or more other substrates to perform a co-digestion process (Esposito et al., 2012). In Table 2.5.1, the methane yields from different substrates are reported (adapted from Raposo et al. (2012)).

2.5.2 Biohydrogen production

Hydrogen is considered an ideal source of energy for the future, since it represents a clean combustible but is also easily convertible to electricity (Yokoi et al., 2002). Biological hydrogen production is related to biogas production for two main reasons: a similar production

process, and the same substrates are suitable These two gaseous products derive from the same biological process that switches on hydrogen production when hydrogen-using microorganisms are inhibited, such as homoacetogens and methanogens; inhibition is commonly achieved through heat treatment of the inoculum to remove all microorganisms, except for spore-forming fermenting bacteria (i.e., species belonging to the families *Clostridiaceae, Streptococcaceae*, *Sporolactobacillaceae*, *Lachnospiraceae,* and *Thermoanaerobacteriacea*) (Angenent et al.,2004). The most common bacteria used in dark fermentation to produce hydrogen are *Clostridium* (Shin et al., 2004) and *Thermoanaerobacterium* (O-Thong et al., 2009; Nitipan et al., 2014). Moreover, several studies have reported successful hydrogen production by mixed cultures in batch or bioreactors (Lin et al., 2004; Prasertsan et al., 2009). The advantages of using mixed cultures for biohydrogen production are several: no need for sterilization, a high adaptive capacity owing to the microbial diversity, the capacity to use a mixture of substrates, and the possibility of obtaining a stable and continuous process (Nitipan et al., 2014).

Table 2.5.1. Methane yields of solid organic substrates (adapted from Raposo et al. (2012))

Solid Organic Substrate	Methane Yield [ml CH_4 g VS_{added}^{-1}]	Reference
Apple Fresh wastes	317	(Buffière et al., 2006)
Banana Peeling	289	(Buffière et al., 2006)
Cabbage Leaves 2mm size	309	(Gunaseelan et al., 2004)
Carrot Peeling	388	(Buffière et al., 2006)
Cauliflower Leaves	341-352	(Zubr, 1986)
Cellulose	356–375	(Owens and Chynoweth, 1993)
Cocksfoot	325	(Mähnert et al., 2005)
Food Wastes	245–510	(Liu et al., 2004)
Fruit and vegetable Wastes	470	(Scaglione et al., 2009)
Glucose	335	(Tong et al., 1990)
Kitchen waste	432	(Neves et al., 2006)
Leather fleshing	490	(Shanmugam et al., 2009)
Lettuce Residues	294	(Buffière et al., 2006)
Maize Residues	317	(Dinuccio et al., 2010)
Mandarin Peels 2 mm size	486	(Gunaseelan et al., 2004)
OFMSW	353	(El-Mashad et al., 2010)
Orange Peeling	297	(Buffière et al., 2006)
Paper and cardboard	109–128	(Pommier et al., 2010)
Pineapple Peel	400	(Shin et al., 2004)
Potato Waste	320 referred to $gVS_{removed}$	(O-Thong et al., 2009)
Rape Oil seed	800–900	(Nitipan et al., 2014)
Rice Straw	347–367	(Lin et al., 2004)
Starch	348	(Nitipan et al., 2014)
Sugar beet	340	(Prasertsan et al., 2009)
Sunflower	428–454	(Ghimire et al., 2015)
Textiles	228	(Levin et al., 2004)
Tomato Skins and seeds	218	(Esposito et al., 2012)
Wheat Straw	267	(Das et L., 2001)
Algal biomass	640	(Zhen et al. 2016)

Furthermore, the same organic substrates, such as solid waste, can be used to produce biogas and biohydrogen, thus converting residues into a source of bio-energies (Angenent et al.,2004). Many processes for hydrogen production have been extensively investigated; among them, hydrogen production by photosynthetic bacteria, algae, and fermentative bacteria is the most interesting because it is environmentally sustainable. In autotrophic conversions, biohydrogen can be produced by photosynthetic microorganisms, i.e., microalgae and photosynthetic bacteria that convert solar energy to hydrogen (Ghimire et al., 2015). Photosynthetic bacteria (e.g., purple non-sulfur bacteria) utilize the end products of dark fermentation, converting them into H_2 via photo fermentation with simultaneous VFA reduction (Levin et al., 2004; Das et L., 2001; Miyake ET AL., 1999; Lo et al., 2008; Chen et al., 2008; Tao et al., 2006). The major limitation of photo-fermentation systems is its poor H_2 production rate due primarily to the slow growth of photosynthetic bacteria and the low light conversion efficiency of photobioreactors (Chen et al., 2008). A photobioreactor (PBR) was developed by Chen et al. (2008) to enhance phototrophic H_2 production by *Rhodopseudomonas palustris* WP3-5 using acetate as the sole carbon source. The photobioreactor was illuminated by combinative light sources, reaching a maximum H_2 yield of 62.3%. Under heterotrophic conditions, two types of fermentation occur: photo fermentation carried out by photosynthetic bacteria and dark fermentation (Pradhan et al., 2015) carried out by anaerobic bacteria that convert carbohydrates into biohydrogen (Ghimire et al., 2015). Different rumen bacteria, such as *Clostridia*, methylotrophs, methanogenic archae, or facultative anaerobic bacteria (*Escherichia coli*, *Enterobacter* spp., *Citrobacter* spp.), and aerobic bacteria (*Alcaligenes* spp., *Bacillus* spp.) have been studied to perform dark fermentation. In particular, *Clostridium butyricum* and *Clostridium articum* produce butyric acid and propionate as major products, respectively, and both products are of interest for hydrogen production (Hawkes et al., 2007). Indeed, photo fermentation takes place under anaerobic conditions involving purple non-sulfur photosynthetic bacteria using light as an energy source for synthesizing hydrogen (Eroglu et al., 2011). The ability of purple non-sulfur bacteria to convert organic acids to biohydrogen is coupled with their ability to synthetize PHB under anaerobic conditions. In fact, Luongo et al. (2016) investigated hydrogen and poly-b-hydroxybutyrate (PHB) production during photofermentative treatment of the effluent from a dark fermentation reactor fed with the organic fraction of municipal solid waste. They compared the hydrogen and PHB production of an adapted culture of *Rhodobacter sphaeroides* AV1b and a mixed consortium of purple non-sulphur bacteria. The mixed cultures resulted in 1.5-fold more H_2 produced than the pure culture (559 and 364 N ml H_2 l^{-1}, respectively). On the contrary, *Rhodobacter sphaeroides*

cultures showed higher PHB productivity (155 mg PHB g COD^{-1}) than the mixed cultures (55 mg PHB g COD^{-1}). As for methane production through anaerobic digestion, biohydrogen can be produced by different bacterial strains using several organic substrates. For example, Cappelletti et al. (2012) focused their study on H_2 production from molasses and cheese whey with the aim of valorizing food industry wastes by their recycling; mesophilic, thermophilic, and hyperthermophilic bacteria were tested to produce H_2. Among them, *Thermotoga* strains showed the most promising results; in particular, *T. neapolitana* was the best performing strain (Table 2.5.2). This result was confirmed by studies conducted on *T. neapolitana* using other organic substrates, such as rice straw (Nguyen et al., 2010), beet pulp pellet, corn starch, and rice flour (Yu and Drapcho, 2011). Such substrates are particularly suitable for producing H_2 thanks to their easy biodegradability and are also convenient because they are present in different carbohydrate-rich wastewaters and agricultural residues (Davila-Vazquez et al., 2008). Other substrates commonly used for biohydrogen production are protein- and fat-rich wastes. A *Clostridium butyricum* strain was studied by Chen et al. (2005) for its ability to produce H_2 from a sucrose-based medium. In particular, *Clostridium butyricum* CGS5 can efficiently produce hydrogen (2.78 mol H_2/mol sucrose) on an iron-containing medium (Chen et al. 2005). The same microbial strain (*C. butyricum* CGS5) was isolated from soil with nine cellulolytic bacterial strains belonging to *Cellulomonas* sp. and *Cellulosimicrobium cellulans* by Lo et al. (2008). Among these strains, only *C. butyricum* CGS5 exhibited efficient H_2 production from rice husk hydrolysates, with a H_2 yield of 17.24 mmol H_2 g $cellulose^{-1}$. Ferchichi et al. (2005) investigated hydrogen production from cheese whey by *Clostridium saccharoperbutylacetonicum*, studying the influence of the initial pH; they found that slightly acidic initial conditions favored a higher H_2 yield than alkaline conditions. The highest hydrogen yield (2.7 mol H_2/mol substrate) was actually obtained at pH 6. Bisaillon et al. (2006) examined hydrogen production by different strains of *Escherichia coli* under different feeding regimes to detect the main limiting factors: strains that showed the highest hydrogen yield (2 mol H_2/mol substrate) when cultured at limiting concentrations of either ammonia or glucose (1 mM NH_4Cl; 0.04% of glucose). Mesophilic bacterium HN001 was tested by Yasuda and Tanisho (2006) as a H_2 producer from starch. In the same work, the authors focused their studies on the influence of temperature, pH, and substrate concentration; the optimal temperature was found to be approximately 37°C, with a hydrogen yield of 2 mol H_2/mol substrate. Liu et al. (2003) investigated H_2 production by mixed cultures in batch experiments using cellulose as a substrate; at the optimal pH of 6.5, the maximum hydrogen yield was 92 ml H_2/g hexose, and an analysis of 16S rDNA sequences showed that the cellulose-degrading

mixed culture was composed of microbes closely affiliated with genus *Thermoanaerobacterium*. Carbohydrate-rich holocellulose of lignocellulosic organic matter can be made available to the H_2 conversion by pretreatment. Examples of lignocellulosic biomass pretreatment methods for hydrogen fermentation were reported by Kumar et al. (2015). They also reported the maximum hydrogen yield associated with pretreatment methods, ranging from 44.9 ml H_2 g^{-1} to 141.29 ml H_2 g^{-1}. The influence of pH was also evaluated by Khanal et al. (2004), who used a mixed microbial culture and starch as a substrate. At the optimal pH of 4.5, the maximum hydrogen yield was 133 ml H_2 g hexose^{-1}. At the same pH value, Fang et al. (2005) reached a maximum hydrogen yield of 210 ml H_2/g hexose using food waste as a substrate. Instead, Valdez-Vazquez et al. (2005) studied the influence of temperature using a mixed culture as the inoculum and mixed waste as a substrate. At 37°C, the maximum hydrogen yield was 210 ml H_2 g hexose^{-1}.

All biotechnological hydrogen production processes have particular limits, since a considerable part of the used substrate is converted into various soluble metabolic products rather than H_2. Thus, the major side product of dark fermentation is a multi-compound mixture of VFAs and other constituents, such as alcohols (Kumar et al., 2016). Therefore, the volatile fatty acid-rich fermentation effluent is a perfect substrate for biologically synthesizing polyesters, e.g., polyhydroxyalkanoate (Albuquerquea et al., 2011; Morgan-Sagastume et al., 2010), which could have an industrial market (Chen et al., 2009).

Table 2.5.2. Hydrogen yields of different substrates (adapted from Li and Fang [114]; Davila-Vazquez et al. (2008)).

Substrate	Strain	Hydrogen Yield	Reference
Sucrose	*Clostridium butyricum* CGS5	2.78 (mol H_2 mol substrate^{-1})	(Chen et al., 2005)
Glucose	*Escherichia coli* strains	2 (mol H_2 mol substrate^{-1})	(Bisaillon et al., 2006)
Glucose	*Thermotoga neapolitana*	1.6 (mol H_2 mol substrate^{-1})	(Cappelletti et al., 2012)
Molasses	*Thermotoga neapolitana*	2.6 (mol H_2 mol substrate^{-1})	(Cappelletti et al., 2012)
Rice straw	*Thermotoga neapolitana*	2.7 (mol H_2 mol substrate^{-1})	(Nguyen et al., 2010)
Cheese whey	*Thermotoga neapolitana*	2.4 (mol H_2 mol substrate^{-1})	(Cappelletti et al., 2012)
Cheese whey	*Clostridium saccharoperbutylacetonicum* ATCC 27021	2.7 (mol H_2 mol substrate^{-1})	(Ferchichi et al., 2005)
Starch	Mesophilic bacterium HN001	2 (mol H_2 mol substrate^{-1})	(Yasuda and Tanisho, 2006)
Starch	Mixed culture from compost	133 (ml H_2/g hexose^{-1})	(Khanal et al., 2004)
Cellulose	Mixed culture from sludge	92 (ml H_2/g hexose^{-1})	(Liu et al., 2003)
Mixed waste	Mixed culture from anaerobic digestion sludge	201 (ml H_2/g hexose^{-1})	(Valdez-Vazquez et al., 2005)
Food waste	Mixed culture from anaerobic digestion sludge	210 (ml H_2 g hexose^{-1})	(Fang et al., 2005)
Acetate	*Rhodopseudomonas palustris* WP3-5 in Photobioreactor	62.3(mol H_2 mol substrate^{-1})	(Chen et al., 2008)
Rice husk	*Clostridium butyricum* CGS5	17.24 (mmol H2 g cellulose^{-1})	(Lo et al. 2008)

2.6. Integrated systems for bioenergy production from industrial and agricultural wastes

Simultaneous production of PHAs and bioenergy from organic wastes

Degradation of biowaste to methane (CH_4) and carbon dioxide is a multiple step process with the possibility of producing H_2 and bioplastics (from volatile fatty acids) as intermediates (Patel et al., 2011). Based on this process, anaerobic digestion can be performed with a two-stage system, where biomass is degraded in the first stage and hydrolysis-acidification occurs. The organic acids produced are processed under aerobic conditions to produce biopolymers and, as an alternative, under anaerobic conditions to produce biogas.

A PHA production system, in its most comprehensive configuration, is composed of four main stages (Figure 2.6), as follows:

1. Feedstock production;
2. Biomass selection;
3. PHA production;
4. PHA extraction

Simplified configurations can be obtained using synthetic substrates (stage 1 is removed from the cycle), using pure culture (stage 2 is removed from the cycle), or using both synthetic substrates and pure culture (stages 1 and 2 are removed from the cycle).

The aims of each stage are listed below:

1. To produce organic acids from complex organic solids (e.g., wastes rich in carbohydrates);
2. To select the microbial strains from the mixed culture that show the highest capacity for PHA accumulation under specific dynamic feeding conditions (Serafim et al., 2008);
3. To produce PHAs using the selected culture;
4. To recover PHAs from microorganisms.

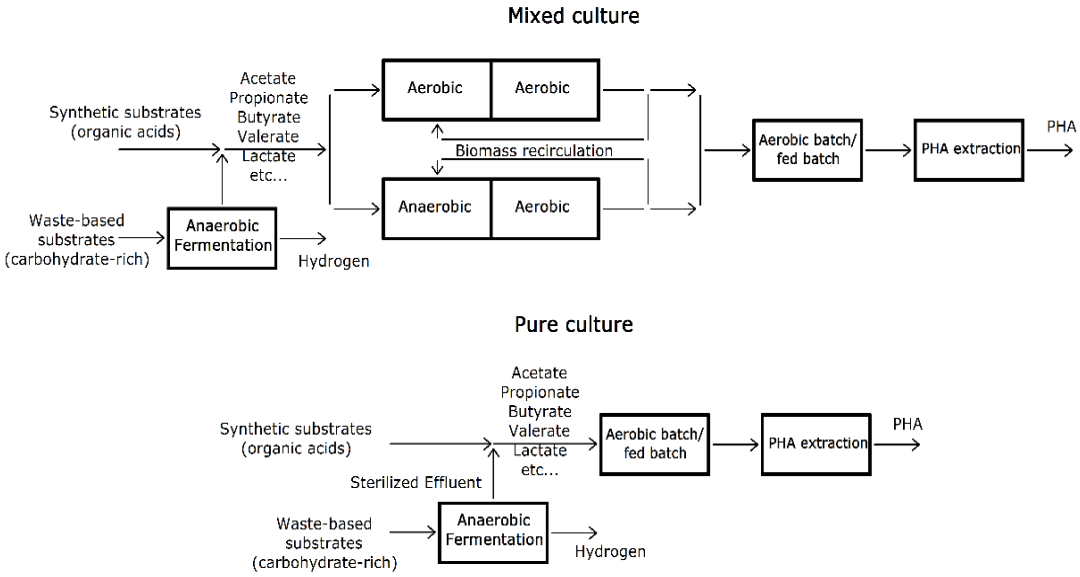

Figure 2.6.1 Cycle of polyhydroxyalkanoates (PHAs) production system (adapted from Serafim et al. 2008).

A dark fermentation process can be successfully used to perform the first stage. This process evolves according to the same sequence of biochemical reactions in the anaerobic digestion process, with the exception of the last stage that is repressed using different strategies (e.g., setting a short hydraulic retention time-HRT, keeping the pH low at 5.5, adding chemical compounds toxic to methanogens, and performing thermal shocks).

The dark fermentation process can be optimized to produce VFAs and consequently H_2 that is a by-product of the biological process and VFAs, varying: (i) the operational conditions (i.e., pH, temperature, HRT, solid retention time–SRT, organic loading rate-OLR); (ii) the configuration of the dark fermentation reactor and feeding system; and (ii) the type of organic waste used to feed the reactor (Figure 2.6.2). The effects of these parameters on VFA production are listed in Table 2.6 (Lee et al., 2014).

Table 2.6. Waste, reactor configuration and operation for the production of VFAs (adapted from Lee et al. (2014)).

Type of waste	Organic content (mgCOD/l)	Reactor type and operating conditions	VFA production	Reference
Waste activated sludge	18.657	Batch, 55°C, pH=8, HRT=9d	368 mg COD $gVSS^{-1}$	(Zhang et al., 2009)
	14.878	Batch, 21°C, HRT=6d	339 mg COD L^{-1}	(Jiang et al., 2007b)
Primary sludge	22.838	Batch, 21°C, HRT=6d	85 mg COD $gVSS^{-1}$	(Ji et al., 2010)
	20.631	Batch, 21°C, pH= 10, room temp, HRT=5d	60 mg COD $(gVSS\ d)^{-1}$	(Wu et al., 2009)
Food waste	91.900	Batch, 37°C, pH=5.5	8950 mg COD L^{-1}	(Elbeshbishy et al., 2011)
	146.1	Batch, 35°C, HRT=5d	5610 mg COD L^{-1}	(Kim et al., 2006)
Kitchen waste	166.18	Batch, 35°C, pH=7, HRT=4d	36 mg L^{-1}	(Zhang et al., 2005)
OFMSW	347.0	Batch, 14-22°C, pH=4-5, HRT=4-4.5d	40 mg g VS^{-1}	(Bolzonella et al., 2005)
	196.7	Plug flow, 37°C, pH=5.7-6.1, HRT=SRT=6d, OLR=38.5 gVS/(L d)	23.110 mg L^{-1}	(Sans et al., 1995)
Palm oil mill	88.0	Semi-continous, 30°C, pH=6.5, HRT=4d	15.300 mg L^{-1}	(Hong et al., 2009)
Olive oil mill	37.0	Packed bed biofilm, 25°C, pH=5.2-5.5, HRT=14d, OLR=26gCOD/(L d)	10.700 mg COD L^{-1}	(Beccari et al., 2009)
Cheese whey	4590	CSTR, 37°C, pH=6, HRT=2.1d	0.84 gVFA-COD g $sCOD^{-1}$	(Bengtsson et al., 2008)

Various microbes, such as *A. eutrophus*, *B.s megaterium, P. oleovorans, A. beijerincki, Rhizobium*, and *Nocardia*, utilize acetic acid, formic acid, and propionic acid as a substrate for PHA production (Kalia et al., 2000). *A. eutrophus* and *A. beijerinckii* were studied by Kalia et al. (2000) and were shown to be capable of accumulating PHAs up to 70% of CDW, under nitrogen and phosphorus limiting conditions, whereas *Pseudomonas* spp. and *Rhizobium* spp. accumulated PHAs at approximately 60% of CDW.

Many other bacterial strains have also been reported to produce PHAs under adverse conditions with different PHA yields. Among them, many purple non-sulfur bacteria, such as *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, *Rhodopseudomonas palustri*s, and *Bacillus* spp., have been reported to produce H_2 and PHA under nutrient-limiting conditions (Saharan et al., 2014).

Patel et al. (2011) investigated the metabolic activities of *Bacillus* strains to transform glucose into H_2 and PHB in two stages. Operating in batch mode, *Bacillus thuringiensis* EGU45 and *B. cereus* EGU44 reached 1.67–1.92 mol H_2/mol glucose, respectively, during the first 3 days. In the next 2 days, *Bacillus thuringiensis* EGU45 was supplemented with residual medium containing glucose, volatile fatty acids, and residual nutrients (nutrient stress condition) and produced a PHB yield of 11.3% of CDW.

R. palustris WP3-5 was studied by Wu et al. (2012) to evaluate possible competition between PHB synthesis and H_2 production, testing cultures on six different substrates, such as acetate, propionate, malate, lactate, glucose, and lactose. The results highlighted that strain WP3-5 could utilize acetate, propionate, malate, and lactate to produce H_2, whereas it was also able to synthesize PHB only on acetate and propionate. PHB synthesis decreased H_2; however, under pH-stress conditions, such a decrease was not observed.

R. palustris was also studied by Vincenzini et al. (1997) to investigate the potential of purple non-sulfur bacteria in the photoproduction of both hydrogen and PHB-containing biomass under limiting amounts of nitrogen. The data demonstrated that under nitrogen-limiting growth conditions, *R. palustris* synthesized 40 mg l^{-1} d^{-1} of PHB and produced 200 ml l^{-1} d of H_2 when the experiments were supplemented with 60 mg l^{-1} d^{-1} of nitrogen.

Yu (2001) performed a two-step integrated system consisting of microbial acidogenesis and acid polymerization from starchy wastewater. In his work, the starchy organic waste was first digested in a thermophilic upflow anaerobic sludge blanket reactor to form acetic (60–80%), propionic (10–30%), and butyric (5–40%) acids. The acids in the effluent solution after microfiltration were polymerized into PHAs by *A. eutrophus* in a second reactor. PHA production from the acid effluent was compared with the production from pure acids in 48 h,

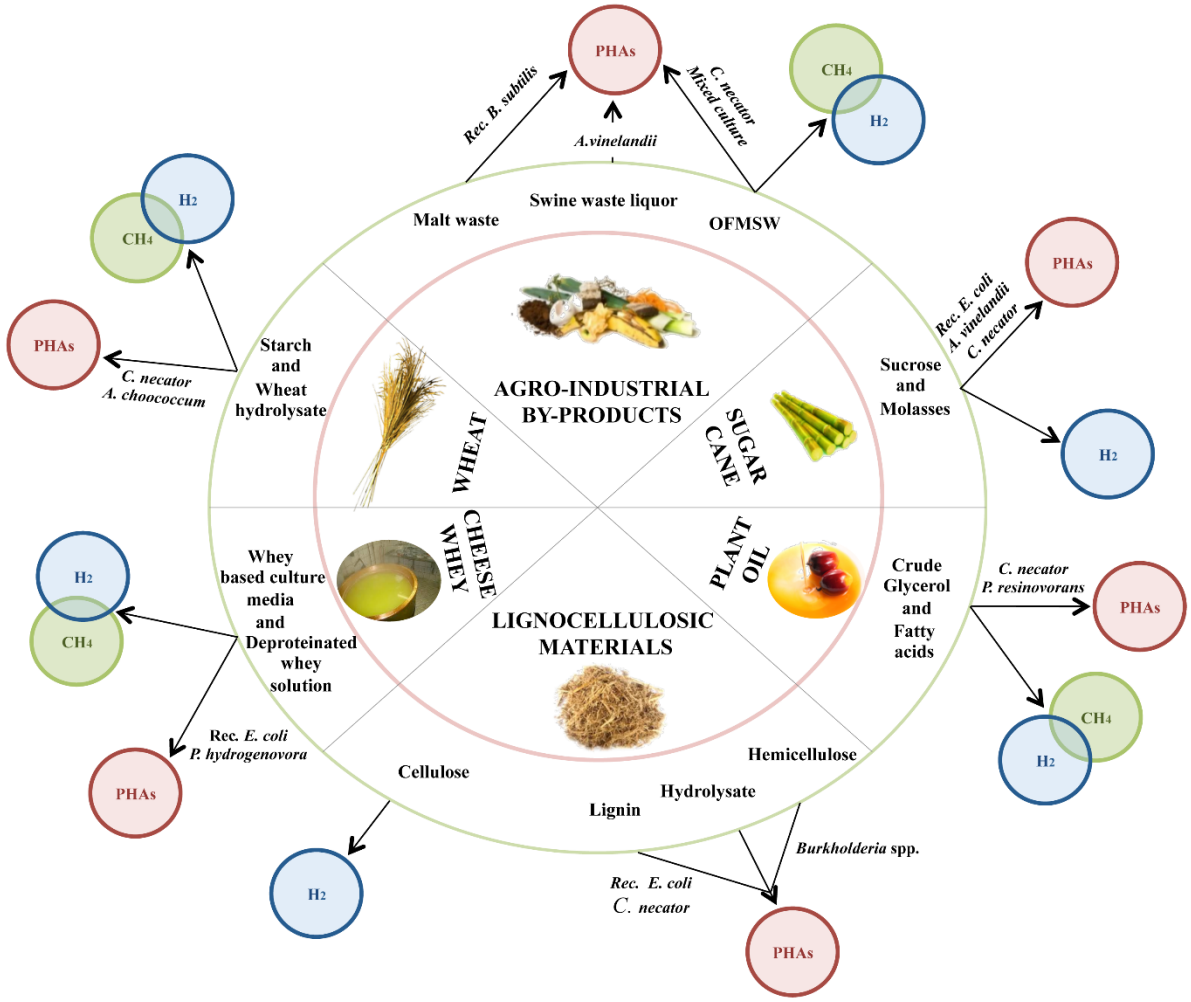

Figure 2.6.2 Sustainable PHAs and bioenergy production from organic wastes and by-products converted by different bacterial species: an overview of the principal process considered in this review.

and the results were very similar. In batch mode, 1.2 g l^{-1} of PHAs was accumulated from acid effluent. Instead, 1.0 and 1.3 g l^{-1} of PHAs was obtained from a mixture of butyric acid and propionic acid in batch and fed-batch mode, respectively.

Albuquerquea et al. (2010) designed another integrated system to valorize the use of wastewater for PHA production. They employed a 2-stage continuous stirred tank reactor (CSTR) system to effectively select PHA-storing organisms using fermented molasses as feedstock. The acidogenic fermentation (step 1) was carried out in a CSTR operated under anaerobic conditions. The reactor effluent was clarified by microfiltration and used as a feedstock for culture selection (step 2) and PHA batch accumulation (step 3). The culture reached a maximum PHA content of 61%.

The best integrated systems developed were based on two-step processes consisting of acidogenic fermentation (operating under anaerobic condition) aimed to produce acid effluent that, after microfiltration, is used in the subsequent aerobic microbial process aimed at PHA polymerization. However, the first step (acidogenic fermentation) is also useful for hydrogen production and could be designed as a dark fermentation process.

2.7 Conclusions

Biological processes can be successfully used in innovative and eco-sustainable technology to convert organic waste into bioenergy and biochemicals, separately or simultaneously. Bioprocesses can provide bioenergy or valuable chemicals and, at the same time, perform pollution control, according to technical feasibility, simplicity, economics, and societal needs. Bio-based plastics can completely replace the conventional ones derived from fossil fuels if the production costs can be reduced, and the use of high-performing bacteria fed with organic wastes and by-products as substrates significantly contribute to achieving this objective.

In this context, different organic substrates and by-products can be used to produce bioenergy (hydrogen and methane) and biopolymers (PHAs). Otherwise, the review highlights the possibility of integrating the two production processes to design a unique system for both energy and biopolymer production. The integrated system is a flexible process that aims: (i) to produce organic acids from complex organic solid wastes rich in carbohydrates; (ii) to use selected microbial strains or mixed cultures that show the highest capacity for PHA accumulation under specific dynamic feeding conditions; and (iii) to produce bioenergy or accumulate PHAs by microorganisms from acidogenic effluents.

This integrated system represents new perspectives on the use of organic waste and by-products, valorizing organic substrates for the production of both bioenergy and PHAs.

CHAPTER 4

Bioenergy production from dairy waste

This chapter has been published as:

Pagliano G. Ventorino V, Panico A, Romano I, Robertiello A, Pirozzi F, Pepe O. The effect of bacterial and archaeal populations on anaerobic process fed with mozzarella cheese whey and buttermilk J Environ Manage. 2018;217:110-122.

4.1. Introduction

In recent years, attention in reducing the pollutant emissions produced by conventional organic waste disposal systems (e.g., landfills) as well as developing technology to convert organic waste into bioenergy and biomaterials has grown.

This new approach to waste management is eco-friendly, easy to be conducted and economical advantageous, mostly for undeveloped and developing countries that have an economic gap with more industrialized countries. This gap is often due to the lack of an available energy source and technological and infrastructural backwardness (Ragazzi et al., 2017), and efforts to reduce it frequently lead to an uncontrolled release of solid and liquid pollutants as well as gaseous emissions into the environment (Riahi et al., 2017). Furthermore, the biotechnological development contributes to replace fossil fuels with biomass (organic waste and/or energy crops) as source of energy and biomaterials, thus preventing the increase of CO_2 in the atmosphere and indirectly taking part to mitigate the global warming (Bauer et al., 2010). For instance, the organic fraction of the municipal solid waste (OFMSW) is successfully and worldwide used for producing enzymes (Clanet et al., 1988), biohythane (Escamilla-Alvarado et al., 2017) and ethanol (Ballesteros et al., 2010); agricultural biomass including corn, woods, sugar, rice and wheat straw, has found a wide use in generating bioalcohols, bio-oil, biogas and biohydrogen (Poggi-Varaldo et al., 2014, Mancini et al., 2016; 2018); and even not readily biodegradable C-based wastes, such as polystyrene (Goff et al., 2007) and polyethylene terephthalate (PET) (Kenny et al., 2012), have resulted to be suitable for polyhydroxyalkanoates (PHA) production. Potential substrates for bioenergy production are cheese whey and buttermilk, by-products of cheese, yogurt, milk and butter processing in dairy factories. Cheese whey represents approximately 80–90% of the total waste volume from dairy factories (Lee et al., 1997) and is the major by-product of mozzarella cheese production. Buttermilk is the liquid left after churning mozzarella cheese. These milk-based wastes have high concentrations of soluble organic matter and are biodegradable, thus suitable for being treated by an anaerobic process that converts them into ethanol, lactic acid, volatile fatty acids (VFAs), H_2 and CH_4. All of them are complementary products of the biological metabolism (Khan et al., 2016) and their production can be associated to one or more bacterial and/or archaeal strains in the system. Although the anaerobic bacteria belonging to the families *Streptococcaceae* and *Enterobacteriaceae* as well as the genera *Clostridium* and *Eubacterium* are the most frequently involved in the anaerobic digestion process (Novaes, 1986; Pagliano et al., 2017), the microflora present in anaerobic digesters is extremely various in species, highly specialized, selected on the base of substrates and inoculum as well as the operating conditions used. When the biological process is fed with dairy wastes, it is expected that:

(i) *Lactobacillus* spp. and *E. coli* are the most common hydrolytic bacteria; (ii) *Acetobacterium* spp., which converts lactate to acetate, are the most common homoacetogenic bacteria (Schug et al., 1987); (iii) archaea are responsible for CH_4 production (Gonzalez-Martinez et al., 2016) from acidogenesis products following the acetoclastic pathway, typical of *Methanosaeta*, *Methanosarcina*, and *Methanothrix* genera, and/or the hydrogenotrophic pathway, typical of *Methanobacterium*, *Methanococcus*, *Methanospirillum*, or *Methanomassiliicoccus* (Gonzalez-Martinez et al., 2016).

The main biochemical reactions involved in the anaerobic degradation of dairy waste with the production of H_2 and CH_4 are listed in Table 4.1 with the relative value standard of Gibbs free energy ($\Delta G°$).

As the end-products of an anaerobic process have different commercial value and industrial use, it is convenient to control the process physically, chemically and microbiologically up to drive it to maximize the production of determined bio-products rather than others (Mohan et al., 2016). Therefore, in order to evaluate the biological and anaerobic conversion of dairy wastes into liquids (e.g., ethanol and lactic acid) and gaseous compounds (e.g., H_2 and CH_4), in this study two series of batch tests were conducted under strictly controlled mesophilic conditions and run with different ratios of substrate and inoculum. In detail, the tests were focused on achieving the following objectives: (i) finding a correlation between the intermediate and end-products of the process (e.g., H_2, CH_4 and VFAs) with the bacterial groups at different times during the process; (ii) understanding the role of microbial groups during the anaerobic biological processes; (iii) governing the microbial activity to achieve a specific target, such as the enhanced production of H_2 and/or CH_4, rather than VFAs, or *viceversa*.

4.2. Materials and methods

4.2.1.Physico-chemical analysis of dairy wastes

Cheese whey and buttermilk were collected from a buffalo mozzarella cheese factory located in Casoria (latitude: 40° 54' 32.62" N and longitude: 14° 17' 37.07" E) in the Campania region (Italy). Dairy wastes were mixed maintaining a ratio of 2:1 (*v/v*) between cheese whey and buttermilk, in

Table 4.1. The main biochemical reactions involved in anaerobic processes with the relative value of standard Gibbs free energy (ΔG°).

Reaction	ΔG° (kJ/mol)	Reference
Glucose to H_2/ethanol/acetate $C_6H_{12}O_6 + 3H_2O \rightarrow 2H_2 + 2CH_3CH_2OH + CH_3COO^- + 2HCO_3^- + 3H^+$	-182	Azbar and Levin, 2012
Glucose to H_2/ethanol/formate/acetate $C_6H_{12}O_6 + 2H_2O \rightarrow H_2 + CH_3CH_2OH + HCOO^- + CH_3COO^- + HCO_3^- + 3H^+$	-183	Azbar and Levin, 2012
Glucose to ethanol $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+$	-196	Azbar and Levin, 2012
Glucose to H_2/acetate $C_6H_{12}O_6 + 4H_2O \rightarrow 4H_2 + 2CH_3COO^- + 2HCO_3^- + 4H^+$	-168	Azbar and Levin, 2012
Glucose to H_2/butyrate $C_6H_{12}O_6 + 2H_2O \rightarrow 2H_2 + 2CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+$	-229	Azbar and Levin, 2012
Glucose to H_2 $C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6HCO_3^- + 6H^+$	+64	Azbar and Levin, 2012
Glucose to H_2/acetate/formate $C_6H_{12}O_6 + 2H_2O \rightarrow 2H_2 + 2CH_3COO^- + 2HCOO^- + 4H^+$	-170	Azbar and Levin, 2012
Glucose to lactate $C_6H_{12}O_6 \rightarrow 2CH_3CH(OH)COO^- + 2H^+$	-172	Azbar and Levin, 2012
Acetate to H_2 $CH_3COO^- + 4H_2O \rightarrow 4H_2 + 2HCO_3^- + H^+$	+116	Azbar and Levin, 2012
H_2 to acetate $4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$	+48.3	Thauer et al., 1977
Butyrate to acetate/H_2 $CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	+88.2	Westermann 1984
Propionate to acetate/H_2 $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+116.4	Westermann 1984
Acetic acid to methane $CH_3COOH \rightarrow CH_4 + CO_2$	-36	Schlegel et al., 2012
H_2 to methane $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-135.6	Voolapalli and Stuckey 1999

order to simulate the standard characteristics of a real dairy waste stream produced from a mozzarella cheese factory. Such mixture was used to conduct the tests. Cheese whey, buttermilk and their mixture were physically and chemically characterized as follows: pH was measured using a HI 221 pH meter (Hanna Instruments Inc., Woonsocket, RI, USA); total titratable acidity (TTA) was calculated as the mL of 0.1 N NaOH/10 mL of sample (AAC Methods, 1975); total solids (TS) and volatile solids (VS) were evaluated as described in the standard methods (APHA, 2005); COD was measured with an ECO08 thermoreactor (VELP Scientifica, Usmate, Italy) and a PF-3 photometer (VELP Scientifica, Usmate, Italy) using kit NANOCOLOR®.

4.2.2. Microbiological analysis of dairy wastes

Bacterial counts were performed on serially diluted cheese whey, buttermilk and their mixture, which were spread on the plate surface containing different media. Total aerobic and anaerobic bacteria were counted on Plate Count Agar (Oxoid, Milan, Italy) and incubated for 48 h at 30°C under either aerobic or anaerobic conditions (Oxoid's Anairogen™ System). Spore-forming bacteria were cultivated on Plate Count Agar after a pretreatment at 80°C for 10 min. The plates were incubated at 30°C for 48 h in aerobic and anaerobic conditions. Lactic acid bacteria (LAB) were counted on MRS (De Man, Rogosa and Sharpe) Agar (Oxoid, Milan, Italy), and the plates were incubated for 48 h at 30°C.

Clostridia were enumerated on Reinforced Clostridial Medium (Oxoid, Milan, Italy), and the plates were incubated for 48 h at 30°C under anaerobic condition.

Enterococci were counted on the selective substrate, Slanetz-Bartley agar (Oxoid, Milan, Italy), after incubation at 37°C for 48 h.

4.2.3. Anaerobic tests set up and monitoring

Five batch anaerobic tests were conducted (Table 4.2): (i) two main tests were filled with 200 mL of dairy wastes mixture and inoculated respectively with 1% *w/v* (test A) and 3% *w/v* (test B) of industrial animal manure pellets (Stalfert N2 - Organazoto Fertilizzanti s.p.a, Pistoia, Italy); (iii) two of three control tests were filled with 200 mL of tap water and inoculated respectively with 1% *w/v* (test 1) and 3% *w/v* (test 2) of industrial animal manure pellets, whereas the remaining test (test 3) was filled with 200 mL of sole dairy wastes mixture and was not inoculated. Tests were conducted in 250 mL GL 45 glass bottles, (Schott Duran, Mainz, Germany). Anaerobic conditions were ensured

Table 4.2. Tests set-up and operating conditions.

	Main tests		Control tests		
	A	**B**	**1**	**2**	**3**
Cheese whey and buttermilk mixture (mL)	200	200	-	-	200
Tap water (mL)	-	-	200	200	
Inoculum (%, *w/v*)	1	3	1	3	-
Working temperature (°C)	38±1	38±1	38±1	38±1	38±1
Repetitions	8	8	3	3	3

by sealing the bottles (working volume of 200 mL) with a 5-mm-thick silicone disc held by a plastic screw cap. All bottles were kept at 38±1 °C in 200 W A-763 submersible heaters (Hagen, Germany). Tests were differently replicated (i.e. from 3 to 8 repetitions).

4.2.3.1. Biological gas production and intermediate products evolution

Biological gas production was measured with a volumetric displacement method (Esposito et al., 2012). The volume of biological gas was measured by connecting each bottle, used as bench scale biological reactors, with a capillary tube to an inverted 1000 mL glass bottle containing an acid solution at 1.5% HCl (Ghimire et al., 2015). The biological gas composition was analyzed in terms of H_2 and CH_4 using a Varian Star 3400 gas chromatograph (Agilent, Santa Clara California, USA) equipped with a Shin Carbon ST 80/100 column and a thermal conductivity detector. Argon was used as the carrier gas with an operating pressure of 20 psi. Biological gas measurements were performed daily during the first week and every three days during the following three weeks of incubation.

Liquid samples were collected at different times during the incubation. Samples were analyzed for lactose, galactose, lactic acid, acetic acid, propionic acid and ethanol by high-performance liquid chromatography (refractive index detector 133; Gilson system; pump 307, column Metacarb 67 h from Varian with 0.4 mL/min flow of 0.01 N H_2SO_4).

4.2.3.2. Monitoring of microbial growth and bacterial community structure by culture-dependent method

Bacterial counts were performed using either generic or selective differential growth media. Representative samples collected from the biological reactors during the incubation were characterized for total aerobic and anaerobic bacteria, aerobic spore-forming bacteria, LAB and clostridia, as described above. Methanogens were counted on agar plates of Medium 119, 120, 141, 334 (Leibniz Institute DSMZ, Germany) according to manufacturer's instructions. The plates were incubated for 7 days at 37°C under strictly anaerobic conditions by using an anaerobic chamber (Whitley DG 250 Anaerobic Workstation, Don Whitley Scientific, Shipley, UK).

4.2.3.3. PCR amplification and DGGE analysis of bacterial 16S rRNA fragments

Total microbial DNA was extracted from samples using the FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch Cedex, France) according to manufacturer's instructions. Genetic fingerprinting of the bacterial populations by DGGE was performed using two different PCR reactions. For the first round, the universal synthetic oligonucleotide primers fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC), described by Weisburg et al. (1991), were used to amplify the bacterial 16S ribosomal RNA (rRNA) gene. PCR mixture and conditions were set according to Ventorino et al. (2016a). In the second round, a nested PCR was done using the primers V3f (GC-CCTACGGGAGGCAGCAG) and V3r (ATTACCGCGGCTGCTGG) (Weisburg et al., 1991), spanning the V3 region of the 16S rRNA. Based on the method of Muyzer et al. (1993), a GC-clamp was added to the forward primer. PCR mixture and conditions were set according to Ventorino et al. (2016b). For the DGGE fingerprint, 25 µL from the nested PCR were analyzed using a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories, Milan, Italy).

DGGE analysis of bacterial communities was performed with a polyacrylamide gel (8% *w/v* acrylamide-bisacrylamide 37:5:1) using a denaturing gradient of 30-60% as described by Ventorino et al. (2013). The electrophoresis was run at 60°C and 200 V for 240 min. After staining the gels for 30 min with SYBR Gold, image analyses were performed in Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, UK).

4.2.3.4. PCR amplification and DGGE analysis of archaeal 16S rRNA fragments

A nested PCR approach was developed in this study to analyze the archaeal community by DGGE analysis using two different PCR reactions: the first PCR was performed using the primers Arch 46F (YTA AGC CAT GCR AGT) according to Øvreås et al. (1997) and Arch 1017R (GGC CAT GCA CCW CCT CTC) according to Barns et al. (1994); the second PCR was subsequently conducted using the amplicons from the first PCR as template and utilizing the primers Arch 344FGC (GC-GAC GGG GHG CAG CAG GCG CGA), as per Raskin et al. (1994), and Univ 522R (GWA TTA CCG CGG CKG CTG) as per Amann et al. (1995). Both PCR conditions were performed as per Akarsubasi et al. (2005). A GC-clamp was added to the forward primer. PCR mixture and conditions were performed as described by Akarsubasi et al. (2005). Finally, amplicons were analyzed by DGGE using a polyacrylamide gel (10% *w/v* acrylamide-bisacrylamide 37.5:1) with a denaturing gradient of 30-70% (Akarsubasi et al., 2005). Electrophoresis was run at 60°C and 200 V for 330 min. After electrophoresis, gels were stained for 30 min with SYBR Gold and bands were visualized and analyzed as described above.

4.2.4. Archaeal DGGE bands sequencing

Archaeal dominant bands were excised from the gel and the eluted DNA was re-amplified using the PCR conditions described above. The amplicons were verified by DGGE using DNA amplified from samples as the control. Several archaea were identified using a molecular marker obtained by a combination of the six PCR products. Actively growing cultures of archaeal strains *Methanococcus voltae* DSM 1537, *Methanobrevibacter ruminantium* DSM 1093, *Methanobacterium congolense* DSM 7095, *Methanosarcina acetivorans* DSM 2834, *Methanosarcina barkeri* DSM 800 and *Methanosarcina mazei* DSM 2053, were provided by Leibniz Institute DSMZ-German (Figure 4.6). These strains were inoculated in tubes closed with a flange-type butyl rubber septum and a screw cap containing four specific liquid grow media (Medium 119, 141, 120, 120a) as per the laboratory's recommendation. Cultures were then incubated in an anaerobic chamber (Whitley DG 250 Anaerobic Workstation) set at 37°C for 7 days. DNA extraction, PCR amplification and DGGE analysis of archaeal DSMZ strain 16S rRNA fragments were performed as previously reported. The procedure was conducted by evaluating the purity and position of the bands for each strain before mixing the PCR products for the marker. All the products that migrated as a single band were purified and

sequenced according to Pepe et al. (2013) and compared to the GenBank nucleotide data library using the BLAST program at the National Center of Biotechnology Information website to determine their closest phylogenetic relatives.

4.2.5. Statistical analyses

A one-way ANOVA followed by a Tukey test for pairwise comparison of means ($p \leq 0.05$) were used to assess the difference in microbial counts at different incubation times. Statistical analyses were performed using the SPSS 21.0 statistical software package (SPSS Inc., Cary, NC, USA) as reported by Ventorino et al. (2017).

Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, England) was used to automatically detect the DGGE bands and perform cluster analysis. The correlation matrix was performed using the method described by Saitou and Nei (1987) and the percentage of similarity of microbial populations present in different samples was estimated according to the average linkage method in the Cluster procedure of Systat 5.2.1.

4.3. Results

4.3.1. Characterization of the dairy waste, their mixture and inoculum

The chemical characteristics of the substrate and inoculum are reported in Table 4.3. COD was higher in cheese whey (124.0±2.8 g L^{-1}) than buttermilk (19.7±0.3 g L^{-1}) and the resulting mixture consequently showed a COD closer to that of cheese whey than buttermilk (81.0±0.8 g L^{-1}). In the mixture TS and VS concentrations were 49.0±0.5 g l-1 and 42.3±0.2 g L^{-1}, respectively. pH was 5.3±0.1 and TTA was 0.8±0.1 °SH.

The microbial characterization of cheese whey, buttermilk, their mixture and the inoculum is shown in Figure 4.1. Initially, total aerobic and anaerobic microorganisms as well as LAB were more abundant in cheese whey (7.8±0.0, 7.2±0.0 and 6.3±0.0 log CFU mL^{-1}, respectively) than in buttermilk (4.0±0.1, 3.8±0.0 and 4.6±0.0 log log CFU mL^{-1}, respectively). In contrast, aerobic spore-

forming bacteria were more numerous in buttermilk (2.9±0.0) than in cheese whey (2.3±0.0 log CFU mL^{-1}).

Table 4.3. Physico-chemical characterists of whey cheese, buttermilk, their mixture and inoculum.

Samples	COD ($g\ L^{-1}$)	TS ($g\ L^{-1}$)	VS ($g\ L^{-1}$)	pH	TTA (°SH)
Cheese whey (W)	124.0±2.8	n.d.*	n.d.*	5.3±0.2	0.8±0.1
Buttermilk (B)	19.7±0.3	n.d.*	n.d.*	5.3±0.1	0.4±0.1
Mix of W and B**	81.0±0.8	49.0±0.5	42.3±0.2	5.3±0.1	0.8±0.1
Inoculum	n.d.*	0.8±0.0	0.4±0.0	n.d.*	n.d.*

*not determined; **(ratio 2:1 *v/v*);

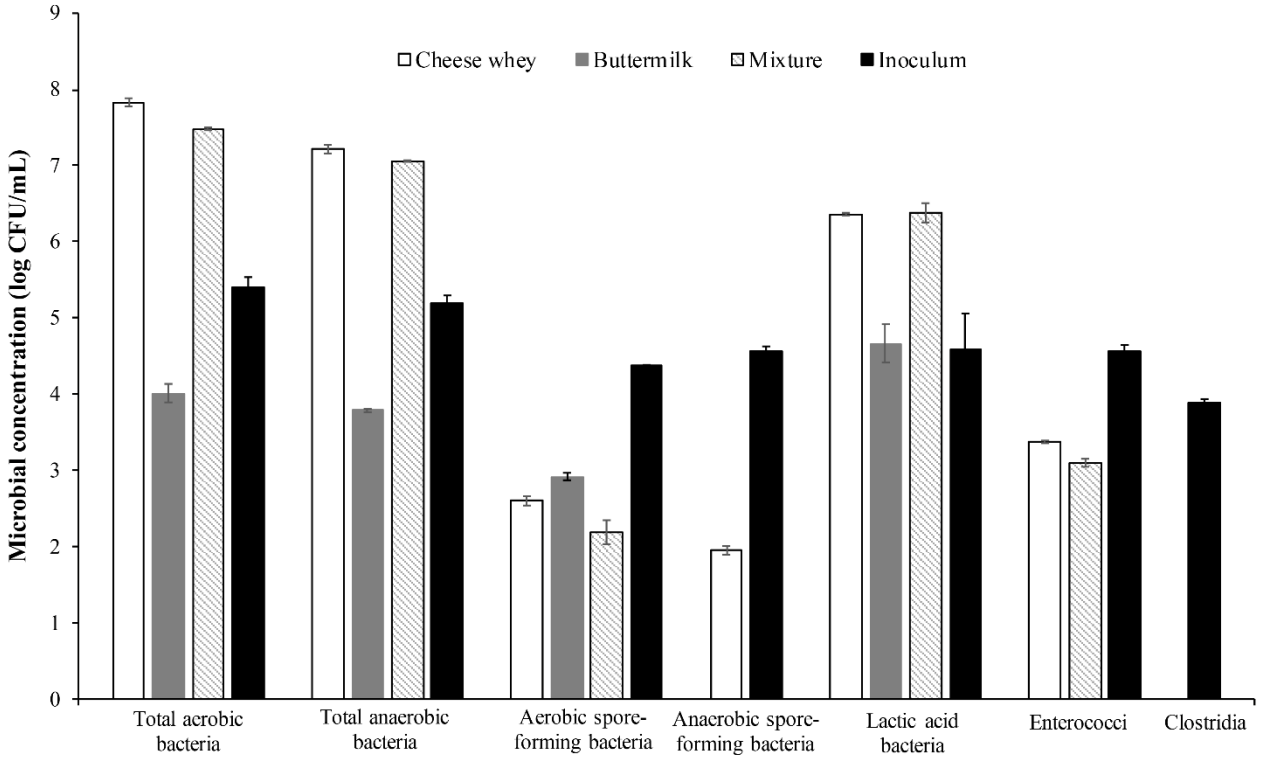

Figure 4.1. Initial microbial characterization of the cheese whey, buttermilk and mixture (ratio 2:1).

Anaerobic spore-forming bacteria (1.9±0.0 log CFU mL^{-1}) and enterococci were only detected in cheese whey (3.8±0. log CFU mL^{-1}) and inoculum (4.6±0.0 log CFU mL^{-1}). The presence of clostridia in the tests was only due to inoculum (3.9±0.0 log CFU mL^{-1}).

4.3.2. Biological gas production and physico-chemical biosystem changes

The gas volumes measured during the incubation period was used to plot the cumulative H_2 and CH_4 curves (Figure 4.2), where the X-axis displays the incubation time, and the Y-axis the corresponding H_2 and CH_4 cumulative production per gram of VS of the initial dairy waste mixture. Moreover, the cumulative production curves were plotted subtracting the H_2 and CH_4 contribution from the inoculum. Approximately 8.9±0.4 mL H_2 g VS^{-1} and 2.2±0.1 mL CH_4 g VS^{-1} were the cumulative productions of H_2 and CH_4 observed in test B after 28 days of incubation at 38°C. The amount of CO_2 (% *v/v*) detected in the gas mixture ranged from 99% (at the beginning of the process) to 68% measured when CH_4 and H_2 production occurred (Table 4.4). The H_2 production rate showed an increasing rate during the first 21 days, followed by a progressive decrease until the end of the incubation time. According to Nielfa et al. (2015), the value of theoretical methane production (BMP_{thCOD}) at laboratory condition is approximately 0.723 L. Under experimental condition used, the methane production achieved was around 0.018 L.

The H_2 production rate was increasing during the first 23 days of incubation, followed by a progressive decrease until the end of the incubation time. Cumulative production curves display a horizontal asymptote representing the maximum experimental production per gram of VS added. H_2 and CH_4 were not detected in test A after 28 days of incubation at 38°C (Figure 4.2) and accordingly CO_2 amount was around 99.9% (*v/v*) (Table 4.4).

Table 4.4. Biological gas composition.

Incubation day		**1**	**2**	**3**	**4**	**5**	**6**	**10**	**13**	**16**	**18**	**20**	**23**	**27**
TEST A														
Gas Composition (%)	**H_2**	0.00	0.00	0.01	0.01	0.01	0.01	0.08	0.05	0.04	0.00	0.01	0.01	0.01
	CH_4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	13.10	3.49	2.57	2.40
	CO_2	99.99	99.99	99.99	99.57	99.57	99.89	99.61	99.93	99.96	86.87	96.50	97.60	97.68
TEST B														
Gas Composition (%)	**H_2**	0.00	0.00	0.00	0.01	0.02	0.38	0.10	2.73	5.63	17.61	19.77	10.05	2.25
	CH_4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.94	14.20	10.72	5.87	0.00
	CO_2	99.99	99.98	99.98	99.98	99.97	99.61	99.89	97.25	92.42	68.18	69.50	84.07	97.74

During the first week, at least 86% of the lactose present initially in the dairy wastes mixture was consumed, and the lactic acid concentration increased up to reach approximately 25 g/L in both tests

A and B. After 14 days the lactic acid concentration decreased in test B (Figure 4.3) and an increase in H_2 production was observed, as shown in Figure 4.2.

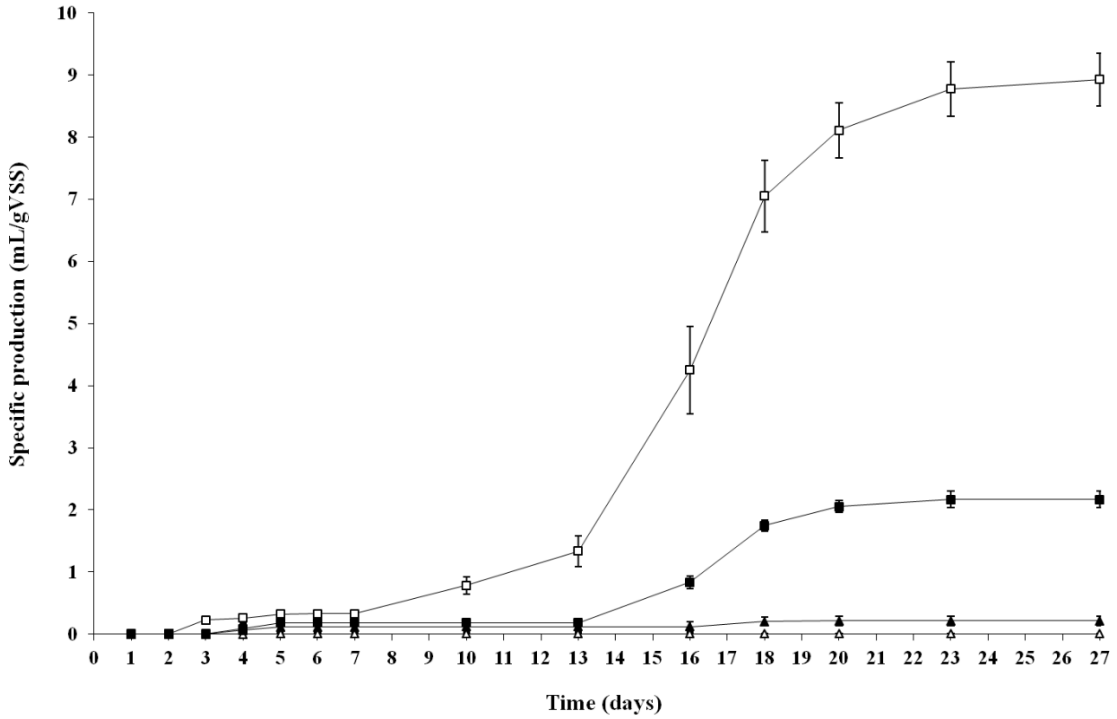

Figure 4.2. H_2 (white symbols) and CH_4 (black symbols) produced during incubation time from test A (triangle) and test B (square).

Whereas, in test A lactic acid concentration remained constant from day 14 to the end of the incubation time, and H_2 and CH_4 were not produced. Ethanol, acetic and propionic acids were only detected in test B after 14 days, at concentrations of 16.4±1.5, 7.3±1.3 and 6.3±1.2 g L^{-1}, respectively (Figure 4.3). Parameters such as pH and TTA were continuously monitored throughout the incubation time. pH in test A dropped to 3.5±0.0 and remained constant until the end of the experiment. Interestingly, in test B, the pH decreased initially (3.9±0.0) and then increased to 5.9±0.0. TTA remained constant, approximately equal to 5.4±0.0 °SH in test A, whereas in test B decreased from 4.0±0.0 °SH to 0.8±0.0 °SH between days 14 and 21, accordingly with the pH and lactose consumption trends. COD in test A moved from the initial value of 79.0±0.1 g L^{-1} to the final of 72.2±0.4 g L^{-1}, whereas, in test B moved from 83.2±0.1 g L^{-1} to 65.0±0.5 g L^{-1}during the incubation time.

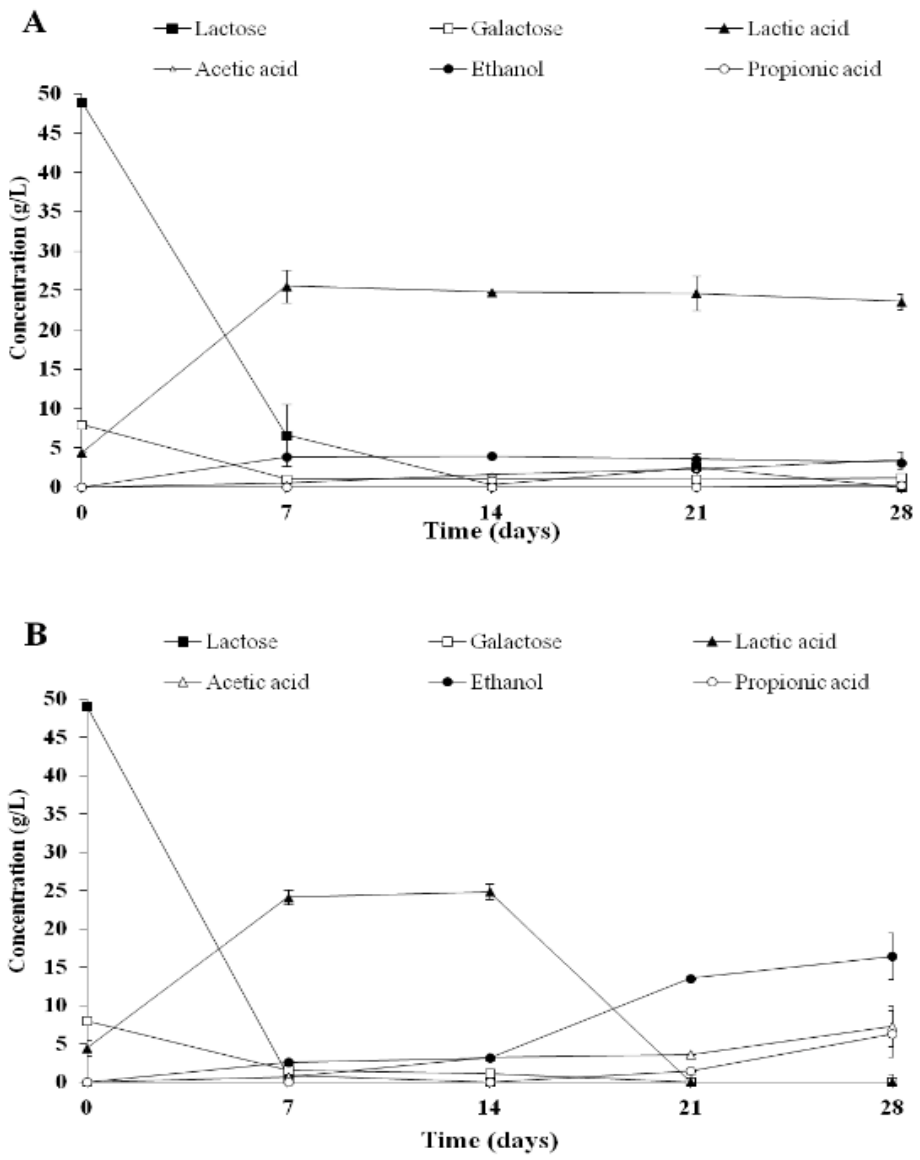

Figure 4.3. Concentrations of lactose, galactose, lactic acid, acetic acid, ethanol and propionic acid in samples collected from test A (A) and test B (B).

4.3.3. Microbial counts during the biodigestion process

Initially, no difference was noticed among the microbial groups in the two tests. Total aerobic and anaerobic bacteria were present in a load of approximately 7-8 log CFU mL^{-1}, whereas the concentration of aerobic and anaerobic spore-forming bacteria and clostridia was approximately 3-4 log CFU mL^{-1} (Figure 4.4).

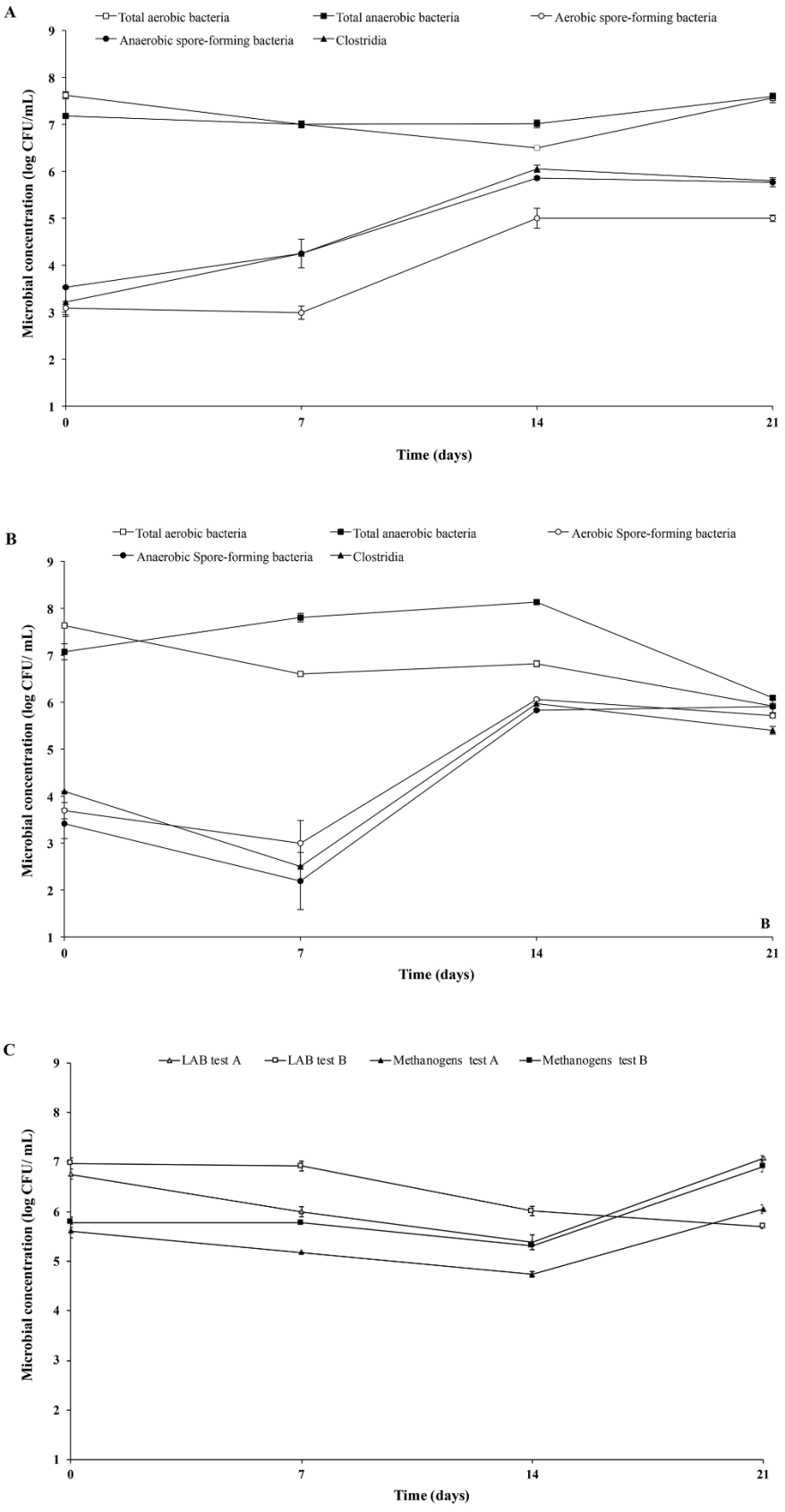

Figure 4.4. Counts of the principal microbial groups in test A (A) and test B (B) and counts of methanogen and LAB (C) in both tests during incubation time.

Total aerobic and anaerobic bacteria remained constant throughout test A, but the anaerobic bacterial load increased after 14 days of incubation in test B (8.1±0.0 log log CFU mL^{-1}). Moreover, at the end of the incubation process, the total aerobic bacteria were more abundant in test A than test B (7.5±0.1 and 5.9±0.1 log log CFU mL^{-1}, respectively). Clostridia and anaerobic spore-forming bacteria increased up to reach approximately 6 log CFU/mL, and keeping constant their concentration, equal to 5.8-5.9 log CFU mL^{-1}, after 21 days of incubation in both tests A and B.After a decrease during the first 14 days (from 6.7±0.1 to 5.4±0.1 log CFU mL^{-1}), LAB increased to 7.0±0.0 log CFU mL^{-1}in test A (p = 0.02), whereas the growth trend in test B showed a decrease from 7.0±0.1 to 5.7±0.0 log CFU mL^{-1} (p = 0.01) after 21 days of incubation (Figure 4.4). This occurred when all lactose was consumed, and the ethanol and organic acid concentrations consequently increased in test B (Figure 4.3). The initial methanogens count was approximately 5.6-5.7 log CFU $mL^{-1}$for both tests A and B (p =0.03). During the incubation period, methanogens increased in test B, and at the end of the experiment, they showed a load of 1 log higher (7.0±0.1 log CFU mL^{-1}, p = 0.00) than in test A, where the methanogen count resulted to be 6.0±0.0 log CFU mL^{-1} after 21 days (Figure 4.4).

4.3.4. Bacterial and archaeal community structure

The DGGE band patterns were representative of samples collected at different times throughout the process. Bacterial and archaeal diversity increased during the anaerobic process in test B, where H_2 and CH_4 production occurred. No difference (similarity of 100%) in the bacterial profile of the samples collected at the initial time and after 7 days was observed in both tests A and B (Figure 4.5). However, cluster analysis showed that the percentage of similarity among the samples within the same test decreased over time, particularly in test B. In detail, from day 14 (T14) to 28 (T28), the similarity level ranged from 76 to 79% and from 81 to 89% in test A and B, respectively (Figure 4.5). However, on the final day (T28), the number of bands in test B increased, indicating the highest bacterial diversity (Figure 4.5). Moreover, the control sample of inoculum (I) had the least similarity (24%, Figure 4.5) compared with samples collected at different days of the incubation time. The DGGE profiles and cluster analysis of the archaeal community showed a significant difference between the two tests, as seen in the marked division of the samples in the cluster analysis (Figure 4.6). The archaeal community remained mostly constant during the first 14 days in test B (similarity 81-84%, Figure 4.6), whereas at days 21 and 28 of the incubation time, a remarkable increase in diversity was found through DGGE profiles, with a similarity of 69% (Figure 4.6). The high similarity

between the inoculum and the samples after 21 days of incubation (91%, Figure 4.6) demonstrated that the animal manure concentration influenced the methanogenic microbial community and determined the higher CH_4 production in test B.

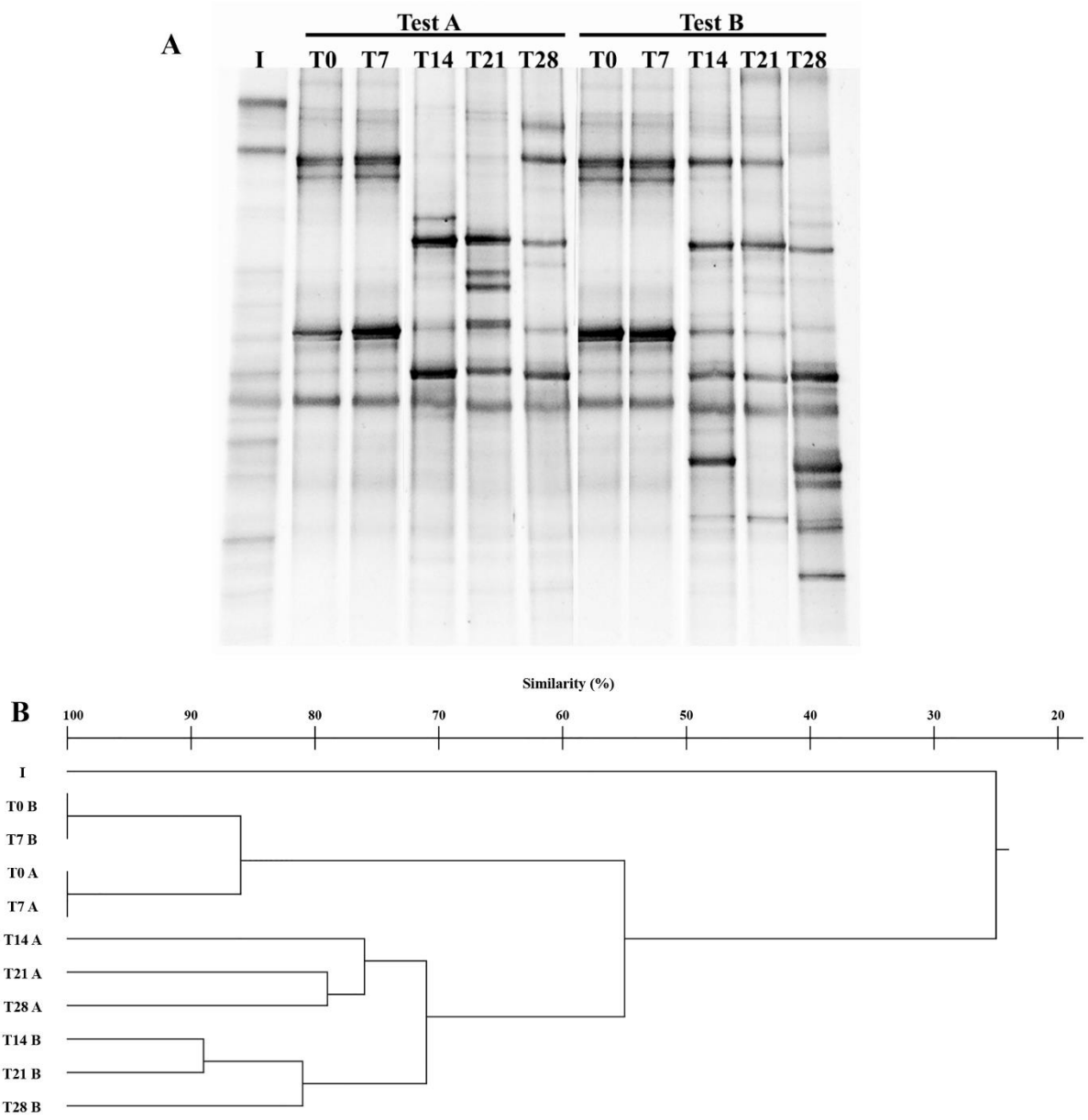

Figure 4.5. Molecular analysis by the culture-independent method (PCR-DGGE) – bacterial community structure. The DGGE band patterns (A) and the cluster analysis (B) were representative of the samples collected from test A and test B at different days of the incubation time. Lanes: 1, animal manure inoculum (I); 2, test A at initial time (T0 A); 3, test A at day 7 (T7 A); 4, test A at day 14 (T14 A); 5, test A at day 21 (T21 A); 6, test A at day 28 (T28 A); 7, test B at initial time (T0 B); 8, test B at day 7 (T7 B); 9, test B at day 14 (T14 B); 10, test B at day 21 (T21 B); 11, test B at day 28 (T28 B).

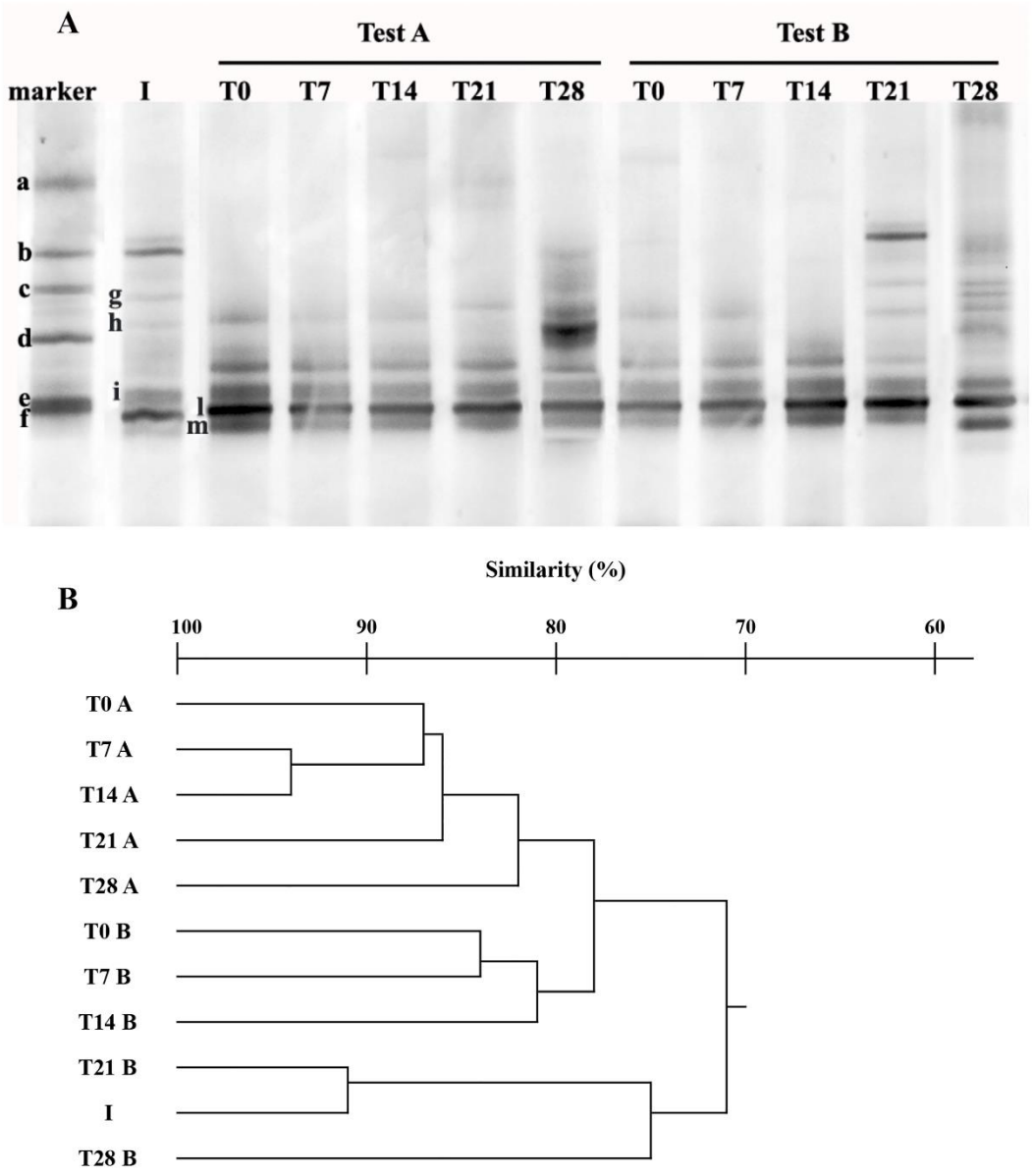

Figure 4.6. Molecular analysis by the culture-independent method (PCR-DGGE) – archaeal community structure. The DGGE band patterns (A) and the cluster analysis (B) were representative of samples collected from test A and test B at different days of the incubation time. Lanes: 1, Marker: a: *Methanococcus voltae*; b: *Methanobrevibacter ruminantium*; c: *Methanobacterium congolense*; d: *Methanosarcina acetivorans*; e: *Methanosarcina barkeri*; f: *Methanosarcina mazei*; 2, animal manure inoculum (I); 3, test A at initial time (T0 A); 4, test A at day 7 (T7 A); 5, test A at day 14 (T14 A); 6, test A at day 21 (T21 A); 7, test A at day 28 (T28 A); 8, test B at initial time (T0 B); 9, test B at day 7 (T7 B); 10, test B at day 14 (T14 B); 11, test B at day 21 (T21 B); 12, test B at day 28 (T28 B).

Table 4.5. Identification based on Blast comparison in the GenBank data libraries of the archaeal bands obtained by PCR-DGGE. The gray color indicates the presence of the bands in different samples.

				Test A					Test B				
Band	**Closest relative species (identity percentage)**	**Accession number**	**Inoculum**	**T0**	**T7**	**T14**	**T21**	**T28**	**T0**	**T7**	**T14**	**T21**	**T28**
a	*Methanococcus voltae* DSM-1537	CP002057.1											
b	*Methanobrevibacter ruminantium* DSM-1093	CP001719.1											
c	*Methanobacterium congolese* DSM-7095	LT607756.1											
d	*Methanosarcina acetivorans* DSM-2834	AE010299.1											
g	*Nitrososphaera* spp. (87%)	NR_134097.1											
h	*Methanobrevibacter* spp. (97%)	NR_074235.1											
i	*Methanoculleus sediminis* (96%)/ *Methanoculleus horonobensis* (96%)	NR_136474.1/ NR_112788.1											
l	*Methanocorpusculum aggregans* (98%)/ *Methanocorpusculum labreanum* (98%)	NR_117749.1/ NR_074173.1											
m	*Methanoculleus hydrogenitrophicus* (98%)/ *Methanoculleus thermophiles* (98%)	NR_116881.1/ NR_028156.1											

In test A, the archaeal diversity remained similar during the anaerobic process (similarity of 86-94%, Figure 4.6) until day 28, when a higher diversity with a similarity of 82% was noticed (Figure 4.6). To identify the archaeal species most involved in the production of H_2 and CH_4, dominant bands were excised from the DGGE gels and identified by sequencing. In addition, the DGGE profiles of the samples were identified by comparing them with the known archaeal bands as a marker. As reported in Table 4.5, several species of methanogens were identified in all samples, whereas others were found only at specific times throughout the process.

Methanobrevibacter ruminantium and *Methanobrevibacter* spp. were recovered from the inoculum and from test B at day 21 (band b and h, Table 4.5), where higher H_2 and CH_4 production occurred, demonstrating the high similarity among these samples (Figure 4.6). Moreover, *Methanosarcina acetivorans* and *Methanobrevibacter* spp. were recognized at day 28 in test A as well as in test B (band d and h, Table 4.5). *Methanoculleus sediminis/Methanoculleus horonobensis*, *Methanocorpusculum aggregans*/*Methanocorpusculum labreanum*, *Methanoculleus hydrogenitrophicus*/*Methanoculleus thermophiles* (band i, l and m) were identified in almost all samples except T28 in test B (Table 4.5).

4.4. Discussion

4.4.1. Characterization of the dairy wastes and inoculum

The chemical characterization of substrates used in this study showed a COD concentration higher than the average value reported in the literature, for instance 100 g L^{-1} (Gelegenis *et al.*, 2007). The high COD in the cheese whey influenced the resulting COD in the mixture, indicating the potential of this substrate as a feed in anaerobic processes (Pilarska et al., 2016) and produce H_2 and CH_4. This aspect is further demonstrated by the large amount of VS measured in the mixture (Hassan and Nelson, 2012). Moreover, cheese whey influenced the pH of the mixture since this waste is typically salty due to the acidity, with pH ranging from 5.2 to 5.4 (Blaschek et al., 2007). Moreover, the relatively high amount of salt in the substrate could inhibit microbial H_2 production (Lee et al., 2012). Combined specific productions of H_2 and CH_4 achieved in this work are relatively low if compared to other works where the same substrate as well as others were used (Table 4.6). For this reason, organic acids were detected at the end of the experiments as organic substance that could be degraded to achieve specific production close to the optimal one.

Table 4.6. Yields of combined production of H_2 and CH_4 from different substrates and under different operating conditions (adapted from Roy et al., 2016).

Substrate	Reactor type I stage	H_2 yield	Reactor type II stage	CH_4 yield	References
Cassava stillage	CSTR	56.6 L kg^{-1} VS	CSTR	249 L kg^{-1} VS	Luo et al. (2010)
OFMSW	CSTR	205 L kg^{-1} VS	FBR	464 L kg^{-1} VS	Chu et al. (2008)
Food waste	Rotating drum reactor	65 L kg^{-1} VS	CSTR	546 L kg^{-1} VS	Wang and Zhao (2009)
Wheat straw hydrolysate	UASB	89 L kg^{-1} VS	UASB	307 L kg^{-1} VS	Kongjan et al. (2011)
Corn stalk	CSTR	79.8 L kg^{-1} VS	CSTR	2272.5 L kg^{-1} VS	Guo et al. (2014)
Algal biomass	Serum bottles	283.4 L kg^{-1} VS	CSTR	253.5 L kg^{-1} VS	Cheng et al. (2014)
Algal biomass	Serum bottles	135 L kg^{-1} VS	Serum bottles	314 L kg^{-1} VS	Wieczorek et al. (2014)
Dairy waste	ASBR	35.6 L kg^{-1} VS	ASBR	627 L kg^{-1} VS	Lateef et al. (2014)
Dairy waste	Batch	8.9±0.4 L kg^{-1} VS	Batch	2.2±0.1 L kg^{-1} VS	this manuscript
Skim latex serum (SLS)	UASB	2.25 L L^{-1}	UASB	6.41 L L^{-1}	Kongjan et al. (2014)
Cassava waste	UASB	0.054 L g^{-1} COD	UASB	0.16 L g^{-1} COD	Intanoo et al. (2014)
Tequila vinasses	SBR	918 L kg^{-1} VS	UASB	0.25 L g^{-1} COD	Buitrón et al. (2014)
Corn silage	UASB	59.4 L kg^{-1} VS	UASB	328 L kg^{-1} VS	Nkemka et al. (2015)

This discrepancy is due to the operating conditions tested in this work, definitely far from the optimal ones as the aim of the authors was to study the natural development of microbial species involved in the anaerobic degradation of dairy waste.

The microbial composition was mostly affected by cheese whey, which determined the high concentration of total aerobic and anaerobic bacteria in the dairy wastes mixture. The microbial community of this substrate was mainly composed of LAB that commonly characterize dairy wastes (Ercolini et al., 2001). Moreover, in cheese whey, the LAB and enterococci concentrations were higher than those generally found in raw milk (Carraro et al., 2011) being them closer to those found in natural whey starter cultures used in cheese industry, which typically range from 6.0 to 7.7 log CFU mL^{-1} and from 3.8 to 5.5 log CFU mL^{-1}, respectively (De Candia et al., 2007). However, despite a relevant LAB load is usually found in acidic environment (pH 4 approximately) (De Candia et al., 2007), in this study, a high LAB concentration (approximately 6 log log CFU mL^{-1}) was detected at a pH around 5.3 due to the fermentation process during the mozzarella cheese production chain (Carvalho et al., 2013). Moreover, enterococci were detected solely in cheese whey and not in buttermilk, consequently, they were present in the dairy wastes mixture (Sodini et al., 2006). The presence of this microbial group is related to H_2 production (Liu et al., 2009).In fact, non-spore-forming species such as *Enterococcus* sp. can contribute to enhanced hydrogen production as reported by Liu et al. 2009 that found these microorganisms in the mixed culture tested. Moreover *Enterococcus* sp. was also detected during operations of a fermentative biohydrogen-producing continuous stirred tank reactor (CSTR) with cheese whey as substrate by Davila-Vazquez et al. (2009).

Since enterococci were also detected in the inoculum, test B could count on a higher initial amount of enterococci than test A and this difference is a possible explanation for the higher H_2 production from test B rather than test A. Furthermore, the most interesting result obtained from the cultural analysis of the inoculum concerns the initial clostridia concentration. The genus *Clostridium* is actually the most common anaerobic population involved in the first steps of the anaerobic digestion process (Pagliano et al., 2017) and it is well known as a H_2 producing bacteria strain (Hung et al., 2011). The presence of this microbial group in tests A and B was exclusively due to inoculum that significantly affected the volume of H_2 produced.

4.4.2. Biological gas production,intermediates and end-products

The microbial analysis highlighted the influence of the inoculum on biological gas production yield. Increasing the amount of inoculum in the ratio with substrate from 1% *w/v* (test A) to 3% *w/v* (test B) resulted in a higher LAB and methanogens concentration. Moreover, H_2 and CH_4 production occurred only in test B, demonstrating that the different amounts of inoculum also affected the environment acidity, which was lower (i.e. pH higher) in test B than test A, thus explaining the difference in biological gas production. The animal manure acted as a buffer due to the presence of ammonium (Ghimire et al., 2017). In fact, when proteins are degraded, ammonium is released forming ammonium bicarbonate, which results in additional buffer capacity (Gallert et al., 1998). Furthermore, also Murto et al. (2004) reported the role of animal manure as buffering agents, however they did not neglect that a high ammonia concentration could result counter-productive for the process performance because it can be toxic to the microorganisms. Actually the presence of high ammonia concentration in the environment can be responsible for the process instability due to changes in the intracellular pH interactions as well as inhibitions of specific enzyme reaction, thus resulting in a lower methane yield (Chen et al., 2008). This inhibitory effect is in general stronger for the acetoclastic methanogens than the hydrogenotrophic ones (Angelidaki and Ahring, 1993). Concerning the microbial changes during the process, the decrease of LAB and lactic acid concentration occurred in test B coupled with methanogen growth toward the end of the process. Furthermore LAB are responsible for acidifying the liquid bulk in the reactors (Terzaghi and Sandine, 1975), because they produce lactic acid by homolactic and heterolactic fermentation processes (Palomba et al., 2012; Pradhan et al., 2017). Therefore the decrease of LAB concentration in test B favored the pH increase and CH_4 production. Indeed, according to Ghaly (1995), low biological gas productivity and CH_4 yield are associated to low pH in the fermented cheese whey, explaining the low productivity in test A. The higher pH value in test B promoted the growth of methanogens that are ecologically related to the animal manure inoculum and are involved in the final step of anaerobic digestion. The LAB load in test A was, actually, 1 log CFU mL^{-1} higher than in test B, thus limiting the CH_4 production.

Comparing results of biological gas with those of organic acids production, lactic acid concentration increased at the beginning of the process in both tests A and B due to lactose consumption during the first 7 days of incubation. Actually, lactose is easily degraded by acidogenic microorganisms during lactic acid fermentation with high lactic acid synthesis. The

degradation of lactose also results in the production of lactic acid, propionate, bioethanol and acetate (Vidal et al., 2000). In fact, the major products of heterolactic fermentation are lactic acid, ethanol, acetate and carbon dioxide produced by LAB through two patterns. With glucose as substrate, these are: (1) the production of lactate, ethanol, and CO_2, with occasional traces of acetate and (2) the formation of glycerol along with lactate, acetate, and CO_2 (DeMoss et al., 1951). As shown in the results of test A, the accumulation of these intermediate products, particularly in the undissociated form, caused the inhibition of several microbial species responsible for biological gas production as reported by Aguilar et al. (1995). For instance, ethanol was produced in large amount from day 14 of incubation in test B, and probably caused an inhibiting effect on the H_2 production in agreement with Hung et al. (2011). Moreover, according to Yang et al. (2013) cannot be excluded that acetate was converted to ethanol in the presence of H_2, thus affecting the trend of acetic acid in Figure 4.3.

Moreover, Noike et al. (2002) observed the decrease or cessation of hydrogen production by different strains of *Clostridium* due to the addition of LAB isolated from the wastes generated in the bean curd manufacturing, reporting that a large amount of lactic acid was also produced. Indeed, lactic acid fermentation occurred in place of hydrogen fermentation promoted by the relationship between LAB and hydrogen producing bacteria, mostly clostridia. The latter consideration explains why test A resulted in a really low H_2 production: actually, less amount of inoculum added resulted in lower clostridia load. On the contrary, the LAB concentration showed a decreasing trend in test B after 14 days of incubation, in correspondence of significant H_2 production.

4.4.3. Effects of microbial community changes on the physico-chemical characteristics of the biological system and viceversa

In test B, an increase in the methanogens concentration was observed during the incubation time, thus leading to a not neglecting biological gas production and lactic acid consumption. Indeed, in test B occurred that LAB, such as *Lactococcus lactis* and *Lactobacillus delbrueckii*, converted lactose into lactic acid, whereas methanogens such as *Methanosarcina* and *Methanosaeta* subsequently converted lactate into acetic acid (Hassan and Nelson, 2012). On the other hand, lactic acid concentration remained constant in test A where the methanogens level was not sufficient to promote biological gas production.

Specifically, *Methanobrevibacter ruminantium* was identified and it is known to be the largest methanogen group in lactating dairy cattle and ovine rumen that can use H_2, CO_2 and formate as substrates for CH_4 production (Hook et al., 2010). In this study, the presence of *Methanobrevibacter ruminantium* was demonstrated by using cultural-independent molecular analysis for the archaeal group, identifying the DGGE bands in the samples collected at the incubation time when H_2 and CH_4 production occurred in test B (T21 B). This result was confirmed by the culture data, since from day 14 the methanogens concentration increased in test B when the pH reached a value of 6. Within the genus *Methanobrevibacter*, only *Methanobrevibacter ruminantium* has been reported to grow at pH 6 (Savant et al., 2002). *Methanobrevibacter ruminantium* was identified in the inoculum as well as in samples collected from test B at day 21, when the pH increased up to 5.8 and biological gas was produced. The pH condition probably selected hydrogenotrophic methanogens (Ventorino et al., 2018) rather than acetoclastic methanogens. *Methanobacteriales* (such as *Methanoculleus* and *Methanocorpusculum*) and *Methanomicrobiales* (such as *Methanobrevibacter* and *Methanobacterium*) were not influenced by pH change according to Kundu et al. (2017).

Archaeal diversity varied throughout the incubation period, and the growth conditions favored several archaeal species that were different from those found in the previously collected samples, such as *Methanosarcina acetivorans*, which was identified in both tests A and B at day 28. *Methanosarcina acetivorans* is actually a metabolically versatile methanogen that grows under extreme energy limitations (Schlegel et al., 2012) intended as generation of ATP. In fact *Methanosarcina acetivorans* is lack in hydrogenase enzyme that reduces protons to hydrogen and thus it has a different electron transport compared with other aceticlastic methanogens (Schlegel et al., 2012).

In summary, the dynamics of the archaeal community structure were greatly influenced by the microbiological features and concentration of the inoculum and consequently affected the performance of the anaerobic process in terms of biological gas production and liquid end-products composition and concentration, according to Hoffmann et al. (2008).

4.5. Practical application and future perspectives

Cheese whey and buttermilk are attractive sources for producing biofuels and bio-based materials as they can be successfully converted in valuable end-products through an anaerobic process, thus conveniently providing a resource-efficient waste management and

environmental protection system. As the performance of a biological process is strictly affected by the microbial community that thrives and diversifies on the substrate used, this research was focused on associating bacterial and archaeal groups to the occurrence of low or high production of different valuable end-products of the process. Results from this study, therefore, can be successfully applied as useful tool to reach a full control of the anaerobic process and steer it toward the production of a specific end-product, rather than others. This work has investigated only few aspects of the interrelations existing in the anaerobic process between microbial community and the group of operating variables composed of substrate, inoculum, environmental conditions and end-products, therefore further studies on this topic are necessary to gain a higher level of knowledge about this key factor of the anaerobic process.

4.6. Conclusions

Cheese whey and buttermilk from mozzarella cheese processing factories are suitable by-products for being valorized in a biorefinery process. In such process, the inoculum amount significantly influenced the bacterial and archaeal communities and consequently the entire performance of the process. This study has demonstrated that the increase of 1 log of methanogens due to the highest inoculum concentration of 3% (*w/v*), improved H_2 and CH_4 production. Microbial analysis has furthermore recognized in *Methanobrevibacter* genus and, in particular *Methanobrevibacter ruminantium* the most dominant species in this specific conditions of anaerobic process fed with a mixture of mozzarella cheese whey and buttermilk.

CHAPTER 5

Bacterial and

archaeal communities dynamics

This chapter reports the content of original paper:

Pagliano G, Ventorino V, Panico A, Romano I, Pirozzi F, Pepe O. Anaerobic process for bioenergy recovery from dairy waste: meta-analysis and enumeration of microbial community related to intermediates production" accepted by Frontiers in Microbiology, section Microbiological Chemistry and Geomicrobiology

5.1 Introduction

In the near future, novel bio-based technologies in waste management can be used to convert organic waste into valuable products such as renewable energy and/or biopolymers through biological processes (Pagliano et al., 2017) with a goal to potentially replace fossil fuels with biomasses and reducing pollutant emissions.

Several organic wastes are potentially suitable to be used as substrates for producing renewable energy vectors (e.g. biohydrogen, biogas and biomethane) through anaerobic biosystems (Raposo et al., 2012; Ghimire et al., 2015; Pagliano et al., 2017). Among them, cheese whey and buttermilk, residues from dairy factories as by-products of cheese, yogurt, milk and butter production process are interesting substrates for their high content of soluble organic matter, i.e. chemical oxygen demand (COD) ranging from 0.1 to 100 g L^{-1} (Prazeres et al., 2014). Besides substrates and operational conditions, microorganisms significantly affect the performance of the anaerobic process (Panico et al., 2014). In fact, the efficiency and stability of this process is entirely dependent upon the syntrophic activity of microorganisms operating in different phases (Li et al., 2009; Vanwonterghem et al., 2014).

Actually, anaerobic digestion process can be conceptually divided into four stages defined by the primary catabolic reactions that occur at each stage: hydrolysis of complex polymers (I, hydrolysis), fermentation of the hydrolysis end-products to volatile fatty acids (VFAs) (II, acidogenesis), conversion of VFAs to acetate and hydrogen (III, acetogenesis), and finally the production of methane from acetate and hydrogen (IV, methanogenesis) (Yu et al., 2010). Therefore, it is important to understand how the raw materials as well as environmental and physical conditions established in the system affect microbial growth and activity, and therefore, the performance of the anaerobic process. Numerous studies using different types of organic wastes have been conducted to better understand the role of the microorganisms involved in each stage and the microbiomes present in the anaerobic reactors (Nelson et al., 2011; Li et al., 2017; Ros et al., 2017; Westerholm et al., 2017). For this purpose, various methods have been applied to investigate the microbial communities or targeted specific groups in anaerobic digesters, including clone library of 16S rRNA genes (Rincón et al., 2008), denaturing gradient gel electrophoresis (DGGE) analysis (Shin et al., 2010; Palatsi et al., 2011; Supaphol et al., 2011; Pagliano et al., 2018; Ventorino et al., 2018) and fluorescence in situ hybridization (FISH) (Braguglia et al., 2012). All these methods, although are highly efficient, analyze a limited number of aspects if compared with the emerging metagenomic approaches based on high-throughput sequencing (HTS) (Yang et al., 2014). Therefore, in this study, the use of a polyphasic approach including HTS in lab-scale batch tests, allowed to elucidate the dynamics of microbiota in different stages of the anaerobic process fed with a mixture of dairy waste from a

mozzarella cheese factory. Culture-independent and culture-dependent approaches coupled with hydrogen and methane production can improve the knowledge concerning this specific anaerobic biosystem. In particular, it is important to elucidate how specific microbial populations can steer the hydrogen and methane production in order to control, also through traditional parameters (pH, TS, VS, COD) (Pontoni et al., 2015), the efficiency of the anaerobic biosystems fed with different wastes and by-products from dairy industry.

5.2 Materials and Methods

5.2.1 Dairy wastes characterization and experimental set up

30 L samples of cheese whey and buttermilk were collected from the production chain of buffalo mozzarella cheese operating at a temperature of approximately 33-37 °C and immediately analyzed for physical-chemical (pH, TTA, TS, VS, COD) and microbial characterization. The pH was measured using a HI 221 pH meter (Hanna Instruments Inc., Woonsocket, RI, USA). Total titratable acidity (TTA) was calculated as the mL of 0.1 N NaOH 10 mL^{-1} of sample (AACC, 1975). Total solids (TS) and volatile solids (VS) were evaluated as described in the standard methods (APHA, 2005). COD was measured with an ECO08 thermoreactor (VELP Scientifica, Usmate, Italy) and a PF-3 photometer (VELP Scientifica) using kit NANOCOLOR®.

Microbiological counts were performed on serially diluted cheese whey, buttermilk and the mixture of them, which were spread on the plate surface containing different media. Heterotrophic aerobic and anaerobic bacteria were counted on Plate Count Agar (Oxoid, Milan, Italy) and incubated for 48 h at 30 °C under either aerobic or anaerobic conditions (Oxoid's Anaerogen™ System, Oxoid). Spore-forming bacteria were cultivated on Plate Count Agar (Oxoid) after a pretreatment at 80 °C for 10 min, and the plates were incubated at 30 °C for 48 h in aerobic or anaerobic conditions. Jactic acid bacteria (LAB) were counted on MRS agar (Oxoid), and the plates were incubated for 48 h at 30 °C. *Clostridia* were enumerated on Reinforced Clostridial Medium (Oxoid), and the plates were incubated for 48 h at 30 °C under anaerobic conditions. Enterococci were counted on the selective substrate, Slanetz-Bartley agar (Oxoid), after incubation at 37 °C for 48 h.

For the experimental plan, 6 L steel vessels were used as anaerobic biodigesters (working volume of 5 L). Each biodigester was equipped with a manometer and valves for biogas collection as well as effluent discharge.

Biodigesters were filled with a mixture of cheese whey and buttermilk (ratio 2:1 v/v, respectively, to simulate the characteristics of a real dairy waste stream produced from a mozzarella cheese factory)

and inoculated with 5% (w/v) of industrial animal manure pellets (Stalfert N2 - Organazoto Fertilizzanti s.p.a, Pistoia, Italy). Tests were performed in duplicate (B1 and B2) at 30 ± 1 °C and for 30 days.

5.2.2 Anaerobic biosystem monitoring

5.2.2.1 Biological gas production and intermediate products evaluation

Biological gas production was measured with a volumetric displacement method (Esposito et al., 2012) using teflon bags (maximum capacity of 10 L) to collect and storage biogas until analyses. The volume of biogas produced was measured by connecting each teflon bag with a capillary tube to an inverted 1000 mL glass bottle containing an acid solution (1.5% HCl) (Ghimire et al., 2015). Gas was detected using a Varian Star 3400 gas chromatograph (Agilent, Santa Clara California, USA) equipped with a Shin Carbon ST 80/100 column and a thermal conductivity detector. Argon was used as the carrier gas with an operating pressure of 20 psi. Gas measurements were performed daily during the first week and every three days during the following three weeks of incubation.
At 0, 7, 14, 21 and 30 days, liquid samples were collected from the biological reactors to analyze the concentration of lactose, galactose, lactic acid, acetic acid, propionic acid and ethanol by high-performance liquid chromatography (HPLC, refractive index detector 133; Gilson system; pump 307, column Metacarb 67 h from Varian with 0.4 mL min^{-1} flow of 0.01 N H_2SO). The pH and TTA were also constantly monitored.

5.2.2.2 Monitoring of microbial growth by cultural dependent analysis

Liquid samples were collected from the biological reactors every week during 30 days of incubation. Bacterial counts were performed using either generic or selective differential growth media and, in particular, representative samples were characterized for heterotrophic aerobic and anaerobic bacteria, aerobic and anaerobic spore-forming bacteria, LAB, *Clostridia* and enterococci, as above described. A one-way ANOVA followed by a Tukey test for pairwise comparison of means ($p \leq 0.05$) were used to assess the difference in microbial counts at different incubation times. Statistical analyses were performed using the SPSS 21.0 statistical software package (SPSS Inc., Cary, NC, USA) as reported by Ventorino et al. (2016).

5.2.3 Microbiota analysis by high-throughput sequencing of the 16S rRNA gene

Total genomic DNA was extracted from the liquid samples collected from the biodigesters using a FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch Cedex, France) according to the manufacturer's instructions.

The microbial diversity was evaluated by amplicon based metagenomics sequencing using the primers S-D-Bact-0341F50 (5'- CCTACGGGNGGCWGCAG -3') and S-D-Bact-0785R50 (5'- GACTACHVGGGTATCTAATCC -3') (Klindworth et al., 2013) for bacterial communities; while for archaea, two different PCR reactions were performed. For the first round, the universal synthetic oligonucleotide primers, Arch 46f (5'- YTA AGC CAT GCR AGT -3') (Øvreas et al., 1997) and Arch 1017r (5'- GGC CAT GCA CCW CCT CTC -3') (Barns et al., 1994), were used as previously reported (Ventorino et al., 2018). For the second round, a nested PCR was performed using the primers Arch516F (5'-TGYCAGCCGCCGCGGTAAHACCVGC -3') and Arch915R (5'- GTGCTCCCCCGCCAATTCCT -3') (Raymann et al., 2017). Amplicon purification, multiplexing and sequencing were carried out by Genomix4Life s.r.l. (Salerno, Italy) as reported in the Illumina 16S Metagenomic Sequencing Library Preparation manual. Sequencing was carried out on a MiSeq platform (Illumina Italy s.r.l., Milan, Italy), leading to 250bp, 2 paired-end reads.

5.2.4 Bioinformatics and data analysis

Row reads were qualitatively analyzed and filtered using PRINSEQ (Schmieder and Edwards, 2011). Low quality reads (Phred score <20) were trimmed and reads shorter than 60 bp were discarded in end-to-end, sensitive mode. Paired-ends reads were merged using FLASH (Magoč and Salzberg, 2011) and sequences were then analyzed using QIIME 1.9.1 software (Caporaso et al., 2010). Operational taxonomy units (OTUs) at 97% sequence identity were picked through a *de novo* approach and the UCLUST method and taxonomic assignment was obtained using the RDP classifier and the Greengenes (McDonald et al., 2012). To avoid biases due to different sequencing depths, OTU tables were rarefied at the lowest number of sequences per sample.

Alpha diversity was evaluated by rarefaction curves, Good's coverage, and Shannon diversity index (Shannon and Weaver, 1949). Beta diversity was also evaluated by UniFrac (Lozupone et al., 2006), and PCoA was generated by QIIME. To test the significant differences, statistical analyses were performed as previously described (Ventorino et al., 2015).

Phylogenetic trees for the representative bacterial and archaeal OTUs detected in this study and sequences downloaded from NCBI were constructed in Mega 4 by the Neighbor-Joining method using a maximum composite likelihood model with 1,000 bootstrap replicates.

5.2.4.1 Data Accessibility

The raw Illumina sequencing data are available in the Sequence Read Archive database of the National Center of Biotechnology Information (SRP155825).

5.3 Results

5.3.1 Characterization of dairy wastes

Viable counts of main bacterial groups were evaluated in samples of cheese whey, buttermilk and their mixture (Figure 5.1). The results showed a microbial load higher in cheese whey than in buttermilk, detected almost for each principal microbial group investigated. Actually, heterotrophic aerobic and anaerobic bacteria as well as LAB showed a higher concentration in the cheese whey (7.28 ± 0.02, 7.34 ± 0.02 and 6.11 ± 0.01 log CFU mL^{-1}, respectively) than in the buttermilk (5.20 ± 0.05, 4.95 ± 0.04 and 4.42 ± 0.06 log CFU mL^{-1}, respectively). Aerobic and anaerobic spore-forming bacteria were 4.08 ± 0.12 and 3.69 ± 0.12 log CFU mL^{-1} as well as 4.11 ± 0.05 and 3.15 ± 0.21 log CFU mL^{-1}, respectively, for cheese whey and buttermilk. Moreover, the *Clostridia* load in cheese whey (3.84 ± 0.33 log CFU mL^{-1}) was approximately two orders of magnitude greater than that recovered in buttermilk (2.02 ± 0.10 log CFU mL^{-1}).

The inoculum showed a heterotrophic aerobic and anaerobic bacteria load of 5.41 ± 0.12 and 5.20 ± 0.90 log CFU mL^{-1}, respectively, whereas, loads of aerobic and anaerobic spore-forming bacteria and LAB were around 4.5 log CFU mL^{-1}.

Enterococci were detected in higher amount in inoculum (4.56 log CFU mL^{-1}) and cheese whey (3.17 log CFU $mL^{-}1$) samples.

Chemical characteristics of substrates and inoculum are listed in Table 5.1. The COD value was higher in cheese whey (74.10 ± 0.40 g L^{-1}) than buttermilk (14.57 ± 0.20 g L^{-1}) and the resulting mixture had a COD value closer to cheese whey than buttermilk (55.45 ± 0.22 g L^{-1}). Obviously, TS and VS concentrations of cheese whey influenced the mixture concentrations resulting in 37.60 ± 0.01 g L^{-1} and 34.53 ± 0.01 g L^{-1}, respectively. The pH measurement did not drop below 5.0 for

cheese whey, buttermilk and their mixture, while the inoculum had a value of 7.8 (Table 5.1); the TTA was 0.80 ± 0.10 °SH in the cheese whey as well as in the mixture but was lower in the buttermilk (0.40 ± 0.10 °SH).

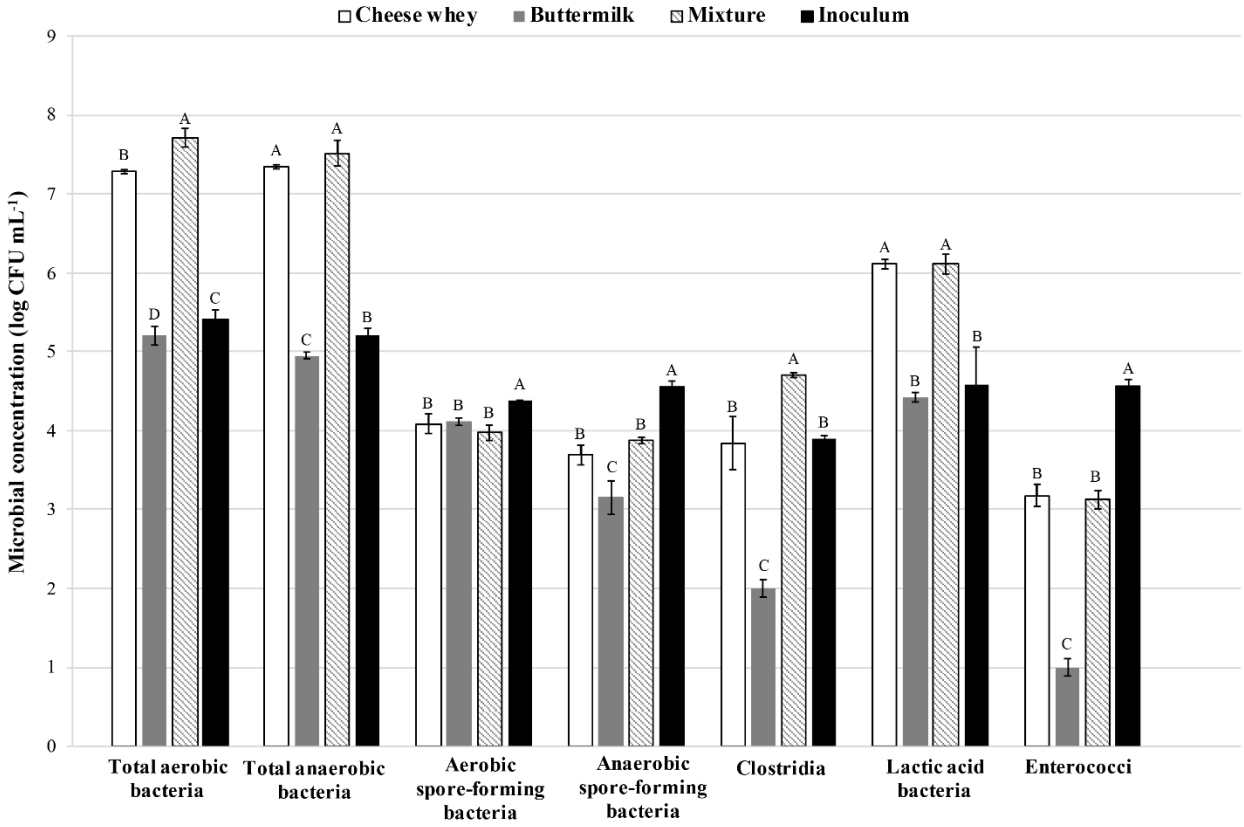

Figure 5.1. Viable counts of main bacterial groups evaluated in samples of cheese whey, buttermilk and their mixture. The error bars represent the means ± SD of two replicates.

Table 5.1. Physico-chemical characterization of cheese whey, buttermilk, their mixture and the animal manure inoculum.

Samples	COD (g L^{-1})	TS (g L^{-1})	VS (g L^{-1})	pH	TTA (°SH)
Cheese whey (W)	74.10 ± 0.40	54.34 ± 0.02	49.37 ± 0.01	5.00 ± 0.10	0.80 ± 0.10
Buttermilk (B)	14.57 ± 0.20	12.07 ± 0.01	9.91 ± 0.01	5.00 ± 0.10	0.40 ± 0.10
Mix of W and B**	55.45 ± 0.22	37.60 ± 0.01	34.53 ± 0.01	5.00 ± 0.10	0.80 ± 0.10
Inoculum***	n.d.*	0.95 ± 0.0	0.43 ± 0.0	7.80 ± 0.10	n.d.*

*not determined; **(ratio 2:1 v/v); ***animal manure.

5.3.2 Biological gas production and biosystem monitoring

Figure 5.2 shows the cumulative volumes of the specific production of H_2 and CH_4 during incubation.

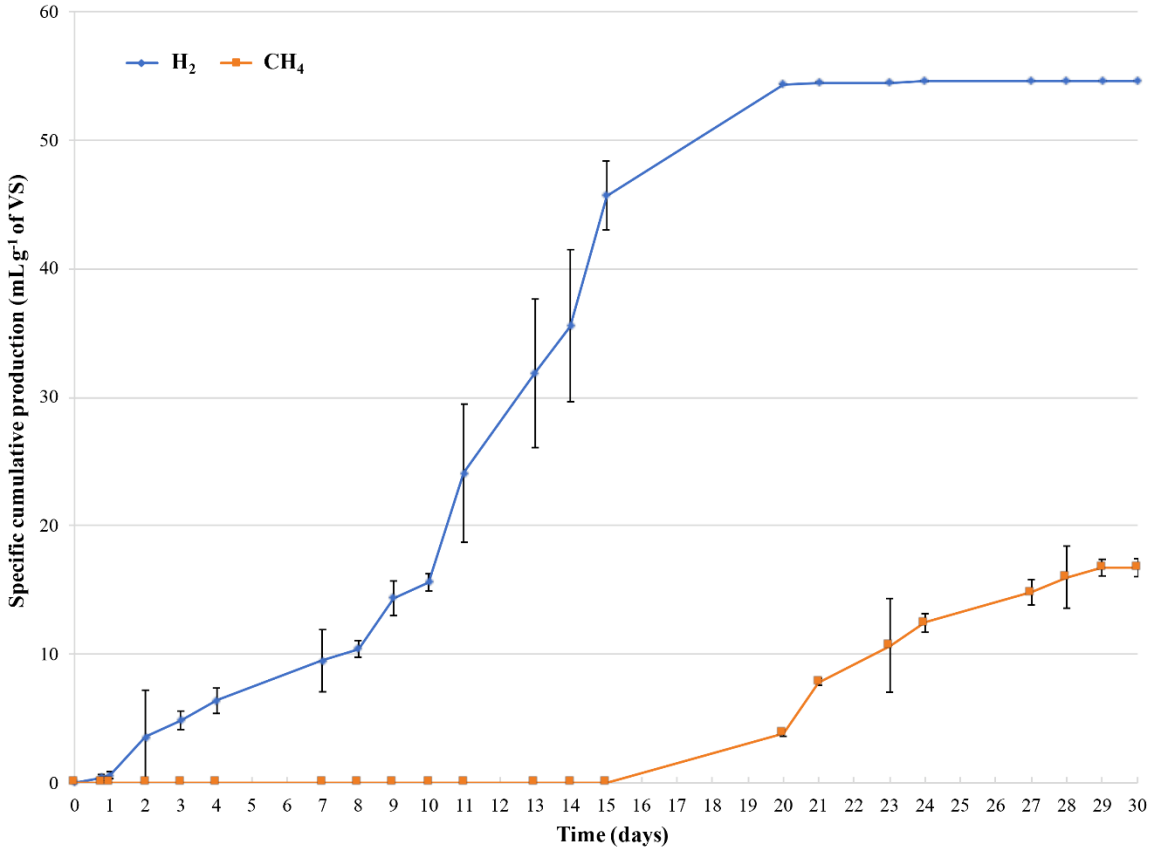

Figure 5.2. Cumulative specific production of H_2 and CH_4 monitored during 30 days of incubation. X-axis displays the day, and the Y-axis is the corresponding cumulative H_2 or CH_4 specific production per gram of the initial concentration of VS of the dairy waste mixture. The error bars represent the means ± SD of two replicates.

After 7 days of incubation, a H_2 production of 9.49 ± 2.41 ml H_2 g^{-1} of VS was observed, increasing up to 54.35±0.1 ml H_2 g^{-1} of VS after 20 days. Regarding to biogas production, CH_4 production was observed from days 15 to 30 of incubation, reaching 16.74 ± 0.71 ml CH_4 g^{-1} of VS (Figure 5.2).The amount of CO_2 (% v/v) detected in the gas mixture ranged from 99% to 85% during the first week of incubation (at the beginning of the process) and from 9% to 12% when CH_4 and H_2 production occurred (data not shown). These results were in good agreement with trends of lactose and lactic acid concentration during incubation (Figure 5.3a). Actually, lactose was consumed and lactic acid concentration increased from 4.66 ± 0.11 g L^{-1} (T0) to 21.03 ± 2.11 g L^{-1} (T7) after 7 days of incubation. At day 14, lactic acid decreased until 0 g L^{-1} and an increase in the pH value up to 6.3 was observed decreasing to 5.1 at the end of the experiment. Whereas, ethanol concentration increased up to 19.69 ± 2.12 g L^{-1} after 14 days, remaining quite constant until day 30 (Figure 5.3a). At day 14, acetic and propionic acids concentrations were 1.92 ± 0.12 and 2.25 ± 0.07 g L^{-1}, respectively, increasing up to 4.11 ± 0.5 g L^{-1} at the end of the process (Figure 5.3b).

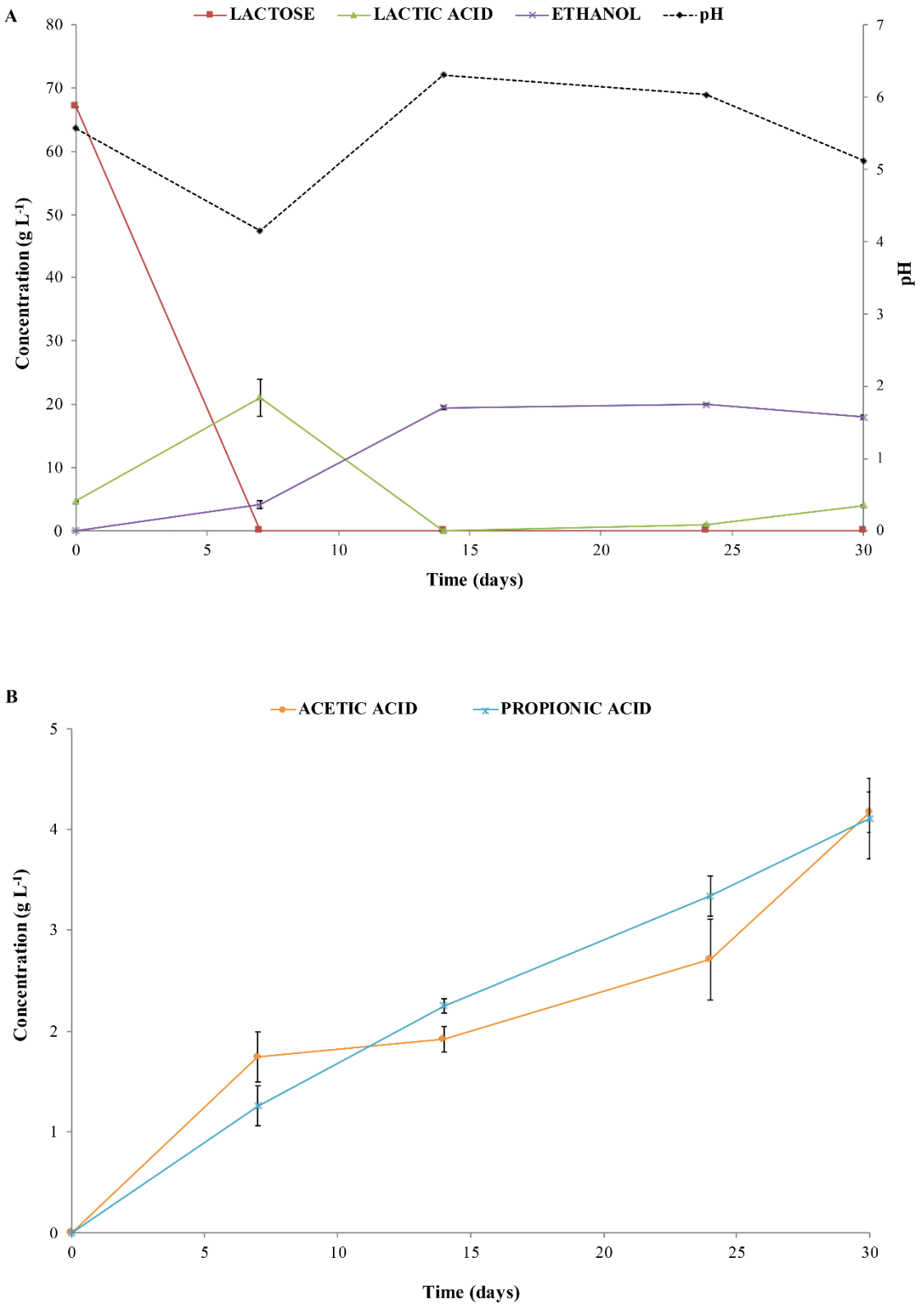

Figure 5.3. pH and concentration of lactose, lactic acid and ethanol (a), and concentration of acetic acid and propionic acid (b) in liquid samples collected from biodigesters at different times of incubation.

5.3.3 Microbial analysis by culture-dependent method

The monitoring of microbial viable counts during incubation time is listed in Table 5.2. The results showed that heterotrophic aerobic and anaerobic bacteria remained constant (approximately 7.2 and 7.4 log CFU mL^{-1}, respectively) during the first week of incubation decreasing until 2.8-2.9 log CFU mL^{-1}, at the end of the biodigestion process ($p < 0.05$). Similarly, aerobic spore-forming bacteria significantly decreased from 4.07 ± 0.09 log CFU mL^{-1} (T0) to 3.17 ± 0.07 log CFU mL^{-1} (T30). Whereas, an increase of 2 log was observed in anaerobic spore-forming bacteria and *Clostridia* loads from the initial time (3.91 ± 0.11 and 4.03 ± 0.36 log CFU mL^{-1}, respectively) to day 14 of incubation (5.17 ± 0.36 and 5.95 ± 0.04 log CFU mL^{-1}, respectively) decreasing again at the end of the incubation time (2.92 ± 0.05 and 3.77 ± 0.05 log CFU mL^{-1}, respectively, $p < 0.05$). Finally, a high load of LAB was detected in the biodigesters until day 7 (6.6-7.6 log CFU mL^{-1}), whereas at day 30 the load dropped showing a concentration of 1.00 ± 0.11 log CFU mL^{-1}.

Table 5.2. Cultural monitoring of bacterial populations during incubation for 30 days at 30°C. The values represent the means ± SD of three replicates. Different letters after the values indicate significant differences ($p < 0.05$).

Microbial groups (log CFU mL^{-1})	**Time**			
	T0*	**T7***	**T14***	**T30***
Heterotrophic aerobic bacteria	7.25 ± 0.14^{A}	7.22 ± 0.18^{A}	4.72 ± 0.67^{B}	2.81 ± 0.07^{C}
Heterotrophic anaerobic bacteria	7.30 ± 0.14^{A}	7.36 ± 0.08^{A}	4.51 ± 0.43^{B}	2.88 ± 0.15^{C}
Aerobic spore-forming bacteria	4.07 ± 0.09^{A}	3.39 ± 0.08^{B}	3.40 ± 0.30^{B}	3.17 ± 0.07^{B}
Anaerobic spore-forming bacteria	3.91 ± 0.11^{B}	3.21 ± 0.09^{C}	5.17 ± 0.36^{A}	2.92 ± 0.05^{C}
Clostridia	4.03 ± 0.36^{C}	5.22 ± 0.36^{B}	5.95 ± 0.04^{A}	3.77 ± 0.05^{C}
Lactic acid bacteria	6.60 ± 0.11^{B}	7.58 ± 0.23^{A}	4.47 ± 0.00^{C}	1.00 ± 0.10^{D}
Enterococci	3.26 ± 0.21^{A}	3.30 ± 0.13^{A}	1.00 ± 0.10^{B}	1.00 ± 0.10^{B}

* T0, mixture of cheese whey and buttermilk inoculated at day 0 of incubation; T7, samples collected after 7 days of incubation; T14, samples collected after 14 days of incubation; T30, samples collected 1 after 30 days of incubation.

5.3.4 High throughput sequencing of PCR-amplified 16S rRNA gene sequences

5.3.4.1 Bacteria

A total of 677,030 high quality reads were analyzed for bacteria. The alpha-diversity was determined by calculating the Shannon diversity index based on OTUs of 97% identity (Table 5.3). The results revealed that the bacterial diversity significantly increased over time showing the highest Shannon diversity index in the biodigesters after 14 days (B1_T14 and B2_T14; $p < 0.05$) and 30 days of incubation (B1_T30 and B2_T30; $P < 0.05$). Good's coverage indicated that 75-78% of the bacterial diversity was described in the samples.

The dynamics of bacterial populations were studied during the anaerobic process through their taxonomic composition detected at family level (Figure 5.4). Analysis of amplicon sequences showed *Streptococcaceae* (59% in Mix_T0 and 47% in MixI_T0) and *Lactobacillaceae* (40% in Mix_T0 and 36.5% in MixI_T0) families as dominant in the initial non-inoculated and inoculated mixture of cheese whey and buttermilk accounting for 99% and 83.5% of the total bacterial biodiversity in Mix_T0 and MixI_T0, respectively (Figure 5.4).

After 7 days of incubation, an increase of the abundance of *Lactobacillaceae* in the biodigesters B1 (67.8%) and B2 (64.9%) coupled with a *Streptococcaceae* decrease (1.9% and 4.1% in B1 and B2, respectively) was observed. Moreover, an increase of the *Clostridiaceae* family by 20% approximately, in both B1 and B2 was observed remaining quite stable until the end of the biodigestion process (around 32% in both B1_T30 and B2_T30, Figure 5.4). In addition to *Clostridiaceae*, *Tissierellaceae* was also a dominant family after 14 days (37.1% and 41.4% in B1 and B2, respectively) and 30 days of incubation (38.3% and 35.4% in B1 and B2, respectively). These two taxa accounted together for around the 70% of the bacterial biodiversity at the end of the anaerobic process (Figure 5.4). To investigate these two dominant bacterial families to a deeper level, a phylogenetic tree was constructed based on the values of OTUs with relative abundances higher than 0.1%. Phylogenetic analysis showed that the main *Clostridiaceae* OTUs, accounted for 2.15% of the total reads, were very similar to each other and were affiliated with *Clostridium thermopalmarium* species (Figure 5.5); while, other two OTUs were affiliated with *C. tyrobutyricum* and *C. clariflavum*. Regarding *Tissierellaceae* family, phylogenetic tree revealed that the main OTUs were affiliated with *Sporanaerobacter acetigenes* species, which accounted for 15.27% of the total reads (Figure 5.5). Among these, OTU denovo78367 and OTU denovo11901 were the most abundant which accounted for 7.35% and 3.63% of total reads, respectively.

Other bacterial taxa belonging to Clostridiales order, such as *Ruminococcaceae*, *Lachnospiraceae*, *Caldicoprobacteraceae*, *Eubacteriaceae* and *Peptococcaceae*, as well as several OTU identified as

Thermoanaerobacteraceae, *Bacillaceae*, *Enterobacteriaceae*, *Pseudonocardiaceae*, *Actinomycetaceae* and *Coriobacteriaceae* were also found to be lesser extent (Figure 5.4). For these taxa, the fluctuating relative abundance among samples does not allow to define a trend during incubation time.

Besides, the principal coordinate analysis (PCoA) of the weighted UniFrac community distances revealed a marked difference between the microbiota in the early and final stages of the anaerobic process identifying three principal groups on the basis of the sampling time (Figure 5.6, panel a). The four samples from biodigesters at days 14 and 30 of incubation (B1_T14, B2_T14, B1_T30 and B2_T30) clustered separately (Figure 5.6, panel a). Moreover, the statistical ANOSIM test showed that the composition of bacterial community in the different samples during anaerobic process was significantly influenced by the sampling time ($p < 0.01$; $R = 0.938$).

Table 5.3. Observed diversity and estimated sample coverage for bacterial and archaeal 16S rRNA amplification from DNA extracted from biodigesters during the anaerobic process. The entire data set was rarefied to 77670 reads or 47510 reads per sample, for bacteria and archaea respectively, before alpha-diversity was calculated.

Sample*	Bacteria			Archaea		
	No. OTUs	Shannon index[a]	Good's coverage (%)	No. OTUs	Shannon index[a]	Good's coverage (%)
Mix_T0	18211.40	6.39^{D}	78.1	--	--	--
MixI_T0	20739.20	7.70^{C}	75.5	6522.00	5.27^{A}	87.7
B1_T7	20132.20	8.51^{B}	77.1	6661.00	5.03^{A}	87.4
B2_T7	20287.00	8.76^{B}	76.8	6264.00	5.51^{A}	88.4
B1_T14	21732.70	9.66^{A}	76.3	4931.00	5.54^{A}	90.9
B2_T14	22777.20	9.80^{A}	74.9	6658.00	5.69^{A}	87.5
B1_T30	22352.40	9.66^{A}	75.2	6215.00	2.82^{B}	88.0
B2_T30	21285.30	9.65^{A}	76.8	6422.00	2.79^{B}	87.6

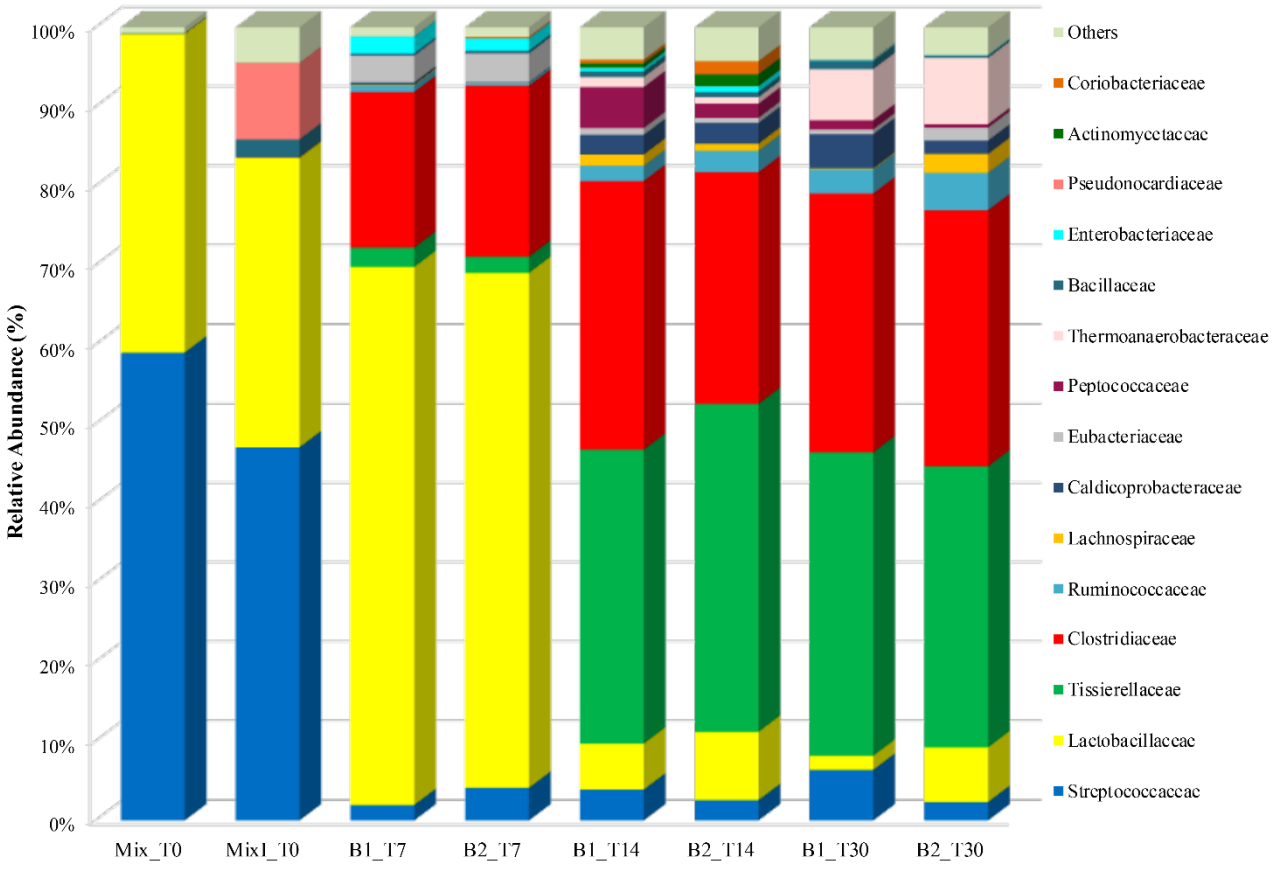

Figure 5.4. Abundance of bacterial families during the anaerobic process. Only OTUs with an incidence > 1% in at least one sample are shown. Abbreviations: Mix_T0, mixture of cheese whey and buttermilk at day 0 of incubation; MixI_T0, mixture of cheese whey and buttermilk inoculated at day 0 of incubation; B1_T7, sample collected from B1 after 7 days of incubation; B2_T7, sample collected from B2 after 7 days of incubation; B1_T14, sample collected from B1 after 14 days of incubation; B2_T14, sample collected from B2 after 14 days of incubation; B1_T30, sample collected from B1 after 30 days of incubation; B2_T30, sample collected from B2 after 30 days of incubation.

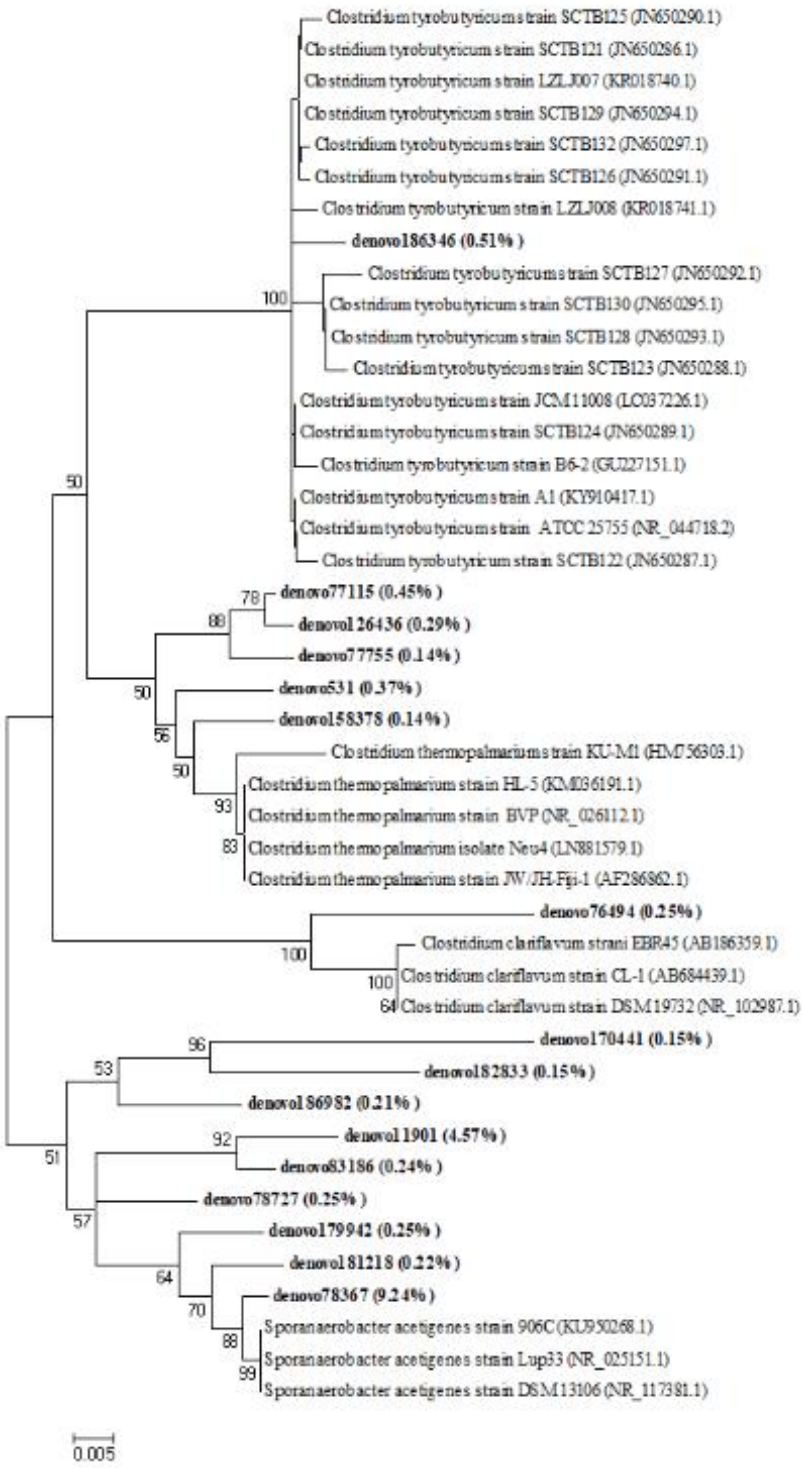

Figure 5.5 Neighbor-joining tree for the representative *Clostridiaceae* and *Tissierellaceae* OTUs (representatives with relative abundance > 0.1%). OTUs from this study were shown in bold reporting in brackets the total relative abundance. The sequence accession numbers of reference sequences from NCBI used for the phylogenetic analysis are shown in *parentheses* following the species name. Bootstrap values (> 50%, expressed as percentages of 1,000 replications) are given at the nodes. The scale bar estimates the number of substitutions per site.

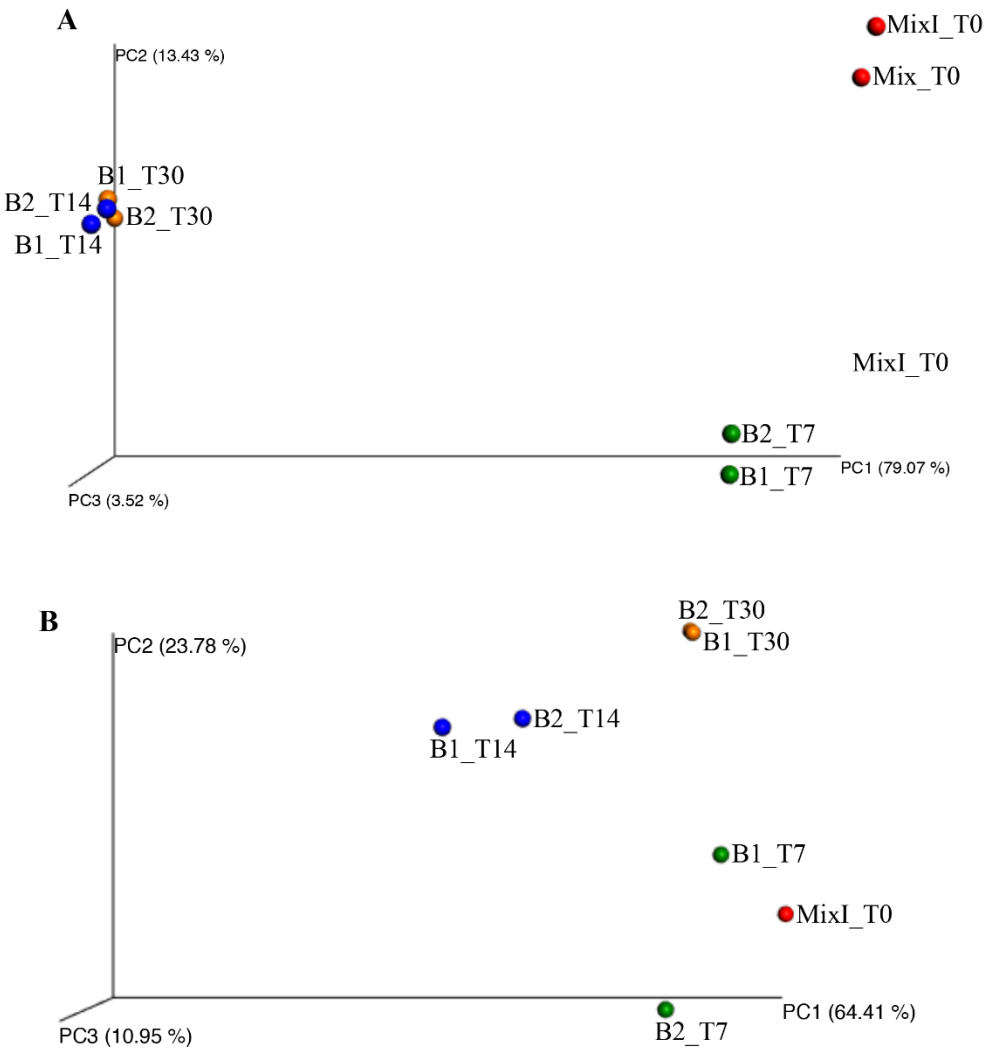

Figure 5.6. Principal Coordinates Analysis of weighted UniFrac distances for bacterial (panel a) and archaeal (panel b) 16S rRNA gene sequence data of samples during the incubation process. Colour label. red: mixture of cheese whey and buttermilk at day 0 of incubation; green: samples collected after 7 days of incubation; blue: samples collected after 14 days of incubation; orange: samples collected after 30 days of incubation.

5.3.4.2 Archaea

A total of 517,142 high quality reads were analyzed for archaea. The alpha-diversity was determined by calculating the Shannon diversity index based on OTUs of 97% identity (Table 5.3). Non-inoculated samples of cheese whey and buttermilk mixture (Mix_T0) was excluded from the analysis since very few archaeal reads were recovered (absolute abundance equal to 97 OTUs). The results

showed that the archaeal diversity significantly increased after 5% of the inoculum was added and then strongly decreased at the end of the anaerobic process (Table 5.3; $p < 0.05$). Whereas, good's coverage indicated that approximately 90% of the archaeal diversity was described in most of the samples.

The archaeal taxa were examined at the genera level to determine the eventual occurrence of any significant shifts in their composition during the incubation time. Relevant results were shown from the samples collected at the initial time of the anaerobic process. Actually, marked differences were observed among inoculated and non-inoculated samples of cheese whey and buttermilk mixture where the relative abundance of archaeal genera was less than 0.1% (Mix_T0, Figure 5.7). Whereas, in the inoculated sample of the same mixture (MixI_T0, Figure 5.7), several archaeal genera were identified, such as *Methanoculleus* (24.7%), *Methanobrevibacte*r (3.4%), *Methanosarcina* (4.8%), *Methanocorpusculum* (2.2%), *Methanobacterium* (1.7%) and *Nitrososphaera* (60.2%). During incubation, non-methanogenic archaea belonging to the *Nitrososphaera* genus decreased from 5.1-2.9% (T14) to < 1% (T30). A similar trend was observed for the methanogenic archaea genera *Methanobrevibacte*r, *Methanosarcina*, *Methanocorpusculum* and *Methanobacterium*, which exhibited an abundance < 1% at the end of the incubation time. Otherwise, *Methanoculleus* was the dominant genus during all the process and its relative abundance increased up to 99.1-99.9% after 30 days of incubation (Figure 5.7). A phylogenetic tree based on the OTUs belonging to this genus showing the relative abundances higher than 0.1% was constructed, in order to evaluate which OTUs became dominant and who were their closest phylogenetic relatives. Phylogenetic analysis showed that the main *Methanoculleus* OTUs were affiliated with *M. thermophilus* (OTU denovo22548 and OTU denovo56442), *M. taiwanensis* (OTU denovo19589), *M. sediminis* (OTU denovo27381), *M. horonobensis* (OTU denovo362) and *Methanoculleus* spp. (OTU denovo26184), accounted for 33.83% of total reads (Figure 5.8). However, the dominant OTU was OTU denovo56442, strongly related to different strains of *M. thermophilus*, which explained most of the methanogenic biodiversity accounting for 32.32% of archaeal total reads (Figure 5.8).

The PCoA of the weighted UniFrac community distances showed a marked difference among the microbiota in the different samples over time. In fact, the sample of the initial inoculated mixture of cheese whey and buttermilk (MixI_T0) and those of the biodigesters at day 7 of incubation cluster together; while the two samples of the biodigesters at day 30 of incubation (B1_T30 and B2_T30) clustered separately (Figure 5.6, panel b). Moreover, the statistical ANOSIM test showed that the composition of archaeal community in the different samples during anaerobic process was significantly influenced by the sampling time ($p < 0.05$; $R = 0.729$).

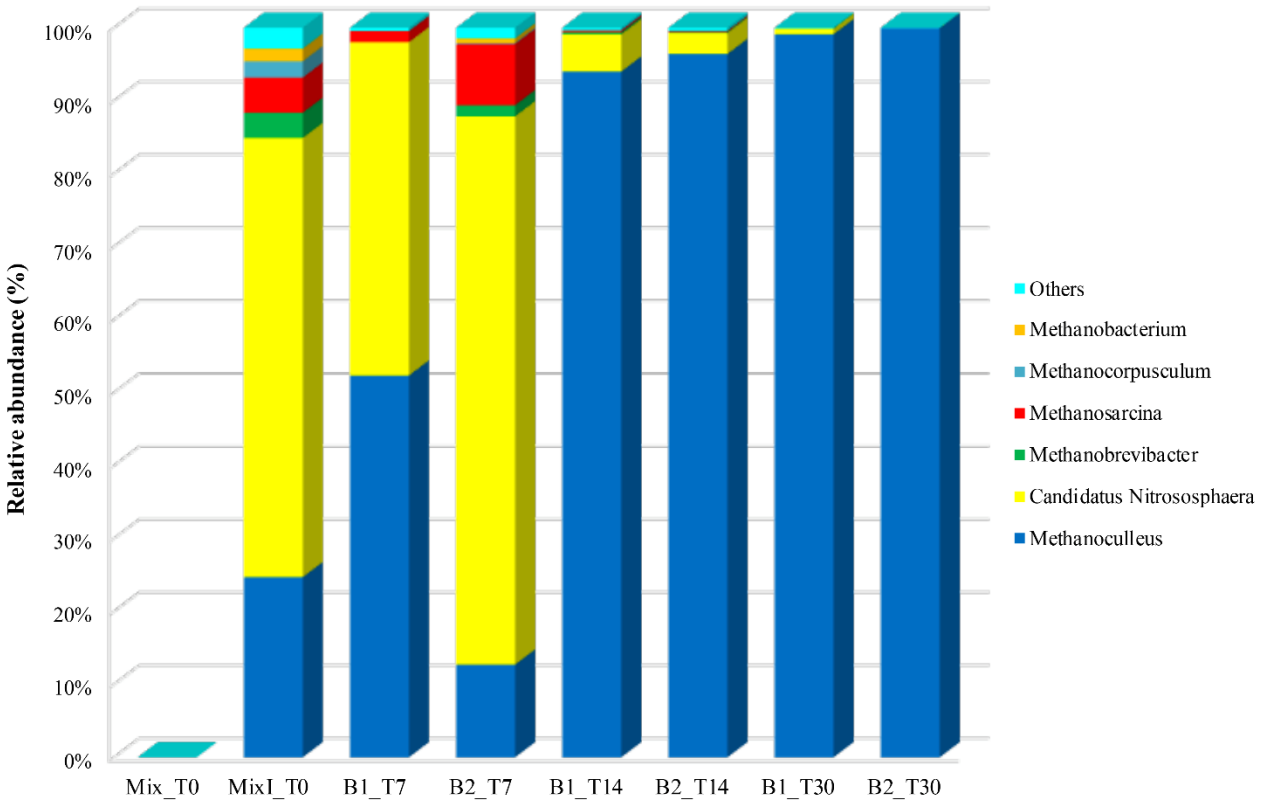

Figure 5.7. Abundance of archaeal genera during the anaerobic process. Only OTUs with an incidence > 1% in at least one sample are shown. Abbreviations: Mix_T0, mixture of cheese whey and buttermilk at day 0 of incubation; MixI_T0, mixture of cheese whey and buttermilk inoculated at day 0 of incubation; B1_T7, sample collected from B1 after 7 days of incubation; B2_T7, sample collected from B2 after 7 days of incubation; B1_T14, sample collected from B1 after 14 days of incubation; B2_T14, sample collected from B2 after 14 days of incubation; B1_T30, sample collected from B1 after 30 days of incubation; B2_T30, sample collected from B2 after 30 days of incubation.

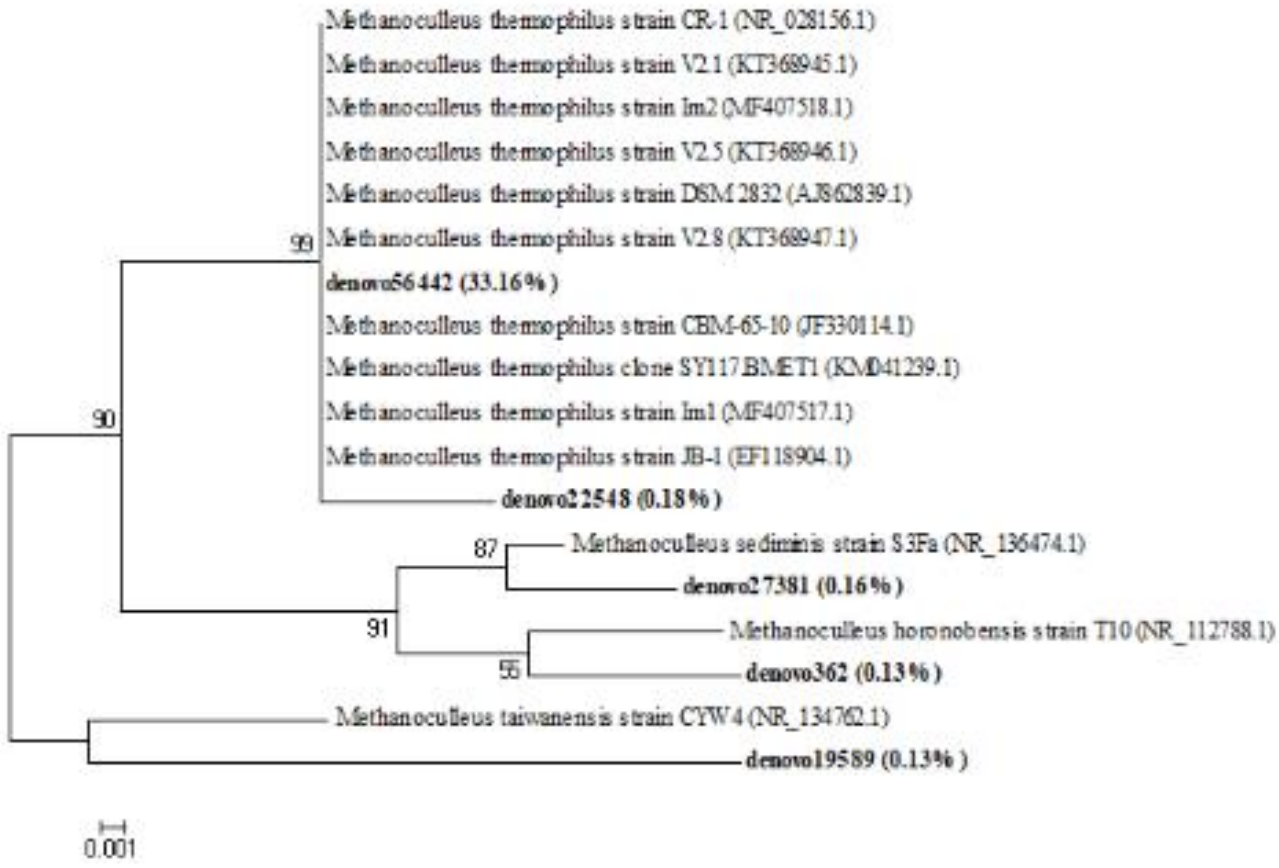

Figure 5.8 Neighbor-joining tree for the representative *Methanoculleus* OTUs (representatives with relative abundance > 0.1%). OTUs from this study were shown in bold reporting in brackets the total relative abundance. The sequence accession numbers of reference sequences from NCBI used for the phylogenetic analysis are shown in *parentheses* following the species name. Bootstrap values (> 50%, expressed as percentages of 1,000 replications) are given at the nodes. The scale bar estimates the number of substitutions per site.

5.4 Discussion

The microbial composition of the initial dairy waste mixture showed a high concentration of viable aerobic and anaerobic bacteria mainly belonging to LAB that commonly thrive in dairy waste (Kasmi et al., 2017). Since LAB produce lactic acid by homolactic and heterolactic fermentation processes (Palomba et al., 2012; Pradhan et al., 2017) they are well adapted to the acidic environment (De Candia et al., 2007), typical of the cheese whey used in this study collected after fermentation and addition of organic acids during the production chain (Carvalho et al., 2013). Physical and chemical characteristics of the cheese whey are, actually, strictly related to the production chain, thus showing a wide range of organic matter concentration as reported in the literature (Ghaly and Singh, 1989;

Ghaly and Kamal, 2004; Farizoglu et al., 2007; Saddoud et al., 2007; Azbar et al., 2009). In this study, cheese whey characteristics were in accordance with Ghaly and Singh (1989) that reported the concentrations of COD and VS equal to 75.8 g L^{-1} and 47.9 g L^{-1}, respectively. The high COD in cheese whey influenced the resulting COD in the mixture, indicating the potential of this substrate for feeding anaerobic process (Carvalho et al., 2013) and producing H_2 and CH_4. During incubation a relevant increase in hydrogen production was observed, simultaneously a decrease of the LAB concentration occurred until to reach a concentration less than 1 log $CFUmL^{-1}$ at the end of the incubation time. The decrease in the LAB concentration was related to the increase in H_2 production proving that lactic fermenters were the main competitors with the H_2 producing microorganisms (Perna et al., 2013). This result was also confirmed by HTS for the bacterial 16S rRNA gene that showed a noticeable decrease (from 67% at day 7 to 6% at day 14) in the relative abundance of the *Lactobacillaceae* family when relevant H_2 production occurred. Accordingly, it has been reported that *Lactobacillus* could not be related to high H_2 production rate (Davila-Vazquez et al., 2009), although Yang et al. (2007) found higher abundance of *Lactobacillus* than *Clostridium* in anaerobic fermentation of cheese processing wastewater, thus reporting *Lactobacillus* related to H_2 production. On the other hand, in this work the increase in H_2 production occurred simultaneously with an increase in *Clostridia* load. Actually, *Clostridia* have been reported to convert lactate into butyrate, CO_2 and H_2 in the presence of acetate (Barlow et al., 1991). Thus, the presence of these microorganisms could favor the production of hydrogen by fermentation of lactic acid followed by H_2 production (Perna et al., 2013). For this reason, lactic acid was not detected at day 14 and an increase of acetic and propionic acids concentration was observed. This cultural approach allowed to acquire information about dynamics of viable bacterial populations which were able to live, grow and die during the biodigestion process of this specific waste. Moreover, in order to obtain more information, a cultural-dependent approach was combined with a cultural-independent molecular method. According to Pandya et al. (2017) these techniques seem to be roughly equivalent and, when used in parallel, it is possible to obtain best results leading to major advances in the reliable knowledge of microbial populations living in an environment. The cultural results were confirmed by the HTS analysis that showed an increase in bacterial families belonging to Clostridiales order, such as *Tissierellaceae* and *Clostridiaceae*, which represented the most abundant bacterial taxa until the end of the incubation time. The selective pressure occurred in the ecosystem due to the presence of inoculum and the chemical-physical conditions of the anaerobic process selected these bacterial taxa, which are well known to be involved in the H_2 production (Navarro-Díaz et al., 2016; Alexandropoulou et al., 2018). In particular, in this study, the most dominant OTUs of *Clostridiaceae* and *Tissierellaceae* were affiliated with *Clostridium* spp. (*C. thermopalmarium*, *C. clariflavum* and

C. tyrobutyricum) and *Sporanaerobacter acetigenes*, respectively, which are commonly detected and isolated in many reactors for CH_4 or H_2 production (Hernandez-Eugenio et al., 2002; Shiratori et al., 2006; Jo et al., 2008; Weiss et al., 2008; Kim et al., 2010; Sasaki 2012; Xia et al., 2014; Kumar et al., 2015; Cibis et al., 2016). The genus *Clostridium* comprised a large number of species that were often used to produce H_2 (Jiang et al., 2013). Among them, *Clostridium tyrobutyricum* has been widely reported to be able to produce significant quantities of H_2 from different sugars (Jiang et al., 2018) as well as it is also capable to utilize lactate as the main substrate for producing H_2 (Wu et al., 2012; Noblecourt et al., 2018). Furthermore, fermentation products of *C. clariflavum* are H_2, CO_2, acetate, lactate, ethanol and a small amount of formate (Shiratori et al., 2009). Whereas *C. thermopalmarium* species are able to ferment sugars into butyric acid producing simultaneously H_2, CO_2, small amounts of acetate, lactate and ethanol (Soh et al., 1991). Geng et al. (2010) demonstrated that the inoculation of *C. thermopalmarium* strain BVP (DSM 5974) increased biohydrogen production rather than the monoculture of *C. thermocellum* from cellulose.

In addition, Navarro-Dìaz et al. (2016), reported the positive relationship between the increased H_2 production with the presence of specific microbial families and genera, such as *Tissierellaceae* that may contribute also to complex substrate degradation because of its putative xylanolytic activity (Niu et al., 2009). *Sporanaerobacter acetigenes* strain Lup 33^T, closely related to the OTU denovo78367, detected at the highest relative abundance, as well as related to the others representative OTUs affiliated to *Tissierellaceae*, was recognized as an acetogenic bacterium able to synthesize a mixture of volatile fatty acids, including acetate, isovalerate and isobutyrate, together with H_2 and CO_2 (Hernandez-Eugenio et al., 2002). Han et al. (2016) reported that *Sporanaerobacter acetigenes* was one of the main contributors for the hydrolysis and acidogenesis stages during anaerobic digestion of food waste-recycling wastewater (Han et al., 2016) as well as it was one of the primary species along with *Clostridium* during semi-continuous fermentation of *C. pyrenoidosa* biomass for H_2 production (Xia et al., 2014). Moreover, the presence of *Ruminococcaceae* members in the samples taken at days 14 and 30 may be also correlated to H_2 production since they are recognized as hydrogen producers and important substrate hydrolyzers (Niu et al., 2009).

In addition, ethanol was also produced in high amount starting from day 14 likely causing an inhibiting effect on hydrogen production (Hung et al., 2011) that therefore could have been higher than observed. This side effect can be related to the presence of *Streptococcaceae*, detected during incubation by using HTS for the bacterial 16S rRNA gene, since genera belonging to this family could produce ethanol thus inhibiting hydrogen production (Ren et al., 2007).

Furthermore, the environmental and physical conditions established in the system were also effective for the selection of the CH_4 producing archaea. First, the archaeal populations' presence in the

biosystem was related to inoculum since the initial samples of the mixture of cheese whey and buttermilk had a relative abundance of archaea less than 0.1%. Secondly, the anaerobic environment in the biodigesters selected archaeal genera causing the decrease of the aerobic *Nitrososphaera* genus also detected in other studies of anaerobic digestion process (Li et al., 2014; Ventorino et al., 2018) even if it could be no able to produce methane (Chen et al., 2012).

During incubation, *Methanoculleus* genus percentage increased achieving very high relative abundance (99% at the end of the experiment) compared with that reported in other studies, such as Di Maria et al. (2017) (85%) and Leven et al. (2007) (18%), related to anaerobic digestion of organic waste. As reported by Poirier et al. (2016), *Methanoculleus* cooperate with acetate oxidizing bacteria belonging to the *Clostridiaceae* family detected in this study by both cultural and molecular approaches.

In agreement with Di Maria et al. (2017), *Methanoculleus* abundance increased during the incubation time and became dominant, whereas *Methanosarcina* decreased, as they are usually dominant in process fed with organic fraction of municipal solid waste (OFMSW) and activated sludge as inoculum (Lin et al., 2012). In this biosystem fed with a mixture of cheese whey and buttermilk, *Methanoculleus* was dominant using a hydrogenotrophic pathway to produce CH_4 causing no organic acids consumption when methane production occurred. This result was in agreement with a previous study in which hydrogenotrophic pathway was identified as the main driver for CH_4 production in batch reactors fed with dairy wastes, although, using different process conditions and a qualitative culture-independent method (DGGE) to microbial identification, *Methanobrevibacter* was found as the genus mostly related to the CH_4 production (Pagliano et al., 2018). Interestingly, in this study, the dominant methanogenic archaeal OTU was affiliated with *M. thermophilus* which is able to produce CH_4 from H_2 or formate (Rivard and Smith, 1982). Recently, this species, with *M. beijingense*, was found to be dominant in a full-scale thermophilic anaerobic digester treating food wastewater (Lee et al., 2017). The other representative OTUs affiliated with *Methanoculleus* genus were closely related to *M. sediminis*, *M. taiwanensis* and *M. horonobensis* previously isolated from sediments near a submarine mud volcano (Chen et al., 2015), deep-sea sediment (Shimizu et al., 2013) and deep subsurface groundwater from a diatomaceous shale formation (Weng et al., 2015), respectively.

Comparing the H_2 and CH_4 production with the literature, the production in this study (54.34 ±0.15 L H_2 Kg $VS^{-1}$and 16.74 ±0.71 L CH_4 Kg VS^{-1}) were higher than that reported by Pagliano et al. (2018) (8.9 L H_2 Kg VS^{-1} and 2.2 L CH_4 Kg VS^{-1}) using dairy waste as substrate and operating in batch mode. Different operating condition can promote the CH_4 production, as reported by Lateef et al. (2014) that studied an anaerobic sequencing batch reactor (ASBR) fed with dairy waste achieving 35.6 L H_2 Kg VS^{-1} and 627 L CH_4 Kg VS^{-1}.

Overall, obtained results highlighted that culture-dependent and independent approaches provided evidence for examining the relationship between bacterial and archaeal populations and biogas production in this biosystem. Besides, metagenomics sequencing technology is important to quantify the different microbial populations occurred in the reactors as well as to better understand the microbial dynamic during the anaerobic process of dairy wastes.

5.5 Conclusions

Anaerobic biosystem was strictly influenced by microbial communities structure and dynamics derived from the inoculum, feedstock and the operating conditions. It represented a sustainable management process for the valorization of abundant wastes and by-products recovered from dairy industry. Polyphasic approach highlighted the function of specific bacterial populations that drove the biohydrogen production. Besides, the inoculation in the reactors with pelleted manure allowed Archaea development, revealing that methane was primarily formed through the hydrogenotrophic pathway, since *Methanoculleus* was the dominant genus during the process.

CHAPTER 8

General discussion

Currently, biological processes represent cost-effective and attractive technologies for disposing different sorts of organic wastes, owing to the prospect of producing renewable energy sources and bioplastics. In fact, growing interest in valorizing wastes was shown by different international organizations and the commission of European community that promote future perspectives for the next years. European Commission estimated that approximately one-third of the EU's 2020 target for renewable energy in transport could be met by using biogas produced from biowaste (European Commission, 2010). Likewise, according to the latest market data compiled by European Bioplastics, global bioplastics production capacity is set to increase from around 2.05 million tons in 2017 to approximately 2.44 million tons in 2022 (European bioplastics, 2017). In order to achieve these objectives, bioprocesses can provide bioenergy and valuable chemicals through high-performing bacteria fed with organic wastes as substrates and, at the same time, keep the pollution under control.

In this context, ***Chapter 2*** shows that different organic wastes and by-products can be used to produce bioenergy (hydrogen and methane) and biopolymers (PHAs), thus highlighting, as a new perspective, the possibility of integrating the two production processes in a unique system for both energy and biopolymer production. The integrated system is aimed to produce hydrogen and/or methane and organic acids from acidogenic effluents, useful to promote the production of PHAs by selected microbial strains or mixed cultures with the highest capacity for PHA accumulation.

As the performance of a biological process is strictly affected by the microbial community that thrives and diversifies on the substrate used, Chapters 3, 4 and 5 were focused on associating bacterial and archaeal groups to the occurrence of low or high production of different valuable end-products of the anaerobic process fed with dairy wastes. As preliminary results, ***Chapter 3*** highlighted the main characteristics of the initial matrices: the mixture of cheese whey and buttermilk was a substrate rich in organic matter and indeed, a suitable substrate for bioenergy production; the commercial animal manure used as inoculum, was recognized as the driver of the anaerobic process. In fact, culture dependent and independent methods demonstrated that bacterial populations strongly changed after anaerobic digestion and were influenced by the inoculum as well as the biogas production. For this reason, the biodigesters inoculated with 5% (w/v) of animal manure resulted in higher biogas production.

In order to limit the use of the inoculum as microbial starter, ***Chapter 4*** showed batch anaerobic tests conducted with a mixture of mozzarella cheese whey and buttermilk, inoculated with 1% and 3% *w/v* industrial animal manure pellets. In agreement with the preliminary experiments, the results confirmed that the biogas production was influenced by the inoculum because H_2

and CH_4 were not detected in test with 1% *w/v* of inoculum. Otherwise, the trend of valuable metabolites of the anaerobic process, such as H_2, CH_4 and VFA were studied in both tests to correlate them with the microbial analysis. Moreover, with the aim to design an integrated system using the anaerobic effluent, is important to observe the organic acids behavior during the process compared with biological gas production. In fact, ethanol, acetic and propionic acids were only detected in the test at 3% *w/v* when H_2 production occurred coupled with a total lactic acid consumption.

The microbial analysis highlighted again the influence of the inoculum on biological gas production yield. Increasing the amount of inoculum resulted in a higher lactic acid bacteria and methanogens concentration. In particular, the archaeal methanogens concentration increased in the test inoculated at 3% (*w/v*) when H_2 and CH_4 production occurred, being one order of magnitude higher than that achieved in the test inoculated at 1% (*w/v*), thus explaining the difference in biological gas production. Obviously, archaeal methanogens were deeply investigated and *Methanobrevibacter ruminantium* and other *Methanobrevibacter* spp. were identified in the inoculum samples and related to the CH_4 production during the process. In fact, the pH condition probably selected hydrogenotrophic methanogens rather than acetoclastic methanogens because *Methanobacteriales*, with the genera *Methanoculleus* and *Methanocorpusculum*, and *Methanomicrobiales*, with the genera *Methanobrevibacter* and *Methanobacterium*, are not influenced by pH change.

To better understand the entire process and the relation of microbiota involved in the anaerobic process with biochemical intermediates, in the ***Chapter 5*** a scale-up of previous experiments confirmed the importance and influence of inoculum to carry out the anaerobic bioprocess. In fact, the dynamics of archaeal populations were strongly related to the inoculum since the relative abundance of some archeal genus detected in the inoculated samples increased during the biodigestion. In particular, among methanogenic archaea, *Methanoculleus* was the dominant genus during all the process especially when the methane production occurred throught hydrogenotrophic pathway, and its relative abundance increased up to 99% at the end of incubation time. This was confirmed, as observed in the previous experiments (Chapter 4), that hydrogenotrophic pathway is dominant when methane production occurred during biodigestion of dairy wastes, causing no organic acids consumption that was useful for the development of the integrated system.

For this reason, in the ***Chapter 6***, the organic acids mixture resulted from the anaerobic experiment ($VFA_{extracted}$) was used as growing substrate for a screening of pure culture of PHB accumulating bacteria in order to study the potentiality of integrating the two biological

processes. From the screening, the growth of *C. necator* DSM 13513 and *P. resinovorans* SA39 was enhanced by synthetic acid mixture (VFA) addition and indeed these strains were chosen for the tests with $VFA_{extracted}$. In these experiments, the percentage of PHB accumulated by *C. necator* DSM 13513 was stimulated by $VFA_{extracted}$ addition reaching 0.50% of PHB after 48 h coupled with an increase in the microbial concentration. In fact, the exposition of *C. necator* to ethanol contained in the $VFA_{extracted}$ at the beginning of the stationary phase did not affect the microbial growth but increased PHB yields about 30% compared with the optimal substrate. On the contrary, *Pseudomonas* SA39 was inhibited by $VFA_{extracted}$ accumulating 0.043% PHB during 24h as a consequence of high ethanol concentrations contained in the $VFA_{extracted}$. Thus, further studies can be useful to increase the H_2 production in the anaerobic phase limiting the ethanol concentration in the effluent used in PHA accumulation tests.

In order to promote low cost processes for PHAs production and to test the influence of the salty waste, in the ***Chapter 7***, mixed microbial cultures (MMC) were selected from the activated sludge of a wastewater treatment plant (Mutela, Portugal) and an organic acid mixture resulting from the effluent of an anaerobic process fed with cheese whey at different level of salinity was used as organic source.

Indeed, the cultures were adapted to two different salinity concentration (20 g NaCl L^{-1} and 10 gNaCl L^{-1}). The amount of the stored polymer was related to salinity concentration as well as their composition. In fact, the MMCs adapted to 20 g NaCl $L^{-1}$was stimulated to PHA accumulation in the assays with salinity lower than the enrichment. Instead, the MMCs adapted to 10 g NaCl L^{-1}achieved the maximum polymer percentage under the same enrichment saline condition. In both cases, the percentage of the PHA accumulated decrease in the tests without NaCl addition demonsting that an adapted MMCs is necessary to valorize a salty waste stream. Clearly, further research will be required to better describe the behavior of MMCs adapted to salinity concentration.

The composition of the biopolymers obtained was also affected by salinity concentration. Anyway, a general behavior for the two MMCs tested was not found. In fact, for the MMCs adapted to 20 g NaCl L^{-1}, HB content in the final biopolymer increased with the increase of salinity concentration in the assays indicating that HB precursors in the feed may be preferentially consumed for maintenance with the increase of salinity concentration. In the experiment with MMCs adapted to 10 g NaCl L^{-1}, a different behavior was observed because the HB percentage decreased with the salinity increase. Otherwise, biopolymer recovery in the form of a thin film were obtained from these tests with good elastomeric properties due to the HV content.

These findings suggest that the approach of integrated system is of direct practical relevance landing to bioenergy and biopolymers production from dairy wastes. This approach could also be applied to different kinds of organic waste representing the opportunity to their valorization through sustainable biopolymers and biofuel production via microbial bio-based process of biorefinery value chain.

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PERSONAL INFORMATION

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Nationality: Italian

Date of birth: 06/02/1989

Gender: Female

Accademic studies and work experience

Current position	PhD candidate at **Department of Agricultural Sciences** of University of Naples Federico II
Name and type of organization providing education	Department of Agricultural Sciences - Division of Microbiology
Main topic	Microbial biotechnologies applied to 2nd and 3rd generation processes for production of biochemical intermediates, bioenergy and biomaterials
Dates	Since May 2018

Training at **FCT Universidade NOVA de Lisboa (Portugal)**

Experimental activities in “Department of Chemistry” laboratory.

Name and type of organization providing education and training	Biochemical Engineering Group (BIOENG), Departamento de Química, Prof. Maria Reis, Dr. Catarina Oliveira.
Principal subjects/occupational skills covered	Polyhydroxyalkanoates (PHAs) production PHAs producing bacteria selection PHAs accumulation Acidogenic fermentation Three-stage process for simultaneous production of bioenergy and biopolymers
Dates	From September 2014 to July 2015

Consultant for STIGE & PARTNERS s.r.l.

Specializing in hydraulic infrastructures design.

The collaboration involved the following projects:
- “Grande Progetto”- Environmental Remediation and Valorisation of the Regi Lagni "Up-grading of Treatment Plant of the Naples" - Final Project;

- “Grande Progetto”- Urban Requalification Naples East – Up-grading of sewage system of Corso San Giovanni - Improvements to the final project;

- Harbour of Naples - Realization of the sewage system - Improvements to the final project;

- Action Plan for the up-grade of the Regional Water System- Adaptation and modernization of the Cercola lifting plant;

	- Assignment of architectural and engineering services in the direction of work and in the coordination of safety during the execution of urban and environmental development works of Via Ferraris, Via Brecce in Sant'Erasmo, Via Gianturco, Via Nuova Of Brecce; - Monitoring the water resources and the realization of the single control unit - Final project;
Dates	From January 2012 to July 2014
Title of qualification awarded	M.Sc in **Environmental Engineering** *summa cum laude*
Title of dissertation	**"**EFFECTS OF DIFFERENT ELECTRON DONORS AND MICROBIAL CULTURE ON AUTOTROPHIC DENITRIFICATION OF SIMULATED MINING WATERS".
Name and type of organization providing education and training	University of Naples Federico II-Engineering college, Naples (Italy)
Principal subjects/occupational skills covered	Waste Water Treatment Plants, Management of Sanitary Environmental Engineering Plants, Emission monitoring, Hydraulics, Soil remediation.
Tutors	Prof. Francesco Pirozzi (University of Naples Federico II, Italy); Prof.Jaakko Puhakka (Tampere University of Technology, Finland)
Dates	From January 2014 to June 2014
Title of qualification awarded	Training at **Tampere University of Technology (Finland)**
	Experimental activities in "Department of Chemistry and Bioengineering" laboratory.
Name and type of organization providing education and training	"Department of Chemistry and Bioengineering"-Tampere University of Technology-Tampere, Finland.
Principal subjects/occupational skills covered	Waste Water Treatment Plants, Sanitary Environmental Engineering, Biological Processes Chemistry.
Dates	From September 2008 to December 2011
Title of qualification awarded	B.Sc in **Environmental Engineering**
Title of dissertation	"BIOGAS PRODUCTION FROM AGRO BASED INDUSTRY".
Name and type of organization providing education and training	University of Naples "Federico II"-Engineering college, Naples (Italy)
Principal subjects/occupational skills covered	Waste Water Treatment Plants, Management of Sanitary Environmental Engineering Plants, Emission monitoring, Hydraulics, Soil remediation.

Conference and Seminars

- Advanced Training Course on Emerging biotechnologies for sustainable waste management and biorefinery development (posters presentation), Napoli 4-5 Aprile 2016, Università degli Studi di Napoli " Federico II";

- XXI IUPAC CHEMRAWN CONFERENCE “Solid Urban Waste Management” (posters presentation), Roma 6-7-8 Aprile 2016, CNR.
- 6th International Symposium on energy from biomass and waste – VENICE 2016 (posters presentation),14-17 novembre 2016, Scuola Grande di san Giovanni Evangelista, Venezia.
- Winter school LIFE/ECOREMED: “Metodi innovativi per l’analisi e la gestione dei siti degradati e/o contaminati”, 13-15 Dicembre 2016, CIRAM, Napoli.
- 1st International ABWET conference: Waste-to-Bioenergy: Applications to Urban Areas (oral presentation), 19-20 Gennaio 2017, Université Paris –Est, Champs sur Marne, Parigi, Francia.
- BioPoliS: un progetto per la bioeconomia e il territorio, 9 Maggio 2017, Centro Congressi dell'Università degli Studi di Napoli Federico II, Napoli.
- BioMAc 2017 Bioreattori a Membrane (MBR) per la depurazione delle Acque, 9-10 Ottobre 2017, Chiesa dei Santi Marcellino e Festo, Napoli.
- IV International Conference on Microbial Diversity – Drivers of microbial diversity (posters presentation), 24-26 Ottobre 2017, Università di Bari Aldo Moro.
- International PhD meeting “Advanced Biological Waste-to-Energy Technologies (ABWET) Marie Skłodowska-Curie” (oral presentation), 12 Gennaio 2017, DICEA Università degli Studi Napoli Federico II.
- International PhD meeting “Advanced Biological Waste-to-Energy Technologies (ABWET) Marie Skłodowska-Curie” (oral presentation), 21 Luglio 2017, DICEA Università degli Studi Napoli Federico II.
- International PhD meeting “Advanced Biological Waste-to-Energy Technologies (ABWET) Marie Skłodowska-Curie” (oral presentation), 10 Novembre 2017, DICEA Università degli Studi Napoli Federico II.
- International PhD meeting “Advanced Biological Waste-to-Energy Technologies (ABWET) Marie Skłodowska-Curie” (oral presentation), 18 Aprile 2018, DICEA Università degli Studi Napoli Federico II.
- Summer School on Computational Analysis From Genomic Diversity to Ecosystem Structure, 3-7 Settembre 2018, Scuola di Agraria - Piazzale delle Cascine, Firenze.
- BioEng Meeting, 16 Settembre 2018, FCT NOVA - CGD Auditorium, Lisbona.
- UCIBIO Annual Meeting, 28-29 Settembre 2018, FCT NOVA - CGD Auditorium, Lisbona.
- “Statistical Data analysis”, Prof. Jihad Abdaliah;
- “Control of bacterial diseases: a successful story”, Prof. Joel Vanneste;
- “Biogenic nanoparticles in the wastewater treatment process”, Prof. Linda Tseng
- “Biohydrogen production from solid waste”, Prof., Hidayet Argun;

Pubblications

Journal articles

Pagliano G., Ventorino V., Panico A., Romano I., Robertiello A., Pirozzi F., Pepe O. (2018). The effect of bacterial and archaeal populations on anaerobic process fed with mozzarella cheese whey and buttermilk. Journal of environmental management 217, 110-122. doi: 10.1016/j.jenvman.2018.03.085. I.F. (2016): 4.010, quartile (2016): Q1.

Ventorino V., Romano I., **Pagliano G**., Robertiello A., Pepe O. (2018). Pre-treatment and inoculum affect the microbial community structure and enhance the biogas reactor performance in a pilot-scale biodigestion of municipal solid waste. Waste Management 73, 69-77. doi: 10.1016/j.wasman.2017.12.005. I.F. (2016): 4.030, quartile (2016): Q1.

Pagliano G., Ventorino V., Panico A., Pepe O. (2017). Integrated systems for biopolymers and bioenergy production from organic waste and by-products: a review of microbial processes. Biotechnology for Biofuels 10, 113. doi: 10.1186/s13068-017-0802-4. I.F. (2016): 5,203, quartile (2016): Q1.

Pagliano G., Ventorino V., Panico A., Romano I., Pirozzi F., Pepe O. Anaerobic process for bioenergy recovery from dairy waste: meta-analysis and enumeration of microbial community related to intermediates production" accepted by Frontiers in Microbiology, section Microbiological Chemistry and Geomicrobiology

Ventorino V., Nicolaus B., Di Donato P., **Pagliano G**., Poli A., Iavarone V., Pepe O. Selection of expolysaccharide-producing *Azotobacter chroococcum* 76A for biopolymer synthesis from vinasse. Waste and Biomass Valorization (WAVE) (submitted article).

Proceedings

Pagliano G., Robertiello A., Ventorino V., Panico A., Pepe O (2016). Evaluation of biogas production from anaerobic digestion of whey and buttermilk. Proceedings Venice2016, Sixth International Symposium on Energy from Biomass and Waste, 14 - 17 November 2016. Great School of St. John the Evangelist, Venice, Italy;

Ventorino V., **Pagliano G**., Nicolaus B., Di Donato P., Taurisano V., Iavarone V., Pepe O. (2016). Agrofood wastes as substrate for bio based polymers synthesis by exopolysaccharides producing bacterial strain. Proceedings Venice2016, Sixth International Symposium on Energy from Biomass and Waste, 14 - 17 November 2016. Great School of St. John the Evangelist, Venice,Italy;

Pagliano G., Panico A., Ventorino V., Romano I., Robertiello A., Comite E., Pirozzi F., Pepe O. (2017). Dairy waste as sourceof bioenergy: evaluation of methane and biohydrogen production coupled with bacterial community characterization. In: Proceedings of the 1st International ABWET Conference: Waste-to-bioenergy: Applications in Urban areas, pp. 76-83. January 19th-20th, 2017 in Champs-sur-Marne Paris, France.(oral comunication);

Pagliano G., Robertiello A., Ventorino V., Panico A., Pepe O. (2017). Bacterial evolution during biodigestion of dairy wastes. In:Proceedings of the IV International Conference on Microbial Diversity – Drivers of microbial diversity, pp.367-368. 24th-26th October 2017, University of Bari Aldo Moro;

Robertiello A., Ventorino V., Romano I., **Pagliano G**., Faraco V, Pepe O. (2017). Preliminary study of adaptation and resistance of *Basfia succiniproducens* to toxic lignocellulosic by-products. In: Proceedings of the IV International Conference on Microbial Diversity – Drivers of microbial diversity, pp.367-368. 24th-26th October 2017, University of Bari Aldo Moro.

Membership

SIMTREA, Italian Society of Agri-Foodand Environmental Microbiology

"Ordine degli Ingegneri" Provincia di Napoli

Research Project

"BioPoliS" Development of green technologies for production of BIOchemicals and their use in preparation and industrial application of POLImeric materials from agricultural biomasses cultivated in a sustainable way in Campania region.

Research project with “IURO s.r.l.” about “Valorization of dairy wastes for bioenergy production”

Mother tongue	**Italian**
Other language	**English**
Reading skills	B2
Writing skills	B2
Verbal skills	B2

Social skills and competences	Good communication skills acquired during the experience as a member of the Board of Degree Course and as student representative.
Organisational skills and competences	Organizer and moderator of technical conferences about waste and water resources.
Computer skills and competences	Microsoft Office, Windows, ArcGIS, AutoCAD, MatLab Qiime

Naples, 19th October 2018

Giorgia Pagliano

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