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STRUCTURE AND DYNAMICS OF MICROBIAL COMMUNITIES IN ANAEROBIC DIGESTION PROCESSES

GIUSEPPE MERLINO
R08382

PhD School Coordinator: Prof. Roberto PRETOLANI

Scientific Supervisor: Prof. Daniele DAFFONCHIO

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Extended Abstract

Nowadays world energy needs rely mostly on fossil fuels (oil, coal and natural gas) which accounts for more than 80% of global energy production. Fossil fuels reserves are estimated to deplete by 2050, moreover the total worldwide energy consumption is expected to rise in the next 25 years of about the 50%. In this context, it arises the need to establish new renewable energetic sources in order to replace fossil fuels and to minimize related climate change impacts, like the increasing concentration of CO_2 in the atmosphere. Biomass, the organic waste derived mainly from agricultural and agro-industrial productions, is being considered as valuable candidate for production of energy mostly because of its plentifully availability. A well-established technology for biomass-to-bioenergy conversion is the production of biogas through anaerobic digestion (AD). This process involves a complex consortium of different functional groups of microbes which, degrading the organic matter, produce biogas composed mainly of methane and carbon dioxide. In the latest 10 years there has been renewed interest for energy production from biomass through AD because of its versatility and potentiality: some advantages are the possibility to solve the problem of waste disposal and the biogas can be used for heating or for electricity. So far, the control and performance of AD process has typically been performed working on operational parameters (such as T, pH, COD, loading rate, etc.). However, recent studies concerning the microbial consortia involved in this complex process have been developing with the final aim to get an exhaustive knowledge of microbiology of the process and how it correlates to the operation of the reactor in order to improve the digester performance making preventive action possible.
The aim of the opening chapter is to summarize the state of the art of the AD process focusing on the potential of application of the culture-independent methods for analyzing microbial communities in anaerobic digesters. An introduction concerning the description of conventional (single-phase) and innovative (two-phase) AD process technologies and the main functional groups of microorganisms is reported. Following, the limitations and strengths of the wide variety of molecular fingerprinting methods currently available are underlined. Key studies illustrating the utility of quantitative real-time PCR assays for elucidating the expression of genes associated with specific activities are also presented. The main statistical tools to analyse and correlate the molecular fingerprints data with operational parameters data are described. The advantages of different statistical approach are shown: descriptive statistical tools, like hierarchical clustering methods, multivariate ordination methods and Microbial

Resource Management (MRM) tool-set are discussed in details, as they can help to better elucidate the structure-to-function relations inside the methanogenic consortium. Recent selected studies illustrating the applicability of statistical analysis methods are presented.

The choice of biomasses to be used as substrates for anaerobic reactors depends from different factors, such as their biochemical methane potential (BMP) as well as availability and cost. In order to evaluate the rate of conversion to methane from different organic substrates, a wide range of research has been dealing with biochemical methane potential (BMP) determinations. Since anaerobic digestion process is linked to microbial community structure and dynamics, recent research is focused on the precisely characterization of overall consortium involved in the AD with the final objective to link microbial community structure to function. To this aim, molecular techniques, like DGGE, and their molecular analysis parameters are valuable tools for microbial ecology interpretation and management. While a wide range of research paper have been developed on the determination of BMP of different substrates, very limited information is available about the description of microbial populations of these AD processes.

The aim of the first work, presented in chapter 2, was to experimentally determine the BMP of different energetic crops and agro-industrial biomasses and, in order to get further insight into the processes, to characterize the microbial communities present both before and after the AD process. Anaerobic BMP was determined using an automated laboratory-scale multi-batch system. Microbial diversity and dynamics were analysed by Denaturing Gradient Gel Electrophoresis (DGGE) and quantification of bacterial and archaeal populations was performed by quantitative real-time PCR (qPCR).

It was demonstrated that high BMP could be obtained from energy crops as well as other agro-industrial residues/by-products. Molecular biology techniques revealed to be useful tools for investigating the structure and diversity of microbial community during AD process. Real-time quantitative PCR revealed that the *Methanosarcinales* were the majority of the archaeal community and that sulphate-reducing bacteria were present at low titers. Denaturing gradient gel electrophoresis (DGGE) showed the dominance of microorganisms affiliated to *Methanosarcina* and *Clostridia*. It was shown that seeding sludge had a fundamental role in determining the basal microbial community with some dominant archaeal and bacterial taxa (*Methanosarcinales*, especially *Methanosarcina* and *Clostridia*) detected by DGGE analysis throughout the course of the process. However, definite changes in the microbial community were observed, suggesting that quantitative changes in the abundance of some key species occurred as response to microbial community adaptation to the different biomasses. Principal component analyses of DGGE profiles indicated that the microbial shifts between the beginning and the end of the AD processes were characterized by significant clustering in the case of *Archaea* whereas most variability was observed for *Bacteria*. In fact, compositional features of biomasses or processing (ensiling) seemed to play a role in the changes of archaeal microbial communities indicating *Archaea* as good indicators for monitoring AD microbial community dynamic. Bacterial communities were richer, more dynamic

and even than archaeal. A statistically significant correlation trend was also identified between archaeal community and BMP indicating that more even archaeal community were associated to higher BMP.

In Italy, many medium- and small-scale biogas plants, usually developed in association with agro-zootechnical farms, were recently built. In these biogas plants, the organic substrates that are used for AD process cover a great variety of biomasses, such as, manure, agro-industrial wastes, energy crops. In particular, this study was focused on the potential use for bioenergy production of by-products and wastes derived from dairy farming. In fact, the exploitation of these products would permit their valorization, solving the problem of waste disposal, and at the same time contributing to the energy self-sustenance of dairy farming.
The aim of the study presented in the third chapter was to evaluate the BMP of whey, alone, and in co-digestion with dairy manure and energy crops. Moreover, considering the importance of the microbial consortia for the performance of the AD process, the microbial community of the digestion tests were characterized by DNA-based molecular methods in order to point out the species associated with methane production. Anaerobic digestion BMP tests were carried out in batch reactors at mesophilic conditions (37°C). Results obtained demonstrated that whey, alone or in co-digestion with others biomasses, gave high values of BMP between 400 and 500 $ml_N CH_4$ g^{-1}TS.
Changes in bacterial and archaeal community structure were monitored both before and after the digestion processes by using the Denaturing Gradient Gel Electrophoresis (DGGE) technique. Qualitative analysis obtained through DGGE experiments showed a general high diversity of Archaea and Bacteria communities. Archaeal populations were dominated by aceticlastic methanogens belonging to genus *Methanosaeta* together with hydrogenotrophic methanogens affiliated to the *Methanomicrobiales* order. In the case of winery wastewaters, a drastic change in the methanogenic community was observed at the end of the process with the dominance of acetotrophic methanogens belonging to genus *Methanosarcina*. In all the test performed, bacterial communities were characterized by high stability and diversity. The main phylogenetic groups observed were *Bacteroidetes*, *Deltaproteobacteria*, *Firmicutes* and *Aminoanaerobia*. Only in rare cases, the presence of peculiar microbial groups was detected, such as *Clostridium butyricum* and *Sintrophomonas* found in digestate of whey and winery wastewaters, respectively.

The conventional AD technology consists of one-stage continuously fed systems; the whole consortium of microbes responsible for the process is kept in a single digester and a delicate balance between the different trophic groups (different physiology, nutritional needs and sensitivity to environmental conditions) must be accomplished. Recently, a two-stage approach with two separate bioreactors in series has been previously proposed, splitting hydrolysis/acidogenesis and methanogenesis and allowing to optimize each phase and producing hydrogen and methane separately from each phase. In recent years, this technological process has been applied to an increasing number of complex feedstock. However, there is a limited knowledge on the microbial characterization of the anaerobic community in two-stage processes. A better

understanding of the ecology and function of the microbial community in these processes is desirable in order to better control and improve the biological processes.

The aim of the work presented in chapter 4 was to characterize and compare the microbial community of a 65-days start-up period of a two-stage hydrogen-methane process (R1 and R2) and of the traditional single-stage anaerobic digestion process (R3), both fed with a mixture of fruit and vegetable wastes and swine slurry. The structure and the dynamics of the microbial population were qualitatively and quantitatively analysed. Denaturing Gradient Gel Electrophoresis (DGGE) was used to investigate the structure and the shifts of bacterial and archaeal communities and to identify the dominant hydrogen-producing bacteria and methanogens. Real-Time PCR was employed to monitor quantitatively the temporal changes of the major functional bacterial groups involved in the anaerobic process: total *Bacteria*, total *Archaea*, hydrogen-producing bacteria, sulphate-reducing bacteria and acetogens.

In both the hydrogen (R1) and methanogenic reactors of two-stage process (R2), all the identified microorganisms matched with uncultured bacteria affiliated to the phylum *Firmicutes*, class *Clostridia*, whereas a greater diversity was observed in the methane reactor of single-stage process with bacteria belonging to the three phyla, *Bacteroidetes*, *Firmicutes* and *Synergistes*. In R1 the DGGE profiles obtained indicated that the bacterial community changed with time especially in the first 15 days of operation. Thereafter, a quite stable bacterial community, composed of very few bands, was established. This bacterial community, associated to the hydrogen production at the steady-state, was composed by bacteria affiliated to *Clostridium senso stricto* or to unclassified *Clostridiales*. *Clostridium* species are well known to ferment from various types of carbohydrates generating mainly acetate, butyrate, hydrogen and carbon dioxide. In the single-stage methanogenic reactor (R3), the community was characterized by a highest microbial diversity. At the steady-state, most of the sequenced dominant bands resulted affiliated to *Bacteroidetes*, bacteria commonly found in anaerobic digesters, capable of fermenting carbohydrates or proteins.

R1 showed a constant archaeal community throughout the process dominated by few species all belonging to *Methanosarcina*. A modest microbial change was observed only in correspondence to a beginning in the accumulation of VFA (>1500 mg acetate/L) and a partial inhibition of the process, resulting in a reduced methane productivity. In R3, during the starting-up, the archaeal community remained identical to that of R1, as it was expected by the use of the same inoculum sludge. Thereafter, it was evidenced a drastic shift of the archaeal population with the establishment of the new community concurring with a stable methane production. The predominant species belonged both to *Methanosarcina* and *Methanosaeta*. From these DGGE findings, it can be inferred that the methane detected was produced mostly by the activity of microorganisms belonging to the *Methanosarcinales* order and mainly by *Methanosarcina spp*.

PCR real-time quantifications showed that in R1 the group of hydrogen-producing bacteria, representing a minimal proportion to total *Bacteria*, varying within percentage 0.05-1.4%, remained almost constant during all the process (106 bacteria per ml). In R2 the maximal number of *Archaea* was reached after 44 day of operation (stable biogas production) with $1,77 \times 10^8$ bacteria per ml. Among methanogens, the dominant group

was the *Methanosarcinales*, which constituted approximately the 50% of the total archaeal concentration. This result is in agreement with the PCR-DGGE data analysis that showed a high abundance of *Methanosarcina*. In R3, both total *Archaea* and *Methanosarcinales* showed a lower bacterial density than R1; *Archaea* did not get up to 107 bacteria per ml. It is likely that the differences in structure and abundance of bacterial and archaeal community between the two anaerobic digesters are responsible for the diverse performance of two anaerobic processes.

Chapter 1

The microbial diversity associated to Anaerobic Digestion processes for energy recovery

1. Introduction

Energy is the power of life. The standard of our living is strictly dependent on the availability of energy and on the its continuous and guaranteed supply. The global consumption of energy in all sectors, from agriculture to transportation and industrial processes, is raising constantly year by year. In the latest 20 years together with the energy consumed by developed countries there is also the energy needs of new rising economies that are striving for improve their standards of living: e.g. in the period from the mid eighties to the mid nineties the US energy consumption has raised 1,7% per year, while China and India, two of the biggest growing economic powers, respectively at 5,3% and 6,6% per year (Ghoniem, 2011).

According to the International Energy Agency (IEA), nowadays world energy requirements are mostly dependent on fossil fuels: these non-renewable sources of energy accounts for close to 80% of global energy production, dominated by natural gas (45,7%) and oil reserves (32,9%) exploitation. Moreover the total worldwide energy consumption is expected to rise by more than 50% in the next 25 years, while the fractional share of the different raw sources in respect to the total amount is not expected to change significantly (Ghoniem, 2011).

This present scenario and the prospect of a future rising consumption of fossil fuels has led to the alarms of irreversible global warming and the associated impacts of climate change due to the emission of Greenhouse gases (GHG), with carbon dioxide (CO_2) being the most important contributor, which are released into the atmosphere as a result of the combustion of fossil fuels and have impacts on global warming (Das and Veziroa, 2001; Hoffert et al., 1998).

Thus an emerging drive towards a more sustainable society, through the establishment of new renewable sources of energy, is extremely necessary and urgent. Biomass, especially organic wastes, is being considered as a valuable candidate as renewable energy source for feasible utilization (Amann et al., 1995). It is environment-friendly, is widely available and its utilization for energy production has a great potential to reduce carbon dioxide emissions and consequently to prevent global warming (Claassen et al., 1999).

The Renewable Intensive Global Energy Scenario (RIGES) suggested that, by 2050, approximately half the world’s current primary energy consumption of about 400 EJ/yr, could be met by biomass and that 60% of the world’s electricity market could be supplied by renewables, of which biomass is a significant component.

There are several pathways enabling the conversion of biomass to bioenergy. Ethanol and hydrogen fermentation and microbial fuel cell (MFC) technology (Logan, 2004; van Haandel, 2005) are only some examples of new approaches for energy generation (Rabaey and Verstraete, 2005). Among all of these new options, methanogenesis in anaerobic digestion (AD) processes, has been studied and is now well established. This technology, which emerged during the seventies, enables the production of biogas mainly composed of methane. In the past 10 years, there has been renewed interest, world-wide, in biomass as an energy source through AD because the methane produced is regarded as a very versatile energy source. It can be used directly for heating and electricity generation or as an alternative gaseous vehicle fuel (Agency for Renewable Resources, Feeding biogas into gas network, 2nd ed. Gülzow, 2006; Agency for Renewable Resources,, Biofuels - a comparative analysis. Gülzow, 2006).

Once produced, biogas is generally composed of ca. 48–65% methane, ca. 36–41% carbon dioxide, up to 17% nitrogen and traces of other gases (Rasi et al., 2007). Both carbon dioxide and methane are GHG and possibly 18% of global warming is thought to be caused by anthropogenically derived methane emissions (Ghosh, 1997). Carbon dioxide released through natural mineralization is considered neutral in GHG terms as the carbon has been recently removed from the atmosphere by plant uptake, to be released again as part of the carbon cycle.

Controlled anaerobic digestion of organic material is therefore to be considered environmentally beneficial in different ways:

- by containing the decomposition processes in a sealed environment, potentially damaging methane is prevented from entering the atmosphere, and subsequent burning of the gas will release carbon-neutral carbon dioxide back to the carbon cycle;
- the energy gained from combustion of methane will displace fossil fuels, reducing the production of carbon dioxide that is not part of the recent carbon cycle;
- less biomass sludge is produced in comparison to other aerobic treatment technologies;
- digestate produced can be used as an improved fertilizer in terms of its availability to plants (Tafdrup, 1995), allowing to reduce dependence on mineral fertilizers (Tambone et al., 2009).

Anaerobic digesters are characterized by complex microbial consortia (Riviere et al., 2009) and culture-independent molecular techniques have demonstrated that the microbial community characteristics can play an important role for a good reactor performance (McHugh et al., 2004). Many studies have rencently postulated that monitoring of the microbial community characteristics could lead to an early detection of operational problems, making preventive action possible (McHugh et al., 2004; Lee et al., 2008; Malin and Illmer, 2008; Rincón et al., 2008; Talbot et al., 2008). However, no direct relationships between microbial community characteristics and process parameters have been established yet (Bouallagui et al., 2005). Thus, more needs to be understood about the microbiology of the process and how it correlates to the operation of the bioreactor.

2. Methanogenic Pathway

Methanogenesis of complex organic materials is a widespread process in anoxic environments where inorganic electron acceptors such as oxygen, nitrate, iron, manganese, and sulfate are absent. Examples of these environments are wetlands, freshwater sediment, and the digestive tracts of animals and insects.

This metabolic pathway is the least exergonic process when compared to aerobic degradation or the alternative anaerobic respirations. Conversion of hexose to methane and carbon dioxide releases only 15 % of the energy that would be available in aerobic degradation, $\Delta G^{0'}$ of -390 kJ mol^{-1} agaist -2.870 kJ mol^{-1} that can be obtained from degradation of glucose to CO_2 and H_2O (where $\Delta G^{0'}$ is the variation on Gibbs Free Energy under standard thermodynamic conditions [298 K, pH 7, solute at 1 M, gases at 105 Pa]).

As a consequence of this small energy gain, the reaction product, methane, stores a major part of the energy available in aerobic biomass conversion. This energy can be exploited subsequently in the presence of oxygen by other organisms, e.g., by aerobic methane oxidizers or by humans in heating and other physical processes.

Anaerobic microbial communities can be classified into two domains, *Bacteria* and *Archaea*, and is accomplished by four physiologically different microbial groups: i) hydrolytic-fermentative bacteria, ii) proton-reducing syntrophic bacteria, iii) hydrogenotrophic methanogens and iv) aceticlastic methanogens. Through the interactions of these microbial groups, the organic materials are eventually mineralized to CH_4 and CO_2.

The first group of bacteria hydrolyze polymers including proteins, polysaccharides, nucleic acids, and lipids to monomers such as amino acids, sugars, nucleotides, and long-chain fatty acids. This microbial group further ferments monomers to reduced compounds (alcohols, short-chain fatty acids, organic acids), H_2 and CO_2. Subsequently reduced products are oxidized to acetate, hydrogen and carbon dioxide by the proton-reducing syntrophic bacteria. H_2 and CO_2 are then converted to CH_4 by the hydrogenotrophic methanogens whereas methanogenic acetate degradation is carried out by the aceticlastic methanogens.

2.1 Syntrophic methanogenesis

During the methanogenic process, oxidation of reduced compounds catalyzed by the proton-reducing microbes is thermodynamically unfavorable. The oxidation of fatty acids is highly endoergonic. The variation of Gibbs Free Energy for the oxidation of butyrate or propionate coupled with proton reduction is +48.1 kJ mol^{-1} and +76.1 kJ mol^{-1}, respectively. These reactions can proceed only through syntrophic interaction between the proton-reducing bacteria and hydrogenotrophic methanogens. These two groups of microorganisms through this interspecies hydrogen transfer are able to keep hydrogen partial pressure low. In this way $\Delta G^{0'}$ of the global process is negative (-135.6 kJ mol^{-1}), meaning a net gain of energy for the microorganisms. For this reason syntrophy is considered to be essential for the oxidation of these substrates.

Several syntrophic fatty acid-oxidazing bacteria including a butyrate oxidizer and a propionate oxidizer have been isolated and characterized (Kamagata and Tamaki 2005). In addition to these syntrophs, it has been discovered that several bacteria can also oxidize acetate syntrophically when hydrogenotrophic and/or formate-utilizing methanogens are present (Hattori et al., 2000; Schnurer et al., 1994).

2.2 Acetotrophic methanogens

Acetate is the precursor of two-thirds of the methane produced in anaerobic bioreactors. At present, two genera of methanogens are known to use acetate as sole energy source: *Methanosarcina* and *Methanosaeta*. *Methanosarcina* rapresents the acetoclastic methanogens. which predominate in many anaerobic ecosystems where organic matter is completely degraded to CH_4 and CO_2. Microorganisms belonging to this group are the most metabolically divers amongst methanogens and have a high growth rate but low affinity for acetate. On the contrary, *Methanosaeta* can be considered a specialist able to grow only on acetate, with higher affinity but lower growth rate than the former. *Methanosaeta* have a much lower minimum threshold for acetate utilization (7-70 µM) than *Methanosarcina* (0.2-1.2 mM), infact species from the genus *Methanosaeta* were found to be the dominant in a variety of laboratory-scale anaerobic continuously mixed reactors at 37 and 55 °C, at low acetate concentrations (Zheng and Raskin, 2000).
Methanosarcina spp. was determined to be the most abundant aceticlastic methanogens in laboratory-scale mesophilic anaerobic bioreactors at high acetate concentrations (Stroot et al. 2001; McMahon et al. 2001). Karakashev et al. (2005) found that the methanogenic diversity was broader in plants operating at mesophilic ranges than the thermophilic plants. The dominance of *Methanosaetaceae* was observed in digesters fed with sludge, while *Methanosarcinaceae* were dominant in manure digesters. Karakashev et al. (2006) also stated that in the absence of *Methanosaetaceae*, the acetate oxidation to H_2/CO_2 with the subsequent generation of methane by hydrogenotrophic methanogens should be the dominant pathway. These results seem to be in agreement with other studies (Shigematsu et al. 2004).
The optimum pH range for acetotrophic methanogens is between 6,6 and 7,3. Acetotrophic methanogens are inhibited strongly below a pH of 6,2, while free ammonia concentrations could also be inhibitory at pH levels above 7,4.

2.3 Hydrogenotrophic methanogens

The hydrogen partial pressure is an important parameter, which defines process stability or upsets in an anaerobic digestion process. Therefore, the activity of the hydrogenotrophic methanogens are crucial for a stable and efficient process performance.
Hydrogenotrophs methanogens constitute the biggest group of methanogenic *Archaea*. They oxidize H_2 and reduce CO_2 to form methane and some of them are also able to oxidize formate. Methanogens of the orders of *Methanobacteriales* and *Methanomicrobiales* belong to this group.

Analysis of bacterial populations in anaerobic reactors by most probable number (MPN) technique under thermophilic and hyperthermophilic (up to max. 70 °C) conditions, in lab-scale continuous reactors indicated that the hydrogenotrophs dominated the acetotrophic methanogens by a factor of 10 to 10.000, presumably due to short HRTs (between 14.2 and 1.25 days) employed. The effect of a temperature was also investigated by Ahring et al. (2001). They found that Hydrogenotrophic methanogens in a digester treating cattle manure were the only microbial group, which exhibited higher specific methanogenic activity (SMA) and unchanged MPN at 65° C, compared to 55 °C, while the activity and the amounts of other methanogens were significantly reduced. The microbial community of a laboratory-scale mesophilic two-phase anaerobic digestion system treating fruit and vegetable wastes was studied (Bouallagui et al. 2005). The species composition seemed to change significantly during the entire study. In the firstphase/acidogenic reactor, *Methanosphaera stadtmanii* and *Methanobrevibacter wolinii* were observed to be the major hydrogenotrophic methanogens. The anaerobic digestion of fodder and sugar beet silage was investigated by Scherer and Lehmann (2004). They found that methanogens known to grow on H_2/CO_2 or formate were found to be dominant at short HRTs between 6.5 and 7.5 days.

3. Anaerobic Digestion Process Technologies

There are several types of reactor in use today, and the design is related to the material to be digested. There are three main groups of anaerobic digestion systems: batch reactors, continuous one-stage systems and continuous two-stage systems.

3.1 Batch Systems

The batch systems are digesters filled once with fresh materials, with or without addition of inoculum, and allowed to go through all degradation steps sequentially. The hallmark of batch systems is the clear separation between a first phase, where acidification proceeds much faster than methanogenesis, and a second phase, where acids are transformed into biogas. Batch systems have not succeeded in taking a substantial market share. However, the specific features of batch processes, such as simple design and process control, robustness towards coarse and heavy contaminants, and lower investment costs make them particularly attractive for developing countries (De Baere, 2000). The dependence of the methane yield on the starting level of digestible organic substances observed in batch digestion tests suggested the operating conditions for the fed-batch or continuous digestion of the materials under consideration. Application of sequencing batch reactor (SBR) technology in anaerobic treatments is of interest because of its inherent operational flexibility, characterised by a high degree of process variability in terms of cycle time and sequence, no requirement for separate clarifiers, and retention of a higher concentration of slow-growing anaerobic bacteria within the reactor (Suthaker et al., 1991).

3.2 Continuous one-stage systems

The conventional technology is actually rapresented by one-stage continuously fed systems. In this reactor design, the acid-forming and the methane-forming microorganisms are kept together in a single digester and there is a delicate balance between these two groups, because both differ widely in terms of physiology, nutritional needs, growth kinetics and sensitivity to environmental conditions (Liu et al., 2006; Zoetemeyer et al., 1982). About 90% of the full scale plants, currently in use in Europe for the anaerobic digestion of organic fraction of municipal solid wastes and biowastes, rely on continuous one-stage systems (Lissens et al., 2001). In this kind of system configuration, combining acidogens and methanogens in one vessel, hydrogen formed by acidogenic metabolism is assimilated by the methanogens to reduce carbon dioxide to methane and water (Poggi-Varalgo et al., 1997). On increasing the feeding rate of the substrate, acidogenic activity, including mainly acetate, carbon dioxide, and hydrogen production, is increased, whereas the methanogenic population cannot increase its activity to the same extent. At a loading rate, were the hydrogen consuming reactions become saturated, accumulation of hydrogen partially inhibits its further formation and consequently more organic electron sink will be formed, causing imbalances and cessation of methane production (Liu et al., 2002a).

3.3 Continuous two-stage systems

The last type of system configuration is the two-stage (or even multi-stage) continuously fed systems. This kind of system design implies a process configuration employing separate reactors for acidification and methanogenesis connected in series, allowing optimization of both processes. A two-stage system can improve the stability of the process compared to one-stage systems, particularly when digesting easily hydrolysable feedstocks (Bouallagui et al., 2005; Mata-Alvarez, 2002). The interesting feature of using two-stage process is to optimize each process separately, leading to a larger overall reaction rate and biogas yield (Blonskaja et al., 2003). Furthermore, a better pathogenic destruction is achieved by a two-stage process, which combines a short hydrolysis stage performing at thermophilic or hyper thermophilic temperatures and methane stage at thermophilic or mesophilic temperatures (Bendixen, 1994). Despite the fact that this kind of system design have a higher performance than single-stage digesters, the two-stage systems have not won inpass as they are more complex from the engineering point of view and thus more expensive to build and maintain.
In a comparison of one- and two-stage thermophilic reactors treating cattle manure (Nielsen et al., 2004), it was found that the two-stage digester had a 6-8% higher specific methane yield and a 9% more effective volatile solids removal than the conventional single stage reactor. Liu et al. (2006) found a 21% increase in methane yield in a two-stage reactor when compared to a single-stage reactor, both operating on municipal solid waste.

4. Molecular Biology Methods

The number of studies dealing with biodiversity of microbial communities has increased exponentially over the last 20 years (Morris et al., 2002). Microbial diversity studies in both natural and engineering systems were limited in the past by the lack of methodological tools and were conducted only using conventional microbiological techniques. These methodologies are based on isolation of pure cultures and have provided extensive information on the biodiversity of microbial communities. However, culturing fails to reproduce the ecological niches and symbiotic relationships encountered in complex natural environments that are required to support the full spectrum of microbial diversity. It is therefore generally accepted nowadays that the number of known prokaryotic species is very small compared to the diversity of microorganisms. About 7000 bacterial species have been described nowadays (DSMZ, 2005), but according to molecular and ecological estimates, the real number must be several orders of magnitude higher (Amann et al., 1995). The conventional microbiological tools enables scientists to detect only ca. 1% of the total microbial communities and this small known fraction can't reflect the composition and variability of the total microbial diversity on Earth.

The disparity between culturable and *in situ* diversity has increased the importance of culture-independent molecular approaches. The possibility of identifying specific populations of microorganisms in their native habitat without the need to isolate them revolutionized microbial ecology and gave rise to various new applications in numerous research fields. Molecular techniques have recently been widely applied to the analysis of communities in anaerobic digesters (Bertin et al., 2004; Rizzi et al., 2006; Koppar and Pullammanappallil, 2008; Palatsi et al., 2010).

This section will briefly outline the most widely molecular tools used for the investigation of microbial ecology of anaerobic digestion processes with a discussion of their limitations and strengths.

4.1 Clone libraries and sequencing

The microbial community composition has been commonly determined by constructing Small Subunit ribosomal RNA (SSU rRNA) clone libraries followed by phylogenetic identification by randomly sequencing the clones or sequencing only representative clones that have been previously clustered using PCR-based screening technologies such as DGGE (Roest et al., 2005) or restriction analysis (Collins et al., 2003). Random sequencing of the complete 16S rRNA genes offers the advantage of better taxonomic resolution, considering that one operational taxonomic unit (OTU) obtained by a fingerprinting method could actually be derived from more than one microbial species. SSU ribosomal DNA clone libraries are made from environmental samples by cloning PCR products from extracted DNA into plasmid vectors. In the anaerobic digestion research area, *Archaea* and *Bacteria* are both important, and both corresponding clone librairies are made separately by choosing domain-specific PCR primer sets, during the initial PCR amplification step. Each clone then contains one rRNA gene that is present

in the environmental sample. This cloning and sequencing approach was first reported by Giovannoni et al. (1990) in an analysis of the diversity of bacterioplankton in Sargasso Sea.
Rarefaction curves are generated by plotting the cumulative number of unique OTUs versus the number of screened clones (Moyer et al., 1994). Such a curve indicates if the community diversity is well represented by the number of clones that have been sequenced, and reveals the approximate total number of different OTUs.
Clone sequence analysis provides information about the phylogenetic identification of members of a microbial community. However, if the primary goal is to monitor changes in the community during a trial, this approach is time-consuming and expensive, compared with real-time PCR assays or fingerprinting techniques.
The cloning/sequencing approach is onerous in terms of money and time but is suitable not only to determine the diversity, but also to get sequence information for the design of signature oligonucleotides that are complementary to interesting target groups, at different the taxonomic level. These newly designed oligonucleotides may be useful in other molecular approach, such as Fluorescence in situ Hybridization (FISH, section 4.7) studies or in the development of real-time PCR assays (section 4.8) for quantification. A recent report (Ariesyady et al., 2007) used the cloning/sequencing approach to design new FISH probes.

4.2 Denaturing gradient gel electrophoresis (DGGE)

The DGGE technology is based on electrophoresis of PCR-amplified fragments of the variable regions of the 16S ribosomal RNA (rRNA) gene, in polyacrylamide gels containing a linearly increasing concentration of denaturant chemical agents (formamide and urea; Muyzer et al., 1993). Amplified DNA fragments may have the same length but with different base-pair sequence, they are characterized by different melting behavior and thus can be resolved by electrophoresis, revealing the diversity of the microbial community.
This methodology has been used widely in environmental microbiology to study diversity, relative abundance and shifts of microbial populations (Muyzer, 1999) in complex systems, including anaerobic bioreactors (Liu et al., 2002b; Roest et al., 2005; Connaughton et al., 2006; Miura et al., 2007).
The taxonomic specificity of the primers used in the PCR amplification process determines which particular groups of bacteria will be analyzed. DGGE bands can be exiced from gel, re-amplified and the PCR-product sequenced. Another advantage is that the DGGE electrophoresis system is less expensive than the automated sequencer required for LH-PCR, T-RFLP and automated RISA (ARISA) methods. However, DNA sequence information from excised gel bands may requires cloning (Kisand and Wikner, 2003; Xing et al, 2008) because of bands co-migration or poor separation of gel bands, especially in the case of complex microbial communities. Furthermore, the size of DGGE bands is usually less than 500bp, so the DNA sequence information obtained from gel bands is limited and phylogenetic identification may be poor in the case of novel sequences having less than 85% identity to known sequences. Other bias are the

gel-to-gel variation and the lesser sensitivity, compared to fingerprints obtained by methods using automated analysis systems, which limit to ensure reproducibility and detection of minor populations and subtle changes.

4.3 Restriction fragment length polymorphism (RFLP) and Terminal-RFLP

RFLP is a simple method based on restriction digestion, with one or more enzymes used either separately or in combination, of the PCR-amplified 16S rRNA total community DNA followed by electrophoretic separation of restriction fragments on high percent agarose or acrylamide gels. Amplification products are either processed as a pool or cloned to achieve separation of individual sequences for further analysis. The restriction digestion of a pool of PCR products and the subsequent separation can result in complex patterns. This approach is to be considered labor- and time-intensive, but it has been successfully used for examining the microbial diversity associated with different natural environment.

Terminal-RFLP (T-RFLP) makes use of the resolution of automated sequencing technology and avoids some of the limitations of RFLP analysis (manual labor, low sensitivity, and low genotypic resolution). Marker genes are PCR-amplified using a fluorescent dye attached to the 5'-end of one of the primers so that the products become labeled (Clement et al, 1997; Osborn et al., 2000). PCR products are subsequently restriction-digested and the mixture of restricted PCR products is physically separated using acrylamide sequencing gels or capillary electrophoresis (CE). In contrast to RFLP, only labeled terminal fragments are detected reducing the complexity of the profiles. The polymorphism is based solely on the fragment length. A size standard labeled with a different fluorophore allows the precise assignment of fragment lengths with single base pair resolution. The main advantages are that the method is relatively simple and that in silico T-RFLP are possible through the implementation of automated fragment length assignment tools, so that the appropriate combination of primers and restriction enzymes are chosen in order to obtain the best resolution at the desired taxonomic level (Padmasiri et al., 2007). Compared with other molecular techniques, e.g. LH-PCR (section 4.5) or RISA (section 4.4), T-RFLP is more time-consuming and more expensive, because the PCR products have to be purified and de-salted before proceeding with the enzymatic restriction digestion step. The reproducibility may also be compromised by the possibility of incomplete restriction digestion. This enzymatic step cannot be controlled because it could happen that some microbial species in the sample have no restriction sites.

Analysis of archeal SSU rRNA gene by T-RFLP has been commonly used to monitor methanogenic population dynamics throughout the bioreactor start-up (Collins et al., 2003), normal operations, and during the development of crisis situations (Scully et al., 2005). Lueders and Friedrich (2003) demonstrated that T-RFLP fingerprints from methanogenic SSU rRNA gene can give a quantitative view of defined template mixtures. They also obtained highly reproducible results from environmental samples. The low diversity of methanogens explains why this is the case. Lueders and Friedrich

(2002) studied the methanogenic populations in rice field soil using the T-RFLP method. By analyzing rDNA and rRNA in parallel, they were able to compare population dynamics and activity shifts.

4.4 Ribosomal intergenic spacer analysis (RISA)

RISA consists in the amplification of the intergenic spacer region (ISR) between 16S and 23S (large subunit) ribosomal genes. This region is variable in both sequence and length due to a lesser selective pressure leading to insertion or deletion events. An advantage over DGGE is that the PCR products are resolved on the basis of size using standard agarose gel electrophoresis, and the primers do not require a GC clamp. RISA profiles can be automated with a DNA sequencer for a higher throughput and a better resolution and named ARISA (Fisher and Triplett, 1999). Two major limitations are: the shorter RISA amplicons are preferentially PCR amplified leading to a quantitative bias and the genome from a single species can contribute to more than one RISA amplicon due to the presence of multiple genomic ribosomal operons. Nevertheless, ARISA has been used to assign specific bacterial and fungal community compositions to different soil types (Ranjard et al., 2001; Hewson and Fuhrman, 2004), and is very sensitive to subtle community shifts. *Archaea* as well as *Bacteria* specific primers can be designed to obtain the diversity for both microbial domains. Castillo-Gonzales and Bruns (2005) used RISA to characterize and to track temporal changes in swine wastewater community in biofilms attached to limestone gravel. Quantitative FISH experiments showed that the concentration of transcribed ISR reflected the activity of the cells more accurately than the 16S and 23S rRNA concentration revealed when using specific probes (Schmid et al. 2001). Therefore, this report suggests that, from the ISR sequences that would be obtained, one might also be able to design primers for quantitative real-time RT-PCR assays that would reflect the metabolic status of key bacteria more appropriately than 16S primers. Future research is needed to determine if transcribed ISR would be a better indicator of metabolic activity than 16S rRNA for biomonitoring of bioreactors.

4.5 Length heterogeneity PCR (LH-PCR)

Length heterogeneity of PCR-amplified SSU rRNA gene (LHPCR) analysis allows distinguishing diverse microorganisms in a community based on natural length variations within SSU rRNA gene variable regions. Different primer sets targeting variable regions of Bacteria may be used in combination to obtain more discriminative concatenated data (Mills et al., 2006). LH-PCR has the advantage of being less laborious and cheaper than T-RFLP because there is no need to digest the PCR product by a restriction enzyme and to desalt before resolving the samples by automated CE. The simplicity of the LH-PCR method explains why more reproducible results are obtained than with the T-RFLP technique (Mills et al., 2003). Bernhard et al. (2005) studied bacterioplankton community shifts by multivariate ordination methods such as correlation coefficients and nonmetric multidimensional scaling (NMDS) and were able

to establish relationships between LH-PCR fragments and water chemistry. Microbial diversity in a thermophilic aerobic biofilm process treating pulp and paper mill wastewater has been monitored by LH-PCR (Tiirola et al., 2003). The study showed that LH-PCR was a valuable method to assess community shifts and recovery after alkaline shocks, even if sequencing studies showed that one LH-PCR amplicon was actually representing a mixture of members from several groups indicating limited phylogenetic resolution. This shortcoming was also noticed for T-RFLP analysis. As stated by Mills et al. (2006), fingerprinting methods have their limitations, but they are useful to monitor community shifts, without knowing exactly the species that are changing. Consequently, LH-PCR is an attractive method due to its simplicity, robustness, and cost-effectiveness compared to T-RFLP.

4.6 Single-strand conformation polymorphism (SSCP)

In SSCP analysis, the electrophoretic mobility of single-stranded DNA in a gel, under non-denaturing conditions, is dependent not only on its length and molecular weight, but also on its three-dimensional state. The folded DNA structure depends on the sequence of the PCR-amplified DNA fragment. An advantage over the DGGE methodology and T-RFLP is that no GC-clamped primer and restriction digestions are required, respectively. However, SSCP analysis has two major shortcomings which are the reannealing of single-stranded DNA during the electrophoretic migration, especially when DNA concentration is high, and the formation of more than one stable conformation resulting in the presence of extra bands (Schwieger and Tebbe, 1998). The lack of consistency between physico-chemical parameters and molecular biology results based on PCR amplification of bacterial genomic DNA could be explained by the assumption that DNA could have been amplified from inactive bacteria at the same extent as metabolically active bacterial groups. SSCP analysis has been used to resolve this issue in an anaerobic digester by comparative analysis of bacterial community rRNA and rDNA (Delbès et al., 2000). Nucleic acid was amplified by PCR or RT-PCR (in the case of rRNA) using fluorescent primers, and the mixtures were resolved by CE. The rRNA analysis has been shown to be useful in investigating the dynamics of activity of the bacterial community facing different perturbations. Bacterial and archeal rRNA and rDNA fingerprints were analyzed (Delbès et al., 2001) in anaerobic digesters seeded with sludges collected from an anaerobic lagoon and industrial-scale anaerobic digesters fed with distillery slops and fed with glucose. Particular bacterial species could be associated with a major but reversible crisis of the process that was reflected by an accumulation of acetate. The rDNA patterns alone could not reveal shifts in relative abundance. Furthermore, the rDNA-based fingerprints showed a higher number of peaks than the rRNA-based patterns, suggesting that some bacterial phylotypes may still be present during a process but are not metabolically active. We observed the same phenomenon in anaerobic digesters communities treating swine manure (Talbot et al., 2004).

4.7 Fluorescence in situ hybridization (FISH)

FISH is based on the microscopic analysis of already defined (at least its SSU rRNA gene sequence) groups of bacteria by a fluorogenic probe targeting SSU rRNA molecules inside cells (Amann et al., 1990). Several studies (Merkel et al., 1999; Araujo et al., 2000; Wu et al., 2001) include FISH results using these same oligonucleotides but the experimental conditions are variable. These probes are still reasonably accurate to target most of the defined phylogenetic groups of methanogenic *Archaea*. However, the availability of new SSU rRNA gene sequence data necessitates a re-evaluation of existing probes with a view of improving their target specificity (Crocetti et al. 2006). These authors published a technical report to evaluate oligonucleotide probes targeting methanogenic *Archaea* at various taxonomic levels and used in FISH. Seven previously published probes (Raskin et al., 1994a) and seven newly designed probes were optimized for use in FISH experiments. Two helper oligonucleotides were designed to target adjacent regions to the MS14114 binding site (probe MS1414 targets *Methanosarcinaceae*) and improved probe access to its binding site. The probes were designed and evaluated by alignment of 3000 sequences from methanogens and other Euryarchaeota using the ARB software package (http://www.arb-home.de). The studied probes targeted *Archaea* at the Order and Family level, and some targeted subgroups. Pure cultures of methanogens and environmental samples from lake sediments and mesophilic anaerobic digesters were used to optimize stringency conditions of FISH experiments, and to determine the specificity. FISH experiments are often performed in combination with one fingerprinting method (most of the time DGGE or T-RFLP) or with a cloning/sequencing approach to quantitatively determine the importance and the spatial distribution of the fingerprint OTUs (or clones) that were found of interest (Chouari et al., 2005b; Collins et al., 2006). In contrast to fingerprinting methods, FISH is limited by the taxonomic specificity of the probes. Nevertheless, FISH is very attractive to verify the level of metabolic activity by analyzing the intensity of fluorescence inside the positive cells.

4.8 Quantitative real-time PCR

Once the genetic identification of key Bacteria and *Archeae* is done, or if candidate bacterial species that are suspected to be of interest in the study are characterized, the design of specific primers is possible for a quantitative monitoring of these microorganisms by real-time PCR technology. In contrast to the conventional end-point detection PCR, quantitative real-time PCR (Q-PCR) technology is based on the detection of fluorescence during amplification of target DNA (Higuchi et al., 1993). The initial amount of target DNA is inversely proportional to the cycle threshold (CT) defined as the moment (or cycle) where the level of fluorescence in the assay is over the baseline fluorescence signal. Q-PCR has better sensitivity and reproducibility than conventional PCR or conventional hybridization techniques and can be easily used in studies requiring a large number of samples. The software included in the Q-PCR system can estimate the initial amount of target DNA. Two main Q-PCR chemistries

have been used in the study of methanogenic bioreactors: the TaqMan and the SYBR Green technologies. The principal advantage of the TaqMan technology is that it is more target-specific than SYBR Green technology which uses only forward and reverse primers. However, the SYBR Green technology is less expensive and the design of primers set is less restrictive than primers and probe set in TaqMan technology. For quantification, a standard curve must be produced from serial 10-fold dilutions of either genomic DNA from a pure culture of target bacteria, PCR-amplified DNA segments, or a plasmid containing target DNA insert. linearized plasmids will give more reproducible results, because eventual plasmid superhelicity can influence amplification efficiency. Once the concentration of standard DNA is determined, the number of SSU rRNA gene copies can be calculated from the average molecular weight of 660 Da for one DNA base pair, the Avogadro's number ($6{,}022 \cdot 10^{23}$ copies/mole), and the number of base pairs of the standard DNA. Q-PCR results are expressed as gene copy number per ml of sample. The amplification efficiency can be calculated from the slope of the standard curve, and should be between 80% and 115%, and the coefficient of determination (R^2) should be more than 0.95 (Zhang and Fang, 2006). A source of variability may come from the fact that DNA extraction and purification efficiencies from samples may be different from time to time.

The reactions that limit the rate and determine the efficiency of methane production in anaerobic bioreactors are typically those at the very end of the process. Methanogenic consortia are hard to obtain by conventional cultivation because of slow growth rate and obligate anaerobiosis. Biological models developed by engineers to effectively control the process operations would be greatly improved by knowledge of the dynamics of each methanogenic genus in absolute numbers. Many studies used quantitative real-time PCR methodology to detect and quantify methanogens at the order and family levels (Yu et al., 2005a, Hori et al., 2006; Yu et al., 2006; Nettmann et al, 2008; Lee et al., 2010). Yu et al. (2005a; Nunoura et al., 2008) designed four TaqMan primers and probe sets that were specific for each of the four orders of methanogens associated with bioreactors: *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*. *Methanosarcinaceae* and *Methanosaetaceae* families have also been quantified with the use of two specific TaqMan primers and probe sets. Two domain-specific primers and probe sets were also designed to quantify total *Archaea* and *Bacteria*. Specificity of the sets was verified in silico from SSU rRNA gene sequences using Probe Match analysis tool from the RDP-II and the PRIMROSE program and estimated experimentally using 28 archeal strains. Results showed satisfactory specificity of these primers and probe sets, and this study represents an important contribution for quantification of methanogens at the order and family taxonomic levels in anaerobic processes or in environmental samples by Q-PCR. The same research team (Yu et al., 2005b) demonstrated that these TaqMan Q-PCR assays were applicable to the determination of the concentrations of aceticlastic methanogens, and correlation with operation condition and performance of various anaerobic digesters was possible. Aceticlastic methanogens were quantified -using *Methanosarcinales*-, *Methanosarcinacea*-, and *Methanosaetacea*-specific primers and probe sets- in samples from various anaerobic processes. Assays with genomic DNA from pure cultures

demonstrated that quantification with the *Methanosarcinales* set was consistent with quantification using the *Methanosaetaceae* or the *Methanosarcinaceae* sets. The results were in accordance with hypothetical growth relationship between the two families competing for acetate utilization. For quantification in continuous processes operated at steady states, hydraulic retention time and acetate concentration are the parameters that are mainly affecting microbial concentrations.

Molecular methods based on SSU rRNA are not suitable to monitor a relevant function (enzymatic activity) occurring in anaerobic bioreactors, especially if this enzyme is found in many distantly related species of organisms. Target genes encoding enzymes that are rate limiting steps for methanogenesis are ideal candidates for identifying metabolic bottlenecks that are sensitive to process changes. Reverse transcription of messenger RNA (mRNA) followed by Q-PCR would quantify the level of transcripts encoding a functional gene. Although the level of mRNA may not always be tightly coupled with potential enzyme activity because of possible post-transcriptional regulation, levels of message are likely to be generally informative of gene expression under specific operational conditions. The following are some functional genes that could be monitored using Q-PCR assays to determine their level of expression in operational anaerobic bioreactors. In anaerobic digestion processes it is interesting to monitor sulfate-reducing bacteria (SRB) due to the fact that methanogenesis predominates in the absence of significant concentrations of sulfate. However in the presence of sulfate, SRB can compete with methanogens for available electron donors such as acetate and hydrogen, and have the potential to inhibit the methanogenic decomposition of waste organic matter, resulting in increased production of H_2S (Gurijala and Suflita, 1993). The *dsr*AB gene coding for α and β subunits of Dissimilatory Sulfite Reductase, an enzyme catalyzing the central energy-conserving step of sulfate respiration by reduction of sulfite to sulfide, is expressed in SRB. Leaphart and Lovell (2001) developed degenerated PCR primers to study the formyltetrahydrofolate synthetase (FTHFS) gene sequence diversity from bacterial DNA extracted from roots of smooth cord grass and from fresh horse manure. FTHFS, an enzyme which catalyzes ATP-dependent activation of formate in the acetyl-CoA pathway, is expressed in homoacetogenic bacteria. They demonstrated that sequence analysis of FTHFS gene sequences is more informative than SSU rRNA gene concerning physiological distinctions among FTHFS-producing organisms. Wawer and Muyzer (1995) reported the use of genes *hyd*, encoding for [Fe]-hydrogenases, which can be used as specific biomarkers of hydrogen producing bacteria (HPB). They used this catabolic genes to assess the genetic diversity of *Desulfovibrio* spp. in environmental samples. Luo et al. (2002) demonstrated that the *mcr* gene which encodes methyl coenzyme M reductase I (a key enzyme in methane production unique to methanogens) was expressed by the thermophilic hydrogen- and formate-utilizing methanogen *Methanothermobacter thermoautotrophicus* strain TM when it was cultured with the syntrophic acetate-oxidizing bacterium *Thermoacetogenium phaeum* type strain, This paper strongly suggests that the mcr gene is expressed in natural methanogenic ecosystems (because hydrogenotrophic methanogens are strictly dependent for growth on syntrophs) and thus may be a gene of interest to study the

metabolic activity of methanogens working in syntrophy with fatty acid oxidizing syntrophs. The results from Q-PCR *mcr*A transcripts assays (Shigematsu et al., 2004) were complementary to the results from Q-PCR SSU rRNA gene assays (Shigematsu et al., 2003), and demonstrated that this gene can be used as a functional marker of methanogens.

5. Statistical methods applied to molecular data

Community-level molecular techniques, as previously shown in section 3, have been widely used in studies to assess the diversity of microbial communities in different environments and to understand their response to changing environmental conditions. The amount of data obtained from these techniques is increasing and the lack of a standardized way to interpret the raw fingerprints makes it difficult to compare between different results. In fact, until recently, the results of these high quality molecular patterns were restricted only to a visual interpretation, neglecting the analytical potential in terms of statistical and ecological significance. Thus, future challenges in the investigation of microbial community of AD processes, as well as other processes or natural environments, will consist of interpreting the observed diversity patterns as a function of contextual operational/environmental parameters.
Most obstacles encountered trying to summarize and further explore large data sets, derived from molecular methods, concern the choice of the adequate mathematical tools to evaluate the data statistically and graphically.

5.1 Multivariate Analysis

Multivariate analysis, as the name indicates, comprises a set of techniques dedicated to the investigation of data sets with more than one variable. This kind of techniques clearly differentiate and are considerably more complex than the corresponding univariate analysis, used when there is only one response variable under consideration. The variables contained in a complex data set may be correlated with each other, and their statistical dependence have to be taken into account when analyzing such data. Thus, multivariate statistical tools, which have been developed by community ecology to investigate the distribution and diversity patterns of plants and animals, could be readily applied in microbial ecology.
Although multivariate analyses of community diversity patterns are well described in the literature, these statistical methods have been used rarely. Thus, the aim of this section is to present the most common multivariate techniques which can be used and integrated into AD microbial community studies, helping to elucidate the correlation between structure, dynamic and functionality of a community with operational parameters of the process.
The typical multivariate data set consist of a matrix of objects (e.g. samples, sites, time periods) in rows and measured variables for those objects in columns. It is of crucial importance to understand what corresponds to objects and variables in the data set. In

general objects are defined a priori by the sampling strategy before making observations and variable measurements. In a typical study focused on the microbial community analysis of an AD process, where samples taken at different time or from reactors treating different substrates are compared on the basis of community fingerprinting techniques, those samples will be the objects while species information and operational/environmental measurements will be considered as variables.

5.1.1 Cluster Analysis

Cluster analysis is a versatile multivariate tool used to group objects into categories based on their dissimilarities. This statistical method allow to minimize within-group variation and maximize between-group variation in order to reveal well-defined categories of objects. The most used clustering methods for inferring evolutionary relatedness are the neighbor-joining (NJ) and the unweighted pair group method using arithmetic averages (UPGMA), classified as distance-based methods, and maximum parsimony (MP) and maximum likelihood (ML), classified as character-based methods. Cluster analysis is very popular in microbial ecology: the grouping of organisms based on their phenotypic or genotypic similarities in order to infer their taxonomic positioning is generally based on this kind of statistical analysis. A common application consists of sorting out clones from environmental samples based on 16S rRNA gene as genotypic marker, because clones or variants are expected to form tight clusters around their parental strains and to be more distinct from other lineages. This ordination method has been widely used in microbial community analysis of AD processes (Cardinali-Rezende et al., 2011; Nayak et al., 2009; Sousa et al., 2007). In a study aimed to elucidate the response of AD microbial community in psychrophilic conditions and perturbed by different temporary shocks, Madden and colleagues (2010) used cluster analysis together with non-metric multidimensional scaling (section 4.1.3) for the interpretation of molecular DGGE fingerprints. They found that microbial succession was independent of the applied shocks, indicating the robustness of the reactor biomass relevant for successful wastewater treatment under the low-temperature conditions. Sasaki et al.(2011) analyzed, by using stable isotopes, the microbial composition and the pathway of acetate degradation in a lab-scale thermophilic continuous-flow stirred-tank reactor operated using artificial garbage slurry. Their results suggest a strong contribution of non-aceticlastic oxidative pathway to acetate degradation, recognized as a rate-limiting step in methanogenic bioreactors. In particular they found an uncultured species, OTU-B1, which showed low sequence similarity (<90%) with the reference sequences in databases, implying that this bacterium may be a novel species of the acetate oxidizer.

5.1.2 Principal Component Analysis (PCA)

This is the oldest and most versatile multivariate method and has been applied to numerous data sets obtained from genotypic fingerprints. PCA calculates synthetic variables (principal components), which are linear combinations of the original

variables of the data set and that account for as much of the variance of the original data as possible. The aim is to represent objects and variables in a new system of coordinates (generally on two axes or dimensions) where the maximum amount of variation from the original data set can be depicted. In recent years PCA approach has been used with increasing frequency in researches aimed at the investigation of AD microbial community.

Ye et al. (2007) monitored the effect of different medium pH on the bacterial community during fermentation step of an AD process fed with vegetable wastes. DGGE was chosen to compare the community structure and the relationship between fermentation product distribution and bacterial community were investigated. The PCA results clearly demonstrated that the microbiota at pH 7 and 8 were more similar to each other and it was also true for pH 4, 5 and 6.Thus, the change of pH effectively influenced the bacterial community structures. Tale and colleagues (2011) used PCA to correlate the specific methanogenic activity (SMA) against propionate of biomasses from various full-scale anaerobic reactors with the DGGE banding patterns for the mcrA gene generated for the same biomass samples. They observed that the biomasses giving the higher SMA values clustered together (derived from reactors treating brewery wastes) and were correlated with species which shared 88-89% and 93-98% sequence similarity to *Methanospirillum hungatei* and *Methanobacterium beijingense* respectively, suggesting these microorganisms to have an important metabolic function leading to higher SMA values.

5.1.3 Non-Metric Multidimensional Scaling (NMDS)

Non-metric multidimensional scaling is a method which reduces complex molecular patterns to a point in a two-dimensional space. By connecting the consecutive points, the relative changes in the bacterial community can be visualized. NMDS is generally efficient at identifying underlying gradients and at representing relationships based on various types of distance measures. The NMDS algorithm ranks distances between objects, and uses these ranks to map the objects nonlinearly onto a simplified, two-dimensional ordination space so as to preserve their ranked differences, and not the original distances. Lee et al. (2010) analyzed quantitative and qualitative methanogen community shifts of a batch AD process treating cheese-processing wastewater. Methanogenic community shifts were statistically correlated with process data using NMDS. The results obtained on the qualitative ordination plot suggested that the shifts were affected by the accumulation and degradation of acidogenic intermediates. On the quantitative ordination plot significant transitions were observed associated with the increase of Methanosarcinaceae and the resulting shift in community structure and dominance. In a previous study the same research team (Lee et al., 2009) was able to correlate the quantitative shifts of the methanogenic community, of a digester fed with synthetic glucose medium, visualized by NMDS plot to the sudden rises or drops of the Methanosarcinaceae populations, the dominant one in the digester. Moreover they compared the microbial community and the performance of this process with two others digesters fed with whey permeate and sewage sludge. Through NMDS analysis it was

clearly observed how the community in three digesters tested produced had different quantitative evolution during the operations. Given that the operating conditions for all trials were identical, the difference in substrate characteristics was likely to be the main factor affecting the direction of community shifts.

5.2 Environmental Interpretation

All the techniques described above can be classified as Exploratory Analysis. This kind of statistical tools are generally aimed at revealing the existence of groups of objects in a data set. When a supplementary matrix of environmental variables is available for those same objects, it is possible to examine whether the observed patterns are related to environmental gradients. In this way it can be possible to reveal the existence of a relationship between community structure and habitat heterogeneity or to identify the main variables affecting bacterial communities when a large set of environmental variables has been conjointly collected.

5.2.1 Redundancy Analysis (RDA)

This is a method in which multiple linear regressions are used to ‘explain’ variation between independent variables (the matrix containing the species data) and dependent variables (the matrix containing environmental data). These operations are performed within the iterative procedure to find the best ordination of the objects. The objective is to represent not only the main patterns of species variation as much as they can be explained by the measured environmental variables but also to display correlation coefficients between each species and each environmental variable in the data set.
RDA was used by Kim et al. (2010) to elucidate the quantitative changes in methanogenic community structure in two anaerobic digestion systems fed with swine wastewater and operated under physic-chemically similar conditions. The results suggest correlations between *Methanobacteriales* and *Methanosarcinales* populations with chemical parameters such as VFAs in both digesters, whereas the correlation between *Methanomicrobiales* and propionate was different: in fact it was stronger in the digester that showed the higher amount of methane produced. Thus the metabolism of propionate by *Methanomicrobiales*, after the depletion of acetate mainly by *Methanosarcinales*, was a crucial factor controlling production of methane.

5.2.2 Canonical Correspondence Analysis (CCA)

The technique is very similar to that of RDA, except that CCA is based on unimodal species-environment relationships whereas RDA is based on linear models. The main goal is to model species response to the environmental variation to enable the estimation of a large number of parameters and the identification of a small number of ordination axes. CCA is particularly adapted for the environmental interpretation of tables of abundance and occurrence of species, and accommodates well the absence of species at certain sites in the data set. Typical questions that are addressed concern the

identification of environmental factors that influence the diversity of bacterial community among large sets of candidate environmental parameters measured for the same samples, when the diversity is determined by genetic fingerprinting techniques. Another interesting feature of the technique is the possibility of determining the specific species that respond to particular environmental variables. In an anaerobic digester fed with mixed wastes, Supaphol and colleagues (2011) investigated the relationship between environmental variables, microbial community structure and putative ecophysiology of the microorganisms, plotting the taxa scores for individual bacteria and *Archaea* (obtained from the sequences of DGGE bands): this enabled the key components of the microbial communities responsible for driving AD process to be identified using multivariate approaches.

5.3 Microbial Resource Management (MRM)

In recent years Marzorati et al. (2008) proposed a theoretical approach allowing the interpretation of molecular fingerprints, through the use of a three parameters tool-set. Born as technique-dependent approach, based on the interpretation only of DGGE gel patterns, MRM has been proposed to be used on other widely used high-throughput molecular techniques (Marzorati et al, 2008; Read et al., 2011).
The first parameter is range-weighted Richness (Rr) and it is used to establish a specific range of values which indicate the richness and genetic diversity of species within a microbial community. In the beginning, Rr was expressed as the total number of bands multiplied by the percentage of denaturing gradient needed to describe the diversity of the sample being analyzed. Dynamics (Dy), the second parameter of the tool-set, was used to determine the rate of change within the same community over a fixed time interval. It refers to the number of species, on average, that are detected to be of significance in a given environment at a certain time point, thus providing a big picture of the dynamics within a community. Recently Dy has been used as a standalone parameter looking at the changing community during bioaugmentation of activated sludge (Bathe et al., 2009). The third parameter is Functional organization (Fo). The Fo of a community should be the result of the action of the microorganisms that are most fitting to the ongoing environmental-microbiological interactions. Thus, this parameter should give an idea of which microorganism tend to become dominant within the structure of the microbial community. However, fingerprinting techniques make use of the differences on the 16S rRNA gene to discriminate among different bacterial species. Thus, it is not always possible to correlate a given functionality with the respective group of microorganisms at the 16S rRNA gene level. As a consequence, Fo fails the original purpose of correlating the distribution of bacteria and their respective role in the overall functionality. In this respect, Read et al. (2011) proposed to rename Fo as Community organization (Co), a parameter that describes the microbial community in terms of degrees of evenness: it describes the species abundance distribution in the microbial community, has a percent value (0=even community, 100=uneven community) and is calculated as the Gini coefficient times 100.

The use of these tools in combination, can help providing with an ecological interpretation of the raw data describing the structure of the community. This has been demonstrated in the last few years on studies carried out in various environments including anaerobic digestion. In a study investigating the correlation of the microbial community structure with reactor functionality in continuous lab-scale anaerobic reactors using MRM concepts, Carballa et al. (2011) found a correlation between Co, Rr and Ripley Index: reactors with low Ripley values (indicative of good performance) typically had also low Co response and high Rr values, suggesting that an even and rich association of Bacteria corresponded with good conversion of fatty acids and thus with a well-functioning process.

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Aim of the Work

Anaerobic digestion is an engineering process which allow the production of energy in the form of biogas. This process is carried out by a complex consortium of microorganisms, which degrade anaerobically the complex initial substrates to obtain as only final product biogas, mainly composed of methane. Anaerobic digestion was discovered during the seventies, but only in the last 20 years had received renewed interest because of the urgent need to find alternatives to the exploitation of non-renewable energy sources. In fact our society is strictly dependent on the continuous availability of energy and the main sources from which this energy is produced are nowadays the fossil fuels reserves. The International Energy Agency (IEA), has estimated that these non-renewable resources accounts for close to 80% of global energy production. It is also important to consider that with the emergence of new fast growing economic powers, the consumption of energy is constantly growing year by year and this is leading to the fast depletion of the non-renewable fossil fuels reserves. Moreover the continuous combustion of oil and natural gas is leading to big environmental issues: greenhouse gases released in the atmosphere are considered to be the main responsible for global warming and the associated impacts of climate change.
In this scenario anaerobic digestion has been pointed as an environmentally-friendly, economic valuable alternative. One of the most interesting possibilities, which makes it so charming, is to couple the production of energy to the treatment of waste materials and wastewater derived from a wide range of industrial and agricultural productive processes.
In recent years numerous studies were focused on anaerobic digestion. In the most of the cases the research has been aimed mainly on the evaluation of the potential yield of a variety of biomasses and on the identification of the optimal operational conditions of the process. However, in spite of this increasing attention on this technology, there still exists relatively few information about the activity, the performance and the interactions of the microbial community of *Archaea* and *Bacteria* involved. In this context, there is the need to gain a more deep knowledge of the microbiology of the process in order to improve the stability, to prevent imbalances and carry corrective measures.
Research on the microbial communities both in natural and engineering systems, in the past, was established only on the limited support of conventional microbiological techniques. These methodologies, based on isolation of pure cultures, fail to describe and deeply investigate the complexity of a microbial ecosystem. In fact has been reported that the conventional microbiological tools enables scientists to detect only ca.

1% of the total microbial communities on Earth. New recently developed high-throughput culture-independent molecular techniques gave a strong new impulse on the investigation of microbial communities in different environment, including anaerobic digestion processes.

Thus the general aim of this PhD thesis was to investigate the microbiology of both batch and continuous, single and two-stage anaerobic systems. The goals were (i) to elucidate the structure of the microbial communities, (ii) to investigate the dynamics, interactions and responses of the key metabolic groups responsible for the degradation of substrates and (iii) to give valuable information on the correlation between structure and function inside the microbial consortiums. To achieve these objectives high-throughput molecular techniques were used. Denaturing gradient gel electrophoresis (DGGE) and Real-Time PCR gave, respectively, qualitative and quantitative valuable information about the structure of the microbial communities, the variation during the process of the proportion and dominance of the different metabolic groups and key microbial species inside the reactors. Ultimately, to further explore the large data sets obtained from these molecular methods, the use of statistical multivariate analysis was evaluated.

Chapter 2

Shifts of microbial community structure during anaerobic digestion of agro-industrial energetic crops and food industry byproducts[1]

1. Introduction

Treatment of organic waste by anaerobic digestion (AD) is an environmental friendly technology for addressing waste disposal with the benefit of energy recovery in the form of biogas (methane or hydrogen) (Chynoweth et al., 2001, Angenent, 2004). Relevant advantages of AD are the reduced sludge release, organic matter stabilization, odor control and pathogen abatement (Van Starkenburg, 1997). Nowadays, AD represents a consolidated technology in many EU countries and the number of biogas plants is estimated to increase, especially for small-scale plants (up to 1 MW).

Biogas plants traditionally treat wastes/biomasses, such as manure, agro-industrial wastes, industrial residues and urban wastewater. For instance, in Italy, biogas plants have usually been developed in association with agro-zootechnical farms, especially in the flat area of Pianura Padana, intensively exploited for livestock farming. During the last years, however, especially in some countries like Germany and Austria, the use of energy crops and crop residues as substrate constantly increased (Weiland, 2010). For the full scale implementation of modern anaerobic digestion processes, involving design, economic and managing issues, a key parameter is the biochemical methane potential (BMP) of the treated biomasses (Angelidaki et al., 2009). In the last 30 years a wide range of research dealt with BMP analysis of different substrates. However, limited information has been recovered on microbial communities of these AD processes including those treating energetic crops.

The AD process involves a consortium of different functional groups of microorganisms establishing a complex system of interactions that drive the overall process performance. In particular, methanogenic *Archaea* deserved particular attention (Ueno et al., 2001, Lee et al., 2010) since they catalyse a final rate-limiting step of the whole AD process. Recent research has been focused on the precise characterization of the involved consortia (Riviere et al., 2009) with the final objective to link microbial community structure to function (Werner et al., 2011). To this aim, molecular techniques, like DGGE, and their molecular analysis parameters are valuable tools for microbial ecology interpretation and management (Marzorati et al., 2008).

The aim of the present study was to experimentally determine the BMP of different

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energetic crops and agro-industrial biomasses and, in order to get further insight into the processes, to characterize the microbial communities present both before and after the AD process. Anaerobic BMP was determined by using an automated laboratory-scale multi-batch system. Microbial diversity and dynamics were analysed by Denaturing Gradient Gel Electrophoresis (DGGE) and quantification of bacterial and archaeal communities was performed by quantitative real-time PCR (qPCR).

2. Experimental

2.1 Bio-methane potential (BMP) determination

BMP experiments were carried out on a 36 batch-digesters system owned by Ente Regionale per i Servizi all'Agricoltura e alle Foreste della Regione Lombardia (ERSAF, Mantova, Italy). The mini-digesters (3 liter working volume) lodged in temperature-controlled chambers and were connected to 2 liters gas meter-bags. The automated, software-controlled analysis apparatus consisted of a gas drying system, a drum-type gas meter (Ritter Apparatebau Gmbh, mod. TGO5) for quantitative gas measurement and a gas-quality analyzer for the determination of the percentage of CO_2, CH_4, H_2S and O_2. Trials were carried out in three consecutive runs under mesophilic conditions (40°C) for 35 days each. Before trials performing, each substrate was analysed for total solids (TS) and volatile solids (VS) (Table 1) according to standard methods (APHA, 1998). Mixtures of biomass and inoculum were prepared with 1:2 ratio, based on TS content. The inoculum consisted of an anaerobic seed sludge collected at three different times from a local biogas plant treating swine slurry. Each biomass, including blank trial (inoculum only), was tested in triplicate.

Biomasses	TS (%)	VS (% TS)	N-tot (% TS)	pH
Maize silage	37.0	95.6	5.6	3.7
Triticale silage	41.0	90.5	1.6	3.9
Sorghum silage	25.0	92.3	2.9	3.8
Green rice grains	86.8	98.2	13.6	6.1
Scrapped rice	86.3	90.0	13.0	6.9
Rice flour	89.9	97.5	18.4	6.7
Dry maize wastes	88.3	95.7	3.3	5.0
Broken soybeans	89.7	94.2	78.0	6.6
Grass mowing	57.5	90.2	4.0	6.8
Whey	6.6	87.0	0.6	6.2
Triple-tomato waste	27.2	95.8	3.9	4.1
Scrapped pasta	89.2	99.1	18.4	5.0
Poultry manure	35.8	15.6	16	7.3

Table 1. Characteristics of the biomasses.

2.2 DNA extraction

One hundred ml from triplicate batch experiments of the same biomass were put together and homogenised with a blender; where required a water dilution (1:1) was done. Aliquots of variable volumes (2-3 ml), to obtain final pellets of 150 mg, were centrifuged (10000×*g*, 30 min, 4°C); the resulting pellets were washed twice with water and centrifuged in the same conditions. DNA extraction (in triplicate) was performed by using the PowerSoil DNA Isolation kit (MoBio Laboratories, Inc., Milan, Italy) according to the manufacturer's instructions. The purified DNA was eluted with 100 µl of elution buffer (10 mM Tris-HCl, pH 8.0) and stored frozen at -20°C until use. Quality of DNA extracted was evaluated by agarose gel electrophoresis.

2.3 PCR-DGGE analyses

The primer sets targeting the 16S rRNA gene of *Bacteria* and *Archaea* were GC-357-F/907-R and GC-ARC787-F/ARC1059-R, respectively (Sass et al., 2001; Hwang et al., 2008). PCR reactions and preparation of polyacrylamide gels (40-60% or 30-70% denaturant gradient) were performed as described previously (Sass et al., 2001; Hwang et al., 2008). Bands excised and eluted from the gels were re-amplified using DGGE primer set without GC clamp and sequenced (Macrogen, Seoul, Korea). The sequences were compared with sequences deposited in the National Center for Biotechnology Information (NCBI) database by using BLAST program. The sequences were phylogenetically classified using the RDP Naive Bayesian rRNA Classifier Version 2.2, March 2010 (http://rdp.cme.msu.edu/classifier/hierarchy.jsp).

2.4 Real-time quantitative PCR (qPCR)

Four qPCR assays targeting *Bacteria*, *Archaea*, *Methanosarcinales* and sulfate-reducing bacteria were used. The primer sets were: Bac357-F/Bac907-R for *Bacteria* (Favia et al., 2007), Arch 931-F/ArchM1100-R for *Archaea* (Einen et al., 2008), Msl812-F/Msl1159-R for *Methanosarcinales* (Yu et al., 2005) and Drs1+-F/Dsr-R for sulfate-reducing (Kondo et al., 2004).All primer pairs used as target gene the 16S rRNA gene, except for sulfate-reducing primer set targeting the *dsr*A gene, codifying for the α subunit of dissimilatory sulfite reductase. PCR SYBR Green reactions were prepared by using the "Brilliant SYBR Green QPCR Master Mix" kit (M-Medical, Stratagene, Milan, Italy). The reaction mix (25 µl) contained: 1 × Brilliant SYBR Green (2.5 mM $MgCl_2$), 0.12 µM of each primer, and 5 µl of template DNA. For *Methanosarcinales*, extra $MgCl_2$ was added to a final concentration of 4.0 mM. The thermal cycling program consisted of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at X°C (X = 58°C for Bac357-F/Bac907-R, 59°C for Drs1+-F/Dsr-R, 64°C for Arch 931-F/ArchM1100-R, 60°C for Msl812-F/Msl1159-R) and 1 min at 72°C. Finally, a melting curve analysis was performed: denaturation of 1 min at 95°C, cooling of 1 min at 55°C and then 95°C again, at a rate of +0.5°C/cycle. Cycle threshold values were calculated using the Biorad real-time software (version 3.0a). Standard curves ranging

from 10 to 10^8 copies of plasmid DNA were used. The standard plasmids were made by cloning into pCRII-TOPO vector (Invitrogen) the specific fragments obtained by PCR amplification of the target genes (see above) using as template DNA isolated from a batch digester sample. The cloned gene fragments were: *Methanobrevibacter sp.* (DQ402034.1) for *Archaea*, *Methanosarcina mazeii LM5* (DQ987528.1) for *Methanosarcinales, Desulfobacterium autotrophicum* (CP001087.1) for sulfate-reducing. *Asaia* sp. (AM404260) was used as target gene in the case of *Bacteria* as previously described (Favia et al., 2007). DNA plasmids were extracted by *E. coli* using the QIAprep Spin Miniprep kit (Qiagen, Milan, Italy) and DNA concentration was evaluated by UV_{260} absorption. Conversion of 16S rRNA gene copy numbers to cell number was done considering the average 16S rRNA gene copy numbers of bacteria (4 copies/cell) and methanogens (2.5 copies/cell) reported in the Ribosomal RNA Database (rrnDB, Lee ZM-P (http://rrndb.mmg.msu.edu/search.php)). In the case of real-time PCR targeting the *dsr*A gene, it was assumed that copy number is equivalent to cell number (Kondo et al, 2004).

2.5 Statistical analyses

Each DGGE gel image was converted into a binary matrix by using the free software ImageJ (Rasband W(http://rbs.info.nih.gov/ij/)). Each binary matrix of *Archaea* and *Bacteria* line profiles was statistically analysed by Principal Component Analysis (PCA) using XLSTAT (vers. 7.5.2 Addinsoft, France) on autoscaled data. DGGE gel images were also analyzed using the Quantity One software (Biorad). Lane background was substracted by the “rolling disk” tool; bands were detected automatically and matched manually. DGGE-based molecular parameters, namely dynamycs (Dy), richness (Rr) and community organization (Co), were calculated as previously described (Mertens et al., 2005, Marzorati et al., 2008). Briefly, Dy was calculated from the similarity matrix (100-% similarity); Rr was the total number of bands multiplied by the percentage of denaturing gradient used; Co was the percentage of Gini coefficient, a value describing the degree of evenness within a community by measuring the normalized area between the Lorenz curve and the perfect evenness line. Statistical significance of molecular parameters and BMP was assessed by test F (ratio regression variance to error variance). Statistical analysis of the qPCR results was performed by ANOVA (analysis of covariance). Statistical significance was considered for p values less than 0.05.

3. Results and Discussion

3.1 BMP from different biomasses

The biogas and methane yields from the tested biomasses are shown in Figure 1. The concentration of methane to the total biogas production varied among the biomasses, ranging from 42% to 68% (results not shown). All the energy crops gave efficient

methane productions, in particular maize silage yielded the highest methane rate (394 ± 20 Nm^3 $t^{-1}VS_{added}$), whereas sorghum and triticale silages showed slightly lower methane yields Methane yields were in the range of methane productions typically found in literature, ranging from about 280 to 420 Nm^3 $t^{-1}VS_{added}$ (Gunaseelan, 1997; Bruni et al, 2010; Herrmann et al., 2011). Methane productions as efficient as silage crops were obtained from some agro-industrial wastes and residues, such as those derived from rice (green rice grains and broken rice), soy (broken soya beans) and maize (dry maize wastes) which produced methane yields higher than 300 Nm^3 $t^{-1}VS_{added}$. These results are not surprising considering the high contents of starch, proteins and fat in these biomasses. Lower methane yields were obtained in the case of rice flour, grass mowing, scrapped pasta and whey with productions rate recorded to average values of 150 Nm^3 $t^{-1}SV_{added}$. High BMP was observed in the case of triple-tomato paste residues (380 ± 9 Nm^3 $t^{-1}VS$) and poultry manure (385 ± 2 Nm^3 $t^{-1}VS_{added}$) in accordance with previous findings and indicating that wastes or agro-industrial by-products that have no market are efficiently suitable and for AD process (Dinuccio et al., 2010; Schievano et al., 2009).

Figure 1. Potential production of methane for all the substrates tested. MS, maize silage; TS, triticale silage; SS, sorghum silage; GRG, green rice grains; SR, scrapped rice; RF, rice flour; DM, dry maize wastes; BS, broken soybeans; GM, grass mowing; W, whey; TTP, triple-tomato paste residues, SP, scrapped pasta; PM, poultry manure. All values are the mean value of triplicate batches with their corresponding standard error and are expressed in Nm^3 $t^{-1}SV$.

3.2 DGGE and phylogenetic analyses of *Archaea* and *Bacteria*

Microbial diversity of archaeal and bacterial communities before and at the end of the AD processes were assessed by DGGE analysis of 16S rRNA gene. The gel images of *Archaea* and *Bacteria* are shown in Fig. 2A and 2A' and Fig. 2B and 2B', and the phylogenetic affiliation of the bacterial and archaeal sequences are reported in Table 2 and Table 3, respectively. A high similarity of the DGGE band patterns among samples

tested with the same inoculum was observed for both *Archaea* and *Bacteria*, indicating the importance of inoculum in the developing of the AD processes.

In the DGGE gel of Fig. 2A, the bands 2, 3 and 4, phylogenetically affiliated to the genus *Methanosarcina*s, were dominant in all samples, both at the beginning and at the end of the processes. Changes occurred and some bands appeared or became more

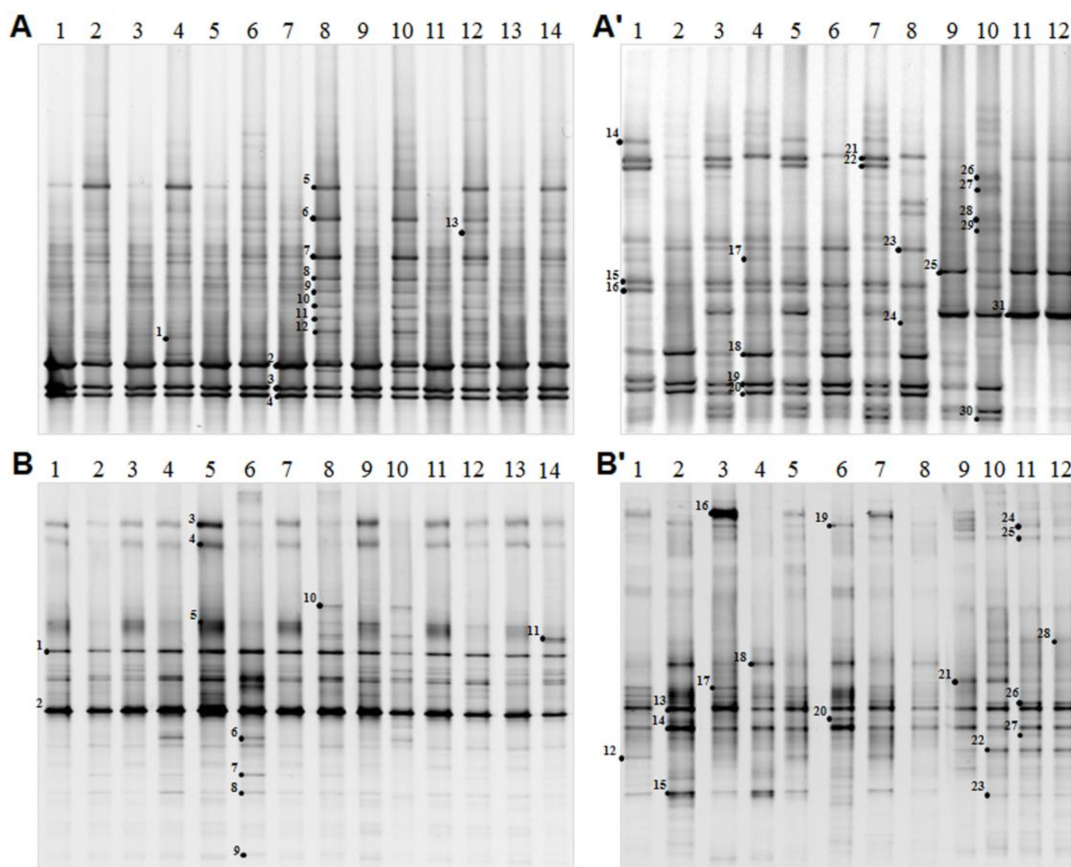

Figure 2. Archaeal (A, A') and bacterial DGGE (B, B') profiles of the 16S rRNA gene PCR products generated from DNA extracted from ingestate (*i*) and digestate (*d*) of substrates analyzed for BMP. A, B) 1-2, green rice grains *i, d*; 3-4, rice flour *i, d*; 5-6, scrapped pasta *i, d*; 7-8, grass mowing *i, d*; 9-10, broken soybeans *i, d*; 11-12, dry maize wastes *i, d*; 13-14, scrapped rice *i, d*. All lanes refers to BMP experiments performed with seed sludge 1. A', B') 1-2, triticale silage *i, d*; 3-4, poultry manure *i, d*; 5-6, sorghum silage *i, d*; 7-8, maize silage *i, d*; 9-10, triple-tomato paste residues *i, d*; 11-12, whey *i, d*. Lanes 1-8 and lanes 9-12 refers to BMP experiments performed with seed sludge 2 and 3, respectively. Dots and numbers indicate the bands sequenced.

intense at the end of the process suggesting that the related methanogens played an important role in methane production

This was the case of band 5, identified by RDP Classifier (confidence threshold of 80%) as *Methanosarcina* and bands 6-12 matching with strain *Methanosarcina* sp. HB-1. The bands showed high intensity in the digestates of grass mowing, broken soybeans e pasta wastes. Band 1 was phylogenetically related to *Methanosarcina barkeri*, whereas band 13 was affiliated to the genus *Methanobrevibacter* by RDP. In DGGE gel of Fig. 2A', two main DGGE band patterns could be recognized, in accordance with the use of two different inoculum sludge. The digestates of AD experiments performed with seed sludge 2 were characterized by the dominance of

three intense bands (18-20), affiliated to *Methanosarcina* genus. Bands 15, 16, 17 and 23 were affiliated to the hydrogenotrophic genus *Methanobrevibacter*. In particular, band 23 resulted in a more intense signal at the end of the process in sorghum and maize silages.

On the contrary, bands 14, 21 and 22, phylogenetically related to uncultured *Archaea* of the family *Methanomicrobiaceae* were observed in the energy crop biomasses and poultry manure only before the onset of the process and disappeared thereafter. The archaeal community of whey and triple-tomato paste (seed sludge 3) showed the prevalence of bands 25 and 31, phylogenetically related to the genus *Methanosaeta*. Whereas the archaeal community remained stable in whey, in the case of triple-tomato paste, a significant band pattern change was observed after the AD process, with a shift from *Methanosaeta* (bands 25 and 31) to *Methanosarcina* (bands 26-30).

The overall data showed a prevalence of the genus *Methanosarcina,* indicating that acetoclastic methanogenesis was the principal pathway of methane production. *Methanosarcina* spp. produce methane from acetate, although some species are more versatile (for example *M. barkeri*) and can also utilize H_2 and CO_2, methylated amines and methanol (Whitman et al., 2006). *Methanosarcina* outcompetes with *Methanosaeta* at high acetate concentrations, in agreement with the higher affinity for acetate of *Methanosarcina* compared to *Methanosaeta* (Jetten et al., 1992).

Almost all the bacterial sequences obtained were affiliated to *Firmicutes* and *Bacteroidetes,* phyla of remarkable importance in the degradation of complex organic matter in anaerobic bioreactors (Riviere et al., 2009, Li et al, 2009 environ microb, Tang et al, 2004). Sequences assigned to the *Clostridia* class (bands 1, 2, 5 11 14, 22, 27) as determined by the RDP Classifier (confidence threshold of 80%), or more specifically to the *Clostridium* genus (bands 13 and 17), whose bacterial members include saccharolytic, but also proteolytic and lipolytic species (Wiegel et al. 2006 cap. Prokaryotes) were frequent in the DGGE profiles. In particular, bands 1, 2, 13 and 14 were dominant with high intensity bands in all samples before and after the process. Assigned to *Firmicutes* were also bands 6, 7, 23, 8, 15, 9, 20. Changes in the DGGE profiles were also observed in the case of *Bacteria*. Particularly, some bands appeared or became more intense at the end of the AD process suggesting that the related microorganisms were actively involved in the process. Band 6, which appeared at the end of the process in rice flour and scrapped pasta, was affiliated to *Syntrophomonas* genus (100%). This syntrophic acetogenic bacteria have an important role in fatty-acid degradation and work in a synergetic way together with hydrogen-scavenging microbes such as hydrogenotrophic methanogens (Zhao et al, 1993, Wu et al, 2006). Bands 7 and 23, strictly related to the genus *Desulfotomaculum* (>99%), were observed in several biomasses with high intensity at the end of the process in scrapped pasta and rice flour. This genus includes a number of gram-positive, spore-formers, sulfate-reducing bacteria, capable of incompletely (to acetate) or completely oxidizing various organic substrates like, H_2, alcohols and short chain fatty acids, by using sulfate as final electron acceptor (Widdel, 2006). Band 9 was phylogenetically closely related to an uncultured *Symbiobacterium* (>98% similarity). To this genus belongs the gram-positive species *S. thermophilum*, previously identified in an anaerobic process

Band	Closest relative (NCBI)	Accession n^{o}.	Identity (%)	Classification*
1	*Methanosarcina barkeri* strain TR-Z13	HQ591417	97.0	*Methanosarcinaceae*
2	*Methanosarcina siciliae* type strain DSM 3028^{T}	FR733698	100	*Methanosarcinaceae*
	Methanosarcina vacuolata type strain DSM1232^{T}	FR733661	100	
3	*Methanosarcina siciliae* type strain DSM 3028^{T}	FR733698	99.6	*Methanosarcinaceae*
	Methanosarcina vacuolata type strain DSM1232^{T}	FR733661	99.6	
4	Uncul. *Methanosarcina* sp. clone W30B	AB489230	99.6	*Methanosarcinaceae*
5	Uncul. archaeon clone F776O8Q02BXGN4	GU855992	96.2	*Methanosarcinaceae*
6-12	*Methanosarcina* sp. HB-1	AB288262	99.0-99.6	*Methanosarcinaceae*
13	Uncul. *Methanobrevibacter* sp. clone RbtMet_14	HM449726	95.3	*Methanobacteriaceae*
14	Uncul. archaeon clone 3A10	HQ678048	100	*Methanomicrobiaceae*
15	Uncul. *Methanobrevibacter* sp. clone RbtMet_33	HM449745	98.0	*Methanobacteriaceae*
16	*Methanobrevibacter smithii* strain ATCC 35061	CP000678	99.5	*Methanobacteriaceae*
17	Uncul. *Methanobrevibacter* sp. CSIRO2.21	AY351487	96.1	*Methanobacteriaceae*
18-19	*Methanosarcina* sp. HB-1	AB288262	98.6-98.7	*Methanosarcinaceae*
20	*Methanosarcina* sp. 48	EF112192	97.4	*Methanosarcinaceae*
21	Uncul. archaeon clone F776O8Q02CFHTT	GU843899	99.0	*Methanomicrobiaceae*
22	Uncul. archaeon clone F776O8Q02CEUWA	GU885092	100	*Methanomicrobiaceae*
23	Uncul. *Methanobrevibacter* sp. clone 26	DQ402034	99.5	*Methanobacteriaceae*
	Uncul. *Methanosphaera* sp. clone 24	DQ402032	99.5	
24	Uncul. *Methanosarcina* sp. gene clone W30B	AB489230	99.1	*Methanosarcinaceae*
25	Uncul. *Methanosaetaceae* isolate DGGE gel band 23	GU734611	97.2	*Methanosaetaceae*
26	*Methanosarcina* sp. HB-1	AB288262	99.1	*Methanosarcinaceae*
	Methanosarcina siciliae strain C2J	U89773	99.1	
27	*Methanosarcina* sp. HB-1	AB288262	97.8	*Methanosarcinaceae*
	Methanosarcina siciliae	U89773	97.8	
28	Uncul. methanogenic archaeon clone SMPFLSS56m_3	FJ982699	99.5	*Methanosarcinaceae*
	Methanosarcina mazeii strain LM5	DQ987528	99.1	
29	Uncul. archaeon clone F776O8Q02BXGN4	GU855992	99.1	*Methanosarcinaceae*
	Methanosarcina mazeii strain LM5	DQ987528	98.6	
30	Uncul. *Methanosarcina* sp. clone A686	JN173201	99.5	*Methanosarcinaceae*
31	Uncul. *Methanosaeta* sp. clone D007024C03	GU179492	99.2	*Methanosaetaceae*

Table 2. Phylogenetic affiliation of the archaeal 16S rRNA sequences from DGGE bands. (*) Family, based on NCBI and RDP Classifier results, is given.

Band	Closest relative (NCBI)	Accession nº.	Identity (%)	Classification*
1	Uncultured bacterium clone MS01639-UBM006	FN985304	99.8	unclassified *Clostridia*
2	Uncultured bacterium clone 50	AB375726	100	unclassified *Clostridia*
3	Uncultured *Bacteroidetes* bacterium clone QEDV2CE03	CU919517	99.4	*Bacteroidia,* unclassified *Porphyromonadaceae*
4	Uncultured *Bacteroidetes* bacterium clone QEDV3DE11	CU919667	99.2	*Bacteroidia,*unclassified *Porphyromonadaceae*
5	Uncultured bacterium clone MS11817-B104	FN993970	100	*Clostridia,* (unclassified *Clostridiales*)
6	*Syntrophomonas* sp. clone D2CL_Bac_16S_Clone14	EU498380	100	*Clostridia, Syntrophomonadaceae*
7	*Desulfotomaculum* sp. DEM-KMe98-6	AJ276560	99.3	*Clostridia, Peptococcaceae*
8	Uncultured *Bacillus* sp. clone De31	HQ183765	97.2	*Bacilli, Bacillaceae*
9	Uncultured *Symbiobacterium* sp. clone SHBZ891	EU639261	99.2	*Clostridia*, Family XVIII incertae sedis
10	Uncultured *Bacteroidetes* bacterium clone G14	EU551114	97.1	(unclassified *Bacteroidetes*)
11	Uncultured bacterium clone M35_D8_L_B_C04	EF586010	100	unclassified *Clostridia*
12	*Alcaligenes* sp. BBTR16	EF471233	99.7	*Betaproteobacteria, Alcaligenaceae*
13	Uncultured *Clostridium* sp. clone BBC810	GQ868409	99.6	*Clostridia, Clostridiaceae*
14	Uncultured bacterium clone 148_BE1_40	FJ825467	100	unclassified *Clostridia*
15	*Bacillus* sp. CHNTR52	DQ337594	98.7	*Bacilli, Bacillaceae*
16	Uncultured bacterium clone LL141-8P23	FJ675660	99.0	*Bacteroidia,* unclassified *Porphyromonadaceae*
17	Uncultured *Clostridium* sp. isolate Marmara9	AM980561	91.8	*Clostridia, Clostridiaceae*
18	Uncultured *Bacteroidetes* clone G14	EU551114	99.6	(unclassified *Bacteroidetes*)
19	*Ruminofilibacter xylanolyticum* strain S1	DQ141183	99.6	*Bacteroidia, Rikenellaceae*
20	*Acetivibrio* sp. enrichment culture clone WSC-3	HM635213	99.8	*Clostridia, Ruminococcaceae*
21	Uncultured WWE1 bacterium clone EEB1CG06	CU918241	99.4	(unclassified *Bacteria*)
22	Uncultured bacterium clone 1-1B-28	JF417919	99.8	unclassified *Clostridia*
23	*Desulfotomaculum* sp. DEM-KMe98-6	AJ276560	99.8	*Clostridia, Peptococcaceae*
24	*Brumimicrobium mesophilum* strain YH207	DQ660382	99.8	*Flavobacteria, Cryomorphaceae*
25	Uncultured *Bacteroidetes* clone QEDR1DA08	CU922385	99.0	(unclassified *Bacteroidetes)*
26	Uncultured *Bacteroidetes* bacterium clone De114 16S	HQ183935	98.0	(unclassified *Bacteroidetes)*
27	*Clostridia* bacterium enrichment culture clone WSC-8	HM635205	99.6	unclassified *Clostridia*
28	Uncultured *Bacteroidetes* clone QEEA3BB03	CU918989	98.2	*Bacteroidia,* unclassified *Porphyromonadaceae*

Table 3. Phylogenetic affiliation of the bacterial 16S rRNA sequences from DGGE bands. (*) Family, based on NCBI and RDP Classifier results, is given.

treating synthetic substrates mimicking energy crops (Pobeheimet al., 2010). This unique bacterium lives in symbiosis with a *Bacillus* sp. strain and uses formate as electron donor during anaerobic respiration (Ueda et al., 2004). Accordingly, an uncultured *Bacillus* was also found in the majority of the samples tested in the first trial (band 8) and in the digestates of triticale silage and poultry manure. *Bacillus* is a widespread genus of gram-positive, spore-forming bacteria with the ability to degrade a wide range of organic compounds, including proteins and carbohydrates (Slepecky and Hemphill, 2005). Band 20, sharing a high sequence identity with *Acetovibrio* sp., was present only in sorghum silage digestate. *Acetivibrio* sp., like *Clostridium* and *Bacillus*, degrades cellulose and has been previously identified in AD processes of municipal solid wastes (Li T. et al., 2009). Other bands detected only after the AD process in some biomasses, such as energy crops and poultry manure, were unclassified *Bacteroidetes* (bands 10 and 18). Uncultured *Bacteroidetes* (bands 25 and 26) were also found in triple-tomato paste and whey and with strong intensity (bands 3 and 4) in all the biomasses samples inoculated with seed sludge 1 (Fig. 2B). Bands 3 and 4 were affiliated to the family *Porphyromonadaceae*, acidogenic bacteria capable of producing various VFA from carbohydrates or proteins and frequently found in anaerobic digesters (Ziganshin et al 2011, Li et al. 2009 envir microb). These bands, however, disappeared at the end of the process suggesting that these microorganisms played a minor role in the degradation of polysaccharides and were displaced by other hydrolytic bacteria. Also assigned to unclassified *Porphyromonadaceae* were bands 16 and 28. Another band observed only before the AD process was band 19, present in energy crops and manure, and assigned to *Ruminofilibacter xylanolyticum,* a rumen bacterium involved in xylan digestion. Specifically detected in whey, was *Brumicrobium* (band 24), a facultative anaerobic bacterium with fermentative metabolism, capable to use lactate as electron donor under anaerobic conditions (Bowman et al., 2003). The only band assigned to *Betaproteobacteria* was band 12, assigned to the genus *Alcaligenes*, a soil and water bacterium capable of dissimilatory denitrification under anaerobic conditions using various carbon sources like monoterpenes, aliphatic sulfonic acids or aromatic compounds (Heyen and Harder, 2000). Classification by the RDP Classifier was possible only at the level of domain in the case of band 21, phylogenetically closely related to an uncultured bacterium from a wastewater sludge.

3.3 Statistical qualitative community analyses

In order to compare the batch AD processes and evaluate the qualitative shifts in archaeal and bacterial community structure, statistical analysis of the DGGE profiles was performed by Principal Component Analysis (PCA).
Multivariate analysis on the two data matrices of *Archaea* are shown in Figure 3A and 3A'. The plots of the two-dimensional scores, defined by PC1 and PC2, accounted for 88% and 66% of the input data variability, meeting the general criteria for reliable analysis. In Figure 3A it can be observed the grouping of the archaeal genetic profiles of all the substrates tested in a very compact cluster (a), indicating that the initial

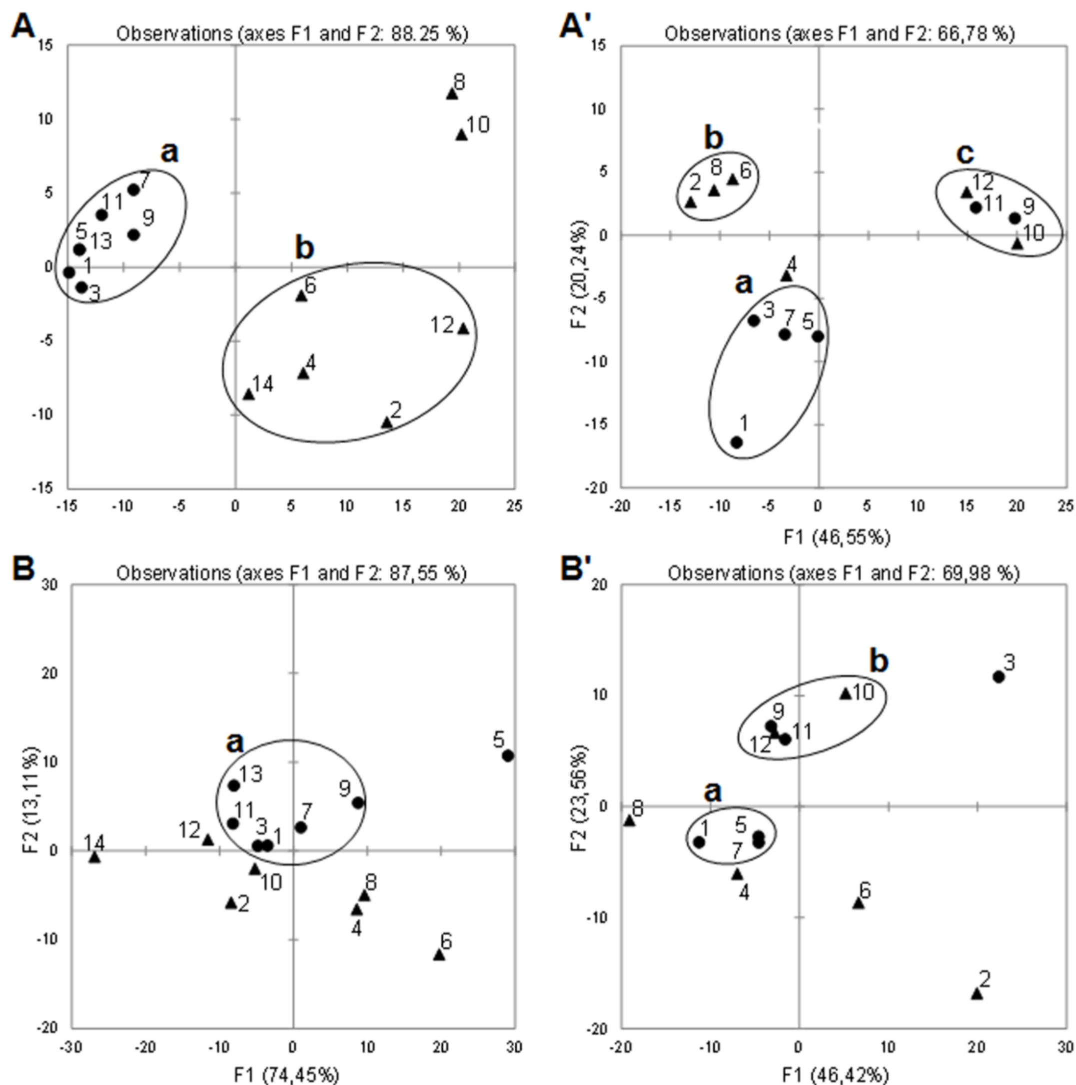

Figure 3. Principal component analysis of archaeal (A, A') and bacterial (B, B´) DGGE profiles. A, B) 1-2, green rice grains; 3-4, rice flour; 5-6, scrapped pasta; 7-8, grass mowing; 9-10, broken soybeans; 11-12, dry maize wastes; 13-14, scrapped rice; A´, B´) 1-2, triticale silage; 3-4, poultry manure; 5-6, sorghum silage; 7-8, maize silage; 9-10, triple-tomato paste residues; 11-12, whey. Dots, ingestate; triangles, digestate. Circles indicating significant clustering are commented in the text.

communities were highly similar to each other before the process, as expected by the use of the same seed sludge. The archaeal communities of substrates changed over time and mostly grouped together at the end of the process locating in quadrant IV (cluster b). Interestingly, all these biomasses (rice biomasses, scrapped pasta and dry maize wastes) were characterized by a high content of starch suggesting that the compositional content of biomasses played a role in determining the changes of community. The PCA of the DGGE samples of run 2 are presented in Figure 3A'. The initial microbial communities of energy crops and poultry manure were located in quadrant III (cluster a), and then, in the case of all the energy crops, shifted over time clustering together at the end of the anaerobic process (cluster b). The high similarity of the final archaeal communities of these three samples could be attributed to the occurrence of lactic acid fermentation, and of intermediates favoring the development of specific dominant methanogens. On the contrary, whey and triple-tomato paste

showed limited shift of the microbial community diversity and clustered in cluster c separated from the other samples in accordance to sequencing results that showed the prevalence of *Methanosaeta*-like methanogens. The resulting plots of the PCA analyses carried on the bacterial DGGE gels are shown in Fig. 3B, and 3B'.
The plots explained 70% or more of the total variance in the data set indicating

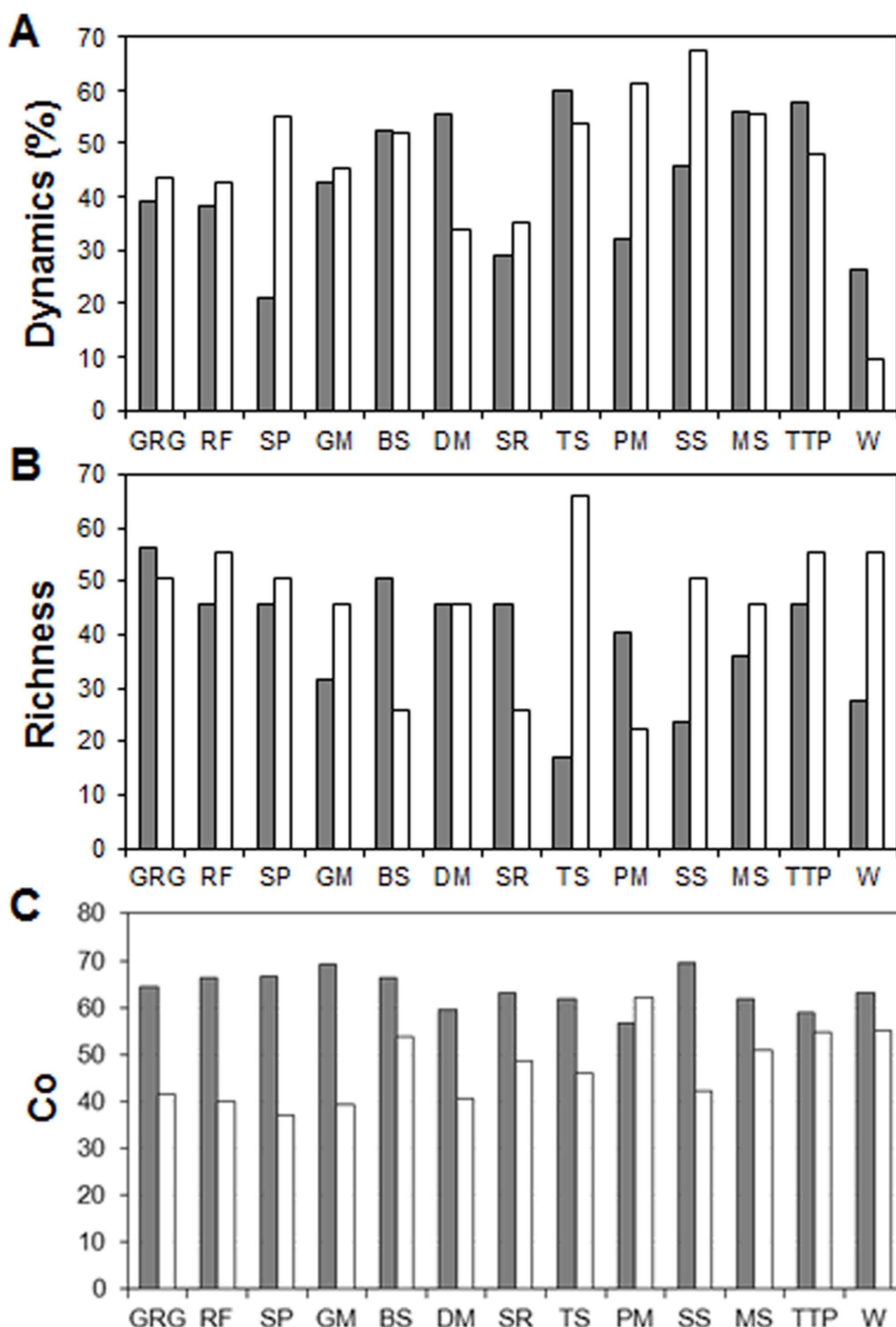

Figure 4. (a) Dynamics, (b) richness and (c) community organization parameters from archaeal (*grey*) and bacterial (*white*) DGGE profiles.

reliability. The PCA analysis of DGGE samples of run 1 is shown in Figure 3B. As already observed for the archaeal community, the initial bacterial community of all the substrates, except scrapped pasta, grouped together (cluster a), however here the shifts of communities were generally moderate and no clear grouping of the final microbial communities could be observed. A similar situation was observed for the initial bacterial community of all silages samples (Fig. 3B', cluster a). In the case of whey and triple-tomato paste, similarly to *Archaea*, initial and final communities grouped together (Fig. 3B', cluster b), with whey exhibiting no change between the beginning and the end of the process. Taken together, PCA analyses showed that, compared to *Archaea*, larger shifts in function of the different matrices occurred for bacterial communities suggesting that diverse bacteria, possibly with different substrate specificity, are involved in the AD process.

The archaeal and bacterial DGGE patterns were further characterized using parameters independent from the DGGE run, namely the dynamics of change (Dy), the richness (Rr) and the microbial community organization (Co) (Fig. 4). The average rate of change and extent of variability (standard deviations) of *Bacteria* and *Archaea* were in the same range (46.4±14.6 for *Bacteria* and 42.7 ±13.1 for *Archaea*), with values lower for the archaeal community as observed previously in studies performed on continuous anaerobic reactors (Fernandez et al, 1999, Carballa et al, 2011). The final bacterial community was in most cases richer than the archaeal one (average values of 45±13 for *Bacteria* and 39±11 for *Archaea*) in accordance with previous findings (Fernandez et al, 1999, Malin and Illmer 2008, Carballa et al, 2011) and with the evident consideration that a wide range of substrates supports the growth of a widest number of species.

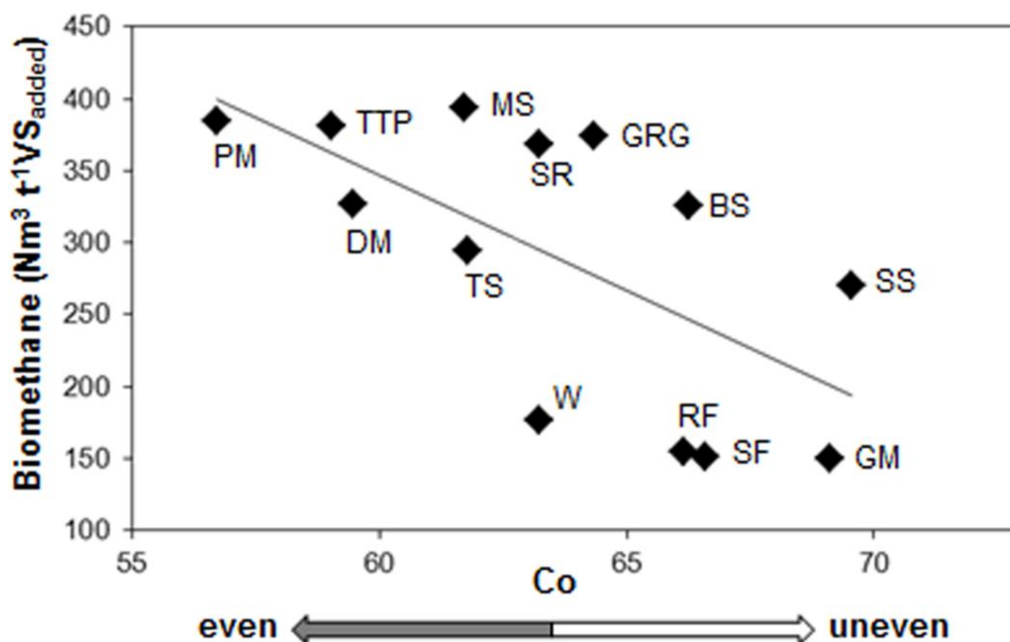

Figure 5. Correlation between community organization (Co) for *Archaea* and biochemical methane potential (BMP). Linear regression equation y = -15,966× + 1304,6; R^2 = 0,4038.

The range of values obtained were those typical of very habitable environments characterized by high microbial diversity (Marzorati et al., 2008). The values of Co were higher for *Archaea* than for *Bacteria* (average Co values of 64±4 for *Archaea* and 47±8 for *Bacteria*). As higher Co values correspond to more uneven microbial community, results indicated that archaeal community was more uneven than bacterial, as observed by Carballa (Carballa et al, 2011). These molecular parameters describing the community structure and diversity were attempted to be correlated with the reactor performance, measured as BMP. Whereas no correlation was found for Dy and Rr, and for Co in the case of Bacteria ($p>0.05$), some statistically significant ($p<0.02$) trend was observed between Co of *Archaea* and BMP (Fig. 5). This trend indicates that communities with higher evenness, i.e. with lower Co, have higher methane production, in agreement with recent findings demonstrating the more robust functional stability of communities with higher evenness, capable of using more parallel metabolic pathways and hence of adapting and responding more efficiently to disturbances (Wittebolle et al., 2009; Werner et al., 2011). However, whereas evenness analysis has proven to be very useful for understanding the relationship between microbial community and its functioning, indication on the AD performance trend may be not so predictive when merely looking at species richness (Werner et al., 2011, Malin and Illmer 2008).

The same can be said for Dy, because well-functioning reactors were observed in stable microbial community (LaPara et al., 2002) as well as in dynamic community (Fernandez et al, 1999), though recent findings seems to indicate that community with higher phylogenetic diversity functioned more efficiently (Werner et al., 2011).

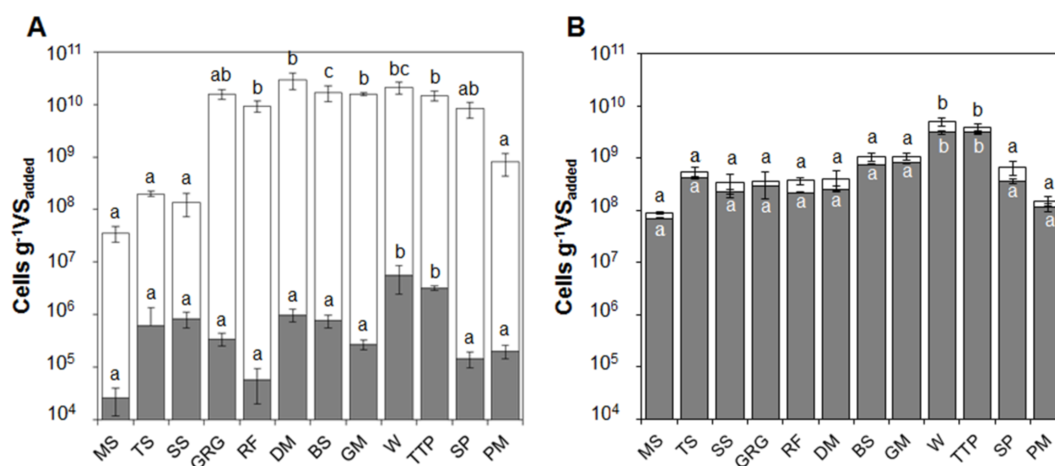

Figure 6. (A) Abundance of sulfate-reducing bacteria (*grey*) to total *Bacteria* (*white*) and (B) of *Methanosarcinales* (*grey*) to total *Archaea* (*white*) at the completion of the AD processes. MS, maize silage; TS, triticale silage; SS, sorghum silage; GRG, green rice grains; RF, rice flour; DM, dry maize wastes; BS, broken soybeans; GM, grass mowing; W, whey; TTP, triple-tomato paste; SP, scrapped pasta; PM, poultry manure. Measurements are the average values of three independent determinations. Values that were significantly different between groups by ANOVA's analysis ($p < 0.05$) are indicated by different letters. No data reported for scrapped rice due to analysis problems.

3.4 qPCR analyses of the *Archaea*

Quantification of *Bacteria*, *Archaea*, *Methanosarcinales* and sulfate-reducing bacteria was performed by real time PCR in order to get further insight into the structure of microbial community at the end of the AD process.
The results are shown in Fiugure 6. Bacteria were present in the different anaerobic biomasses at concentrations ranging from 10^8 to 10^{10} cells g^{-1} VS_{added}, a variability confirmed by ANOVA analysis (Fig. 6A). The sulfate-reducing bacteria were always $10^2/10^5$-fold lower than *Bacteria* and were present in the order of magnitude of 10^5 g^{-1} VS_{added} (Figure 6A). *Archaea* were relatively stable for all the samples and were enumerated at values around 10^9 cells g^{-1} VS_{added} (Figure 6B), thus several order magnitude higher than sulfate-reducing bacteria which may compete with hydrogenotrophic methanogens for hydrogen utilization. The *Methanosarcinales* represented the majority of the archaeal community (up to 80% of total *Archaea*) (Fig. 6B), confirming the data of the DGGE analyses. The ANOVA analyses performed on *Archaea*, *Methanosarcinales* and sulfate-reducing bacteria showed that all the quantification values were not significantly different, except for whey and triple-tomato paste. In the case of *Bacteria*, no apparent correlation with DGGE data could be deduced. However, matrices containing relatively low titers of *Bacteria* (silages and poultry manure) were those in which hydrolysis and fermentation process were previously carried out. On the whole, however, no evident correlation was found between qPCR values and BMP determinations.

4. Conclusions

In this study it was demonstrated that high BMP could be obtained from energy crops as well as other agro-industrial residues/by-products. Molecular biology techniques revealed to be useful tools for investigating the structure and diversity of microbial community during AD process, allowing identifying the dominant species associated with the biogas production. It was shown that seeding sludge had an important role in determining the basal microbial community with some dominant archaeal and bacterial taxa (*Methanosarcinales* and *Clostridia*) detected by DGGE analysis throughout the course of the process. However, definite changes in the microbial community were observed, suggesting that quantitative changes in the abundance of some key species occurred as response to microbial community adaptation to the different biomasses. In particular, compositional features of biomasses or processing (ensiling) seemed to play a role in the changes of archaeal microbial communities indicating *Archaea* as good indicators for monitoring AD microbial community dynamics. A correlation trend was identified between archaeal community and BMP indicating that more even archaeal community were associated to higher BMP.

acknowledgements

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Chapter 3

Energetic yield and microbial characterization of Anaerobic Digestion processes of different dairy and agricultural wastes[1]

1. Introduction

Whey is one of the main waste products of dairy industry. Approximately 50% of the whey produced worldwide every year is discharged in the environment (Saddoud A. et al., 2007). Uncontrolled disposal of this waste can cause serious environmental problems: alteration of chemical and physical structure of the soil, reduction of crop yields and serious groundwater pollution issues. This is important considering that dairy industry represents one of the most influential economic sectors worldwide, also because of the strong rise in the demand for milk and milk products in many countries which led to steady growth in the productivity of dairy business.
The principal components of whey are lactose, proteins and mineral salts. This waste is characterized by a high organic content concentration and this make such biomass suitable for anaerobic digestion (AD) technology with the advantage of energy generation in the form of methane. This scenario may represent a significant gain of resources, particularly in Italy for the huge number of small and medium-size dairy factories which produce typically high-quality cheeses and which may exploit whey as a potential renewable resource at zero cost and zero environmental impacts.
AD is a well-established technology, which enables the production of energy in the form of biogas, mainly composed of methane ad carbon dioxide. The process that takes part in anaerobic digesters is called methanogenesis. A complex consortium of different bacterial and archaeal microorganisms in absence of oxygen, are able to degrade gradually the initial organic material breaking it down to biogas.
Few studies have focused on whey as feeding material for AD process. Demirel et al. (2005) showed that AD of dairy wastewater is possible with satisfactory results. However, in some studies Malaspina et al. (1996) and Mockaitis et al. (2006) stated that whey is a difficult substrate to treat because of the lack of alkalinity, its high organic content concentration and the tendency to acidify very rapidly. Recently has been reported that co-digestion of whey with manure was proved to be possible up to 50% participation of whey, by volume (Gelegenis et al, 2007).
Limited traditional culture-dependent methods do not allow to identify and characterize most of the microorganisms present in the complex anaerobic digestion community responsible for the conversion of the organic matter in methane and carbon dioxide.

[1] Manuscript in preparation for submission as: Energetic yield and microbial characterization of Anaerobic Digestion processes of different dairy and agricultural wastes. Merlino G., Rizzi A., Cabassi G., Cattaneo , Borin S. and Daffonchio D.

However, recently developed molecular techniques have provided powerful tools to investigate anaerobic communities without cultivation In this study, anaerobic co-digestion of cheese whey with dairy manure and other biomasses was studied. Experiments were carried out in batch reactors at mesophilic conditions (37°C) for the evaluation of biochemical methane potential (BMP). Changes in bacterial and archaeal community structure were monitored both before and after the digestion process using Denaturing Gradient Gel Electrophoresis (DGGE) technique. This molecular method is effective in detecting microbial community shifts and in identifying the phylogenetic affiliation of microbial populations in mixed culture systems (Ueno Y., et al., 2001).

2. Materials and Methods

2.1 Experimental system

The Automated Methane Potential Test System (AMPTS; Bioprocess control, Sweden) was used to carry out the BMP experiments of this research. This is a multi-batch lab-scale equipment which allow on-line measurements of ultra-low biogas and bio-methane flows produced from the anaerobic digestion.
The instrument setup can be divided into 3 units: 15 batch vials (500 ml of operative volume) containing the sample with anaerobic inoculums are incubated in a controlled-temperature bath. The media in each vial is mixed by a slow rotating agitator. The biogas produced from each vial passes through an individual vial (CO_2-fixing unit), containing an alkali NaOH solution. Several gas fractions, such CO_2 and H_2S, are removed allowing only CH_4 to pass through the NaOH solution without change. A pH indicator is used so the pH level of the solution can be monitored in order to ensure a sufficiently high OH^- concentration for taking away CO_2 and H_2S. Thus, CH_4 gas released from CO_2-fixing unit is analyzed using a wet gas flow measuring device with a multi-flow cell arrangement, 1 cell for each batch vial. This measuring device works according to the principle of liquid displacement and can monitor very low gas flow, where a digital pulse is generated when a defined volume of gas flows through the device. A data acquisition system is used together with the flow cells in order to record, display and calculate data, as well as analyze the results.

2.2 Inoculum

Two samples, taken in different moments, of the same digestate from the Anaerobic Digester of Activeted Sludge of a Wastewater Treatment Plant (WWTP) were used as bacterial inoculum for two different trials of anaerobic digestion experiments. The inoculum samples were processed in the same way. Firstly they were sieved through a 0.5 mm sieve; than they were per-incubated in anaerobic conditions in order to deplete the residual biodegradable organic content, releasing the gas produced. This has be done by storing the digestate typically for 5 days at mesophilic temperature (37°C) corresponding to the process temperature from where the inoculum was taken from.

2.3 Substrates for Bio-Methane Potential (BMP) tests

The choice of the substrates to be used in BMP trials was dependent on the biomasses availability in a typical small or medium-size Italian dairy company. Thus the first option for our experiments was whey, as one of the most abundant and continuously produced byproduct of milk processing. This waste has been tested alone or in co-digestion with other typical biomasses of a dairy company, but less abundant, both in terms of continuous availability and/or quantity. For this reason two energy crops residues, i.e. sorghum and triticale silage, where chosen. We further choose to test in co-digestion with whey also dairy manure, which is typically produced in high quantities in a dairy farm, to test the possibility of maintaining the optimal pH conditions for methanogenesis as stated by Gelegenis et al (2007). Chemical characterization of biomasses used in our trials is shown in Table 1.

Biomasses	**pH**	**SO_4^{2-}**	**PO_4^{3-}-P**	**NH_4^+-N**	**NT**	**COD**
Inoculum	8.0	61.1	148.3	643.3	9053.3	12.5
Whey	5.0	1250.0	190.3	101.4	871.3	71.7

Table 1. Characteristics of the biomasses. All measure are expressed in mg l^{-1}. COD is expressed in g l^{-1}.

2.4 Bio-Methane Potential tests

All trials were carried out under mesophilic conditions (37°C) until no methane production was detected, typically for a period of 30 days. Each trial was performed in triplicate and was composed of a mixture of biomass and inoculum with 1:2 ratio on total solids content. The blank trial (inoculum only) was also carried out in triplicate.

2.5 Analytical methods

Each substrate and the inoculum were analyzed for pH, total solids (TS), volatile solids (VS), chemical oxygen demand (COD), total nitrogen (TN), ammonia nitrogen (NH_4^+-N), sulfate (SO_4^{2-}) and phosphate (PO_4^{3-}-P) (Table 1).
TS and VS were measured according to the procedures in Standard Methods (APHA, 1998). The pH was measured by a Jenway 3510 pH meter (Bibby Scientific, UK). COD, total nitrogen, NH_4^+-N, sulphate and PO_4^{3-}-P analysis were carried out using Lange DR 2800 spectrophotometer and cuvette tests from Hach-Lange (Germany). When necessary the samples were diluted to reach the correct concentration range for the measurement.

2.6 Sampling

Samples from each batch reactor were collected both at the beginning and at the end of each trial. The samples were centrifuged (15 minutes, 4000 rpm, +4°C) in a centrifuge model 5804R (Eppendorf, Germany) in order to obtain a pellet, which was washed with physiological solution and re-centrifuged at the same condition above. In order to obtain

a final pellet of 100 mg, variable volumes (2-3 ml) of starting samples were used for centrifugation. The pellets were stored at -20°C until DNA extraction.

DNA Extraction

Total community DNA was extracted from the pellet of bioreactor samples using PowerSoil DNA Isolation Kit (MoBio Laboratories, USA) following the manufacturer guidelines. The purified DNA was eluted with 100 µl of elution buffer (10 mM Tris-HCl, pH 8.0) and stored frozen at -20°C until use. All DNA were extracted in triplicate. Quality of DNA was evaluated by agarose gel electrophoresis.

2.7 Archeael and Bacterial Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

The 16S rRNA gene of *Archaea* was PCR amplified using specific primers ARC787F and ARC1059R (Hwang et al., 2008) with an expected PCR product of about 300 bp. A 40-bp GC-clamp was added at the 5' end of ARC787F to stabilize the melting behavior of PCR fragments (Muyzer et al., 1993). PCR amplification was carried out in a final volume of 50 µl reaction mix by using the "FastStart HiFi PCR System dNTPack" kit following the manufacturer's indications (Roche Applied Science, Germany). One µl of the extracted DNA was used as template for amplification. The PCR program was performed as described by Hwang et al. (2008).
Bacterial 16S rRNA gene fragments (expected length of 630 bp) were amplified by using primers GC357f, containing a 40-bp GC clamp, and 907r (Sass et al., 2001). PCR amplification was performed as described previously by Sass et al. (2001).
The PCR products were loaded onto 7% polyacrylamide gels with a 40-60% and 30-70% gradient for *Bacteria* and *Archaea* respectively (100% denaturant corresponding to 7M urea and 40% [v/v] formamide) and run for 17h at 90 V in 1× TAE buffer at a constant temperature of 60°C using a D-Code electrophoresis system (BioRad, USA). Gels were prepared with a gradient maker (BioRad, USA) according to the manufacturer's instructions. Gels were stained with SYBR Green I Nucleic A (Invitrogen) and documented with the GelDoc 2000 apparatus (BioRad) by using the Diversity Database software (BioRad). Bands of interest were excised from the gels, transferred to 50 µl of 10 mM Tris-HCl pH 8 solution and incubated at 37°C for 4 hours. Eluted DNA (5 µl aliquots) were re-amplified by standard PCR conditions using DGGE primer set without GC clamp. The obtained PCR products were purified and sequenced (Macrogen, Korea). The sequences were compared with sequences deposited in the National Center for Biotechnology Information (NCBI) database by using BLAST program. Sequence alignment and neighbor-joining Phylogenetic tree construction were carried out using MEGA software, version 5.0 (Tamura et al., 2011).

3. Results and Discussion

3.1 BMP results

Two different runs were carried out at mesophilic temperature of 37 °C, up to no methane production was detected by the gas cell-flow measuring device of AMPTS.
In the first run of BMP determination, whey alone was tested.

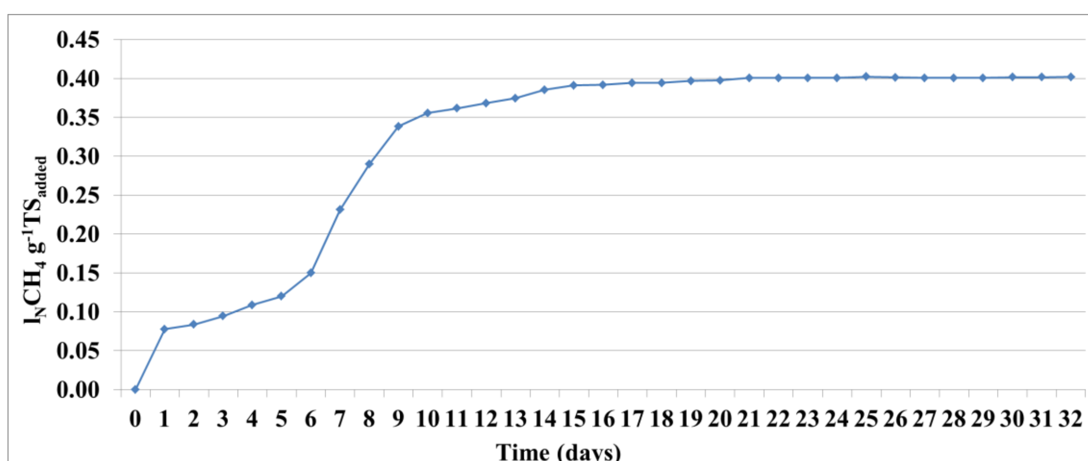

Figure 1. Methane cumulative production (l_N $g^{-1}TS_{added}$) obtained during the BMP trial of whey, after deduction of cumulative production of blank.

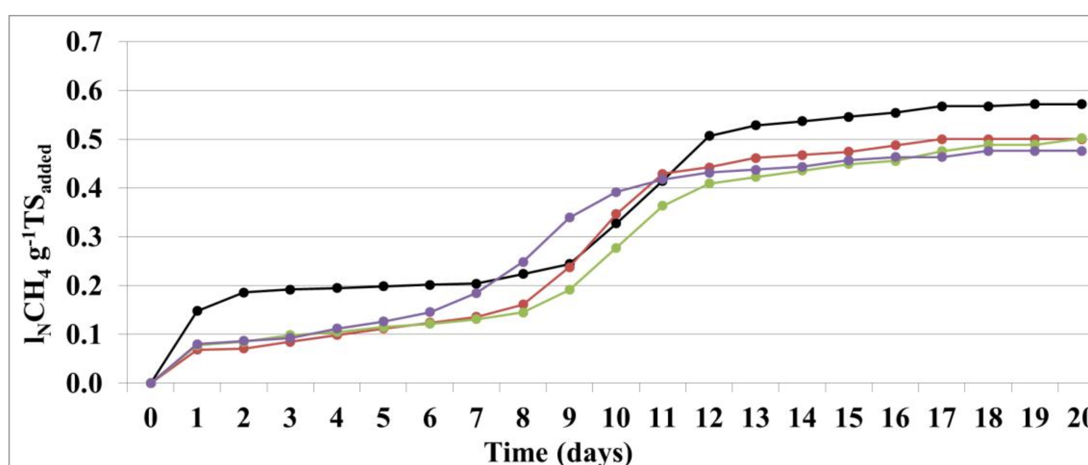

Figure 2. Methane cumulative production (l_N $g^{-1}TS_{added}$) obtained during the BMP trial of whey in co-digestion with other biomasses, after deduction of cumulative production of blank.

Whey **Whey & Manure** **Whey & Triticale Silage** **Whey & Sorghum Silage**

In Figure 1 the cumulative production of methane (l_N $g^{-1}TS_{added}$) after deducting the production of the blank (inoculum only) are shown. An initial lag phase between day 1 and 5 can be observed, after which a fast rising in methane production was detected. After 21 days no remarkable methane generation was observed. The final volume of CH_4 produced during this trial was of 400 ml_N $g^{-1}TS_{added}$.
In the second trial, the potential methane production of whey in co-digestion with dairy manure, triticale and sorghum silage was tested (Figure 2). Similar production curves were obtained in this second trial. In this case was observed a longer lag phase

compared to the first BMP experiment (between day 1 and 7). Subsequently the plateau was reached at day 20 as was observed for the first trial. In general the cumulative production obtained with whey alone or in co-digestion gave similar final volume of methane of about 500 ml_N $g^{-1}TS_{added}$.

3.2 DGGE and *Archaea* and *Bacteria* and phylogenetic analysis

Microbial diversity of archaeal and bacterial communities present in the samples at the beginning and after the AD process were assessed by DGGE analysis of 16S rRNA gene.

For what concerns *Archaea* DGGE gel (Figure 3a), high similarity was clearly appreciable both between the beginning and ending time of the AD process of a same substrate and also between DGGE profiles of different substrates. It is interesting to notice that also the genetic fingerprints of the two different inoculum used in our tests were compatible. In fact the samples of inoculum used in our BMP trials were taken from the same WWTP, even if in different moments. This suggests that the dominant species were already present in the inoculum and were consolidated during the digestion process.

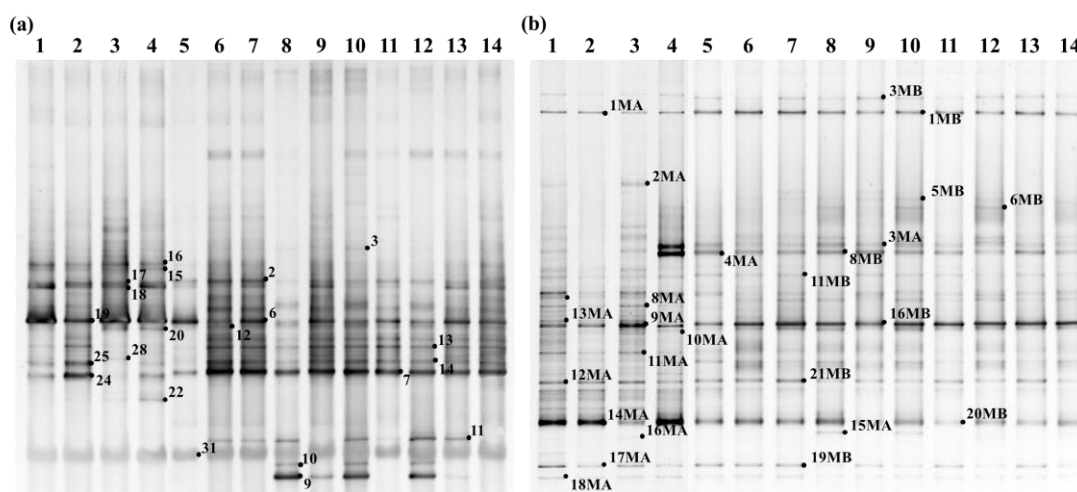

Figure 3. Archaeal (a) and bacterial (b) DGGE profiles of the 16S rRNA gene PCR products generated from DNA extracted from ingestate (*i*) and digestate (*d*) of substrates analyzed for BMP. Samples 1 to 4 are relative to the first trial, while samples 5 to 14 are relative to the second trial. 1-2, inoculum 1ST *i, d*; 3-4, whey 1ST *i, d*; 5-6, inoculum 2ND *i, d*; 7-8, whey 2ND *i, d*; 9-10, whey & dairy manure *i, d*; 11-12, whey & triticale silage *i, d*; 13-14, whey & sorghum silage *i, d*. Dots and numbers indicate the bands sequenced.

DGGE bands were excised from the gel and then sequenced. The phylogenetic affiliation of the sequences and the phylogenetic tree constructed to visualized the relationship of the band sequences to the database sequences are reported in Table 2 and Figure 4, respectively. Microorganisms strictly affiliated to *Methanomicrobiales* group (similarity higher than 98%) were found in all the samples (bands 12, 13, 14, 25 and 31). Bands 11 and 22 were only found at the end of the process in the samples of whey anaerobically digested alone. Sequence identification of these bands showed similarity with methanogens belonging to *Methanobacteriales* group for band 11 and to genus

Methanobacterium for band 22. These two groups include hydrogenotrophic methanogens, which are able use mainly H_2 and CO_2 as substrates, but also formate and sometimes alcohols.

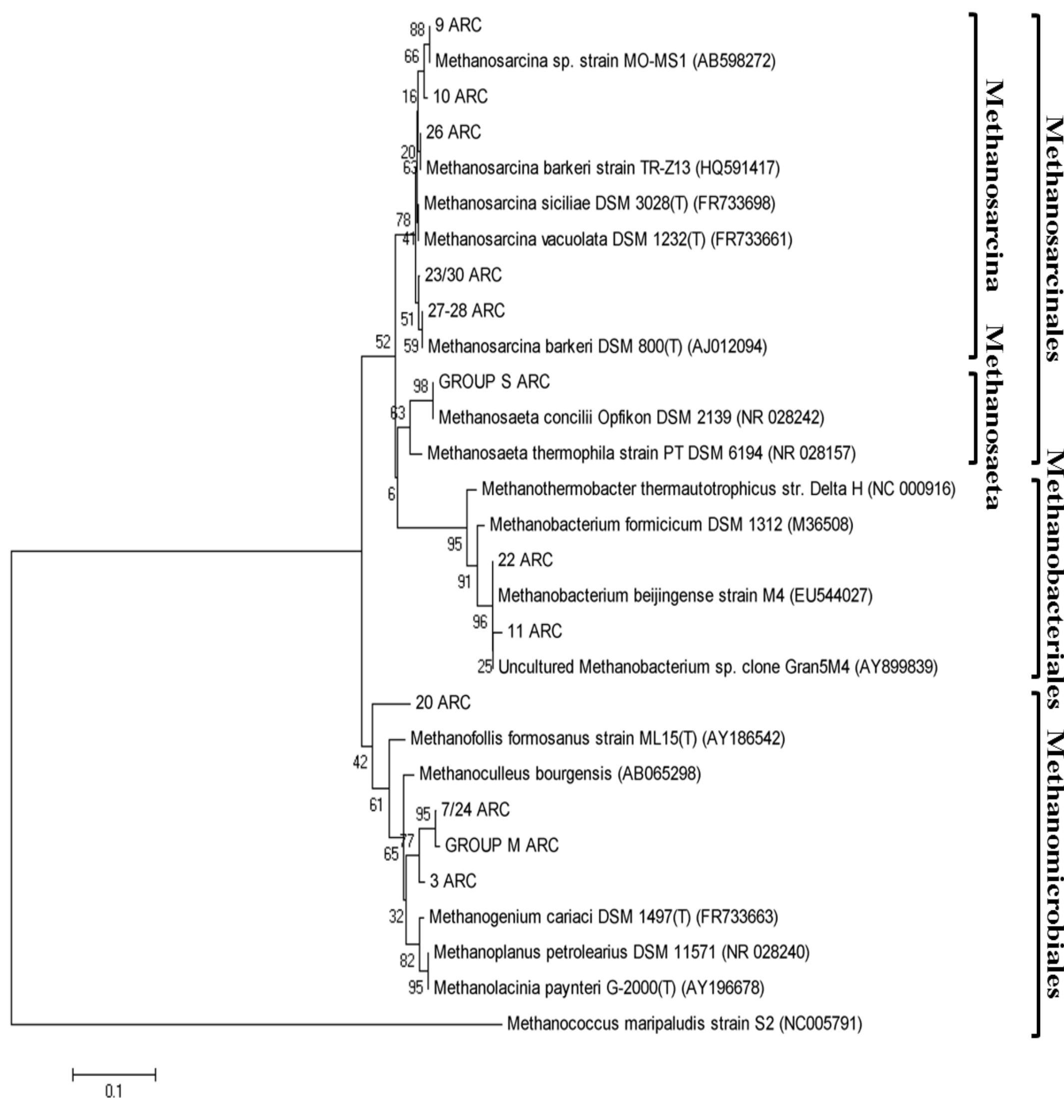

Figure 4. Phylogenetic tree showing the phylogenetic relationships of archaeal 16S rRNA sequences with reference sequences deposited at the GenBank database. The tree was construct using the Maximum Likelihood algorithm and the Tamura Nei parameter correction and was bootstrapped 2000 times.

Microorganisms belonging to genus *Methanosarcina* were identified in digastate samples of whey alone and in co-digestion with dairy manure or triticale silage. Similarly to bands 11 and 22, the presence of *Methanosarcina*-belonging species at the end of the AD process suggests the importance of these microorganisms in methane production. The sequences of these bands were closely affiliated to species *Methanosarcina barkeri*, *Methanosarcina vacuolata* and *Methanosarcina siciliae*. Genus *Methanosarcina*, as Genus *Methanosaeta*, comprises acetoclastic methanogens, which are the main responsible for CH_4 production in typical anaerobic digestion processes (Demirel and Scherer, 2008). *Methanosaeta* is able to grow only on acetate, while *Methanosarcina* is more versatile and can use other substrates, as H_2 and CO_2, methanol and methylamine.

Band	Closest relative (NCBI)	Accession n^{o}.	Identity (%)
2-15-16-17-18	*Methanosata concilii Opfikon*	NR028242	99.5-99.8
3	Uncultured *Methanomicrobiales archaeon*	CU917241	98.0
	Methanolinea tarda strain sk0808-3	FJ155846	97.0
6-19	*Methanosaeta concilii* Opfikon	NR_028242	100
7-24	Uncultured *Methanosarcina* sp. clone W30B	CU916955	100
	Uncultured *Methanomicrobiaceae* archaeon isolate	GU734610	98.3
	Uncultured *Methanolinea* sp.	EU857626	98.0
9	*Methanosarcina* sp. MO-MS1	AB598272	100
10	*Methanosarcina* sp. HB-1	AB598272	98.7
	Methanosarcina barkeri strain TR-Z13	AJ012094	98.5
11	Uncultured *Methanobacteriales* archaeon	CU916723	98.6
12-13-14-25-31	Uncultured *Methanomicrobiales* archaeon	CU916955	98.5-99.5
20	Uncultured *Methanomicrobiales* archaeon	CU917455	94.7
22	*Methanobacterium beijingense* strain M4	EU544027	99.5
	Uncultured *Methanobacterium* sp. Clone Gran5M4	AY899839	99.5
23-30	*Methanosarcina siciliae*. Type strain DSM3028 (T)	FR733698	99.1
	Methanosarcina vacuolata type strain DSM1232 (T)	FR733661	99.1
26	*Methanosarcina barker* strain TR-Z13	HQ591417	99.1
27-28	*Methanosarcina barkeri* type strain DSM 800 (T)	AJ012094	99.0-99.5

Table 2. Phylogenetic affiliation of the archaeal 16S rRNA sequences from DGGE bands.

Band	Closest relative (NCBI)	Accession n^{o}.	Identity (%)
6MA	*Comamonas* sp. strain SFCD1	AY134850	99.4
8MA	Uncultured Betaproteobacteria from clone QEEB1DH12	CU917876	99.6
	Diaphorobacter oryzae strain RF21 EU342380	EU342380	97.9
10MA	*Azovibrio* sp. BS20-3	AF011349	97.5
11MA	*Syntrophomonas* sp. TB-6	AB098336	99.8
12MB/21MB	Uncultured Firmicutes bacterium clone QEEB1CF08	CU918340	100
20MB/14MB	*Coprothermobacter* sp. GK5	AB537980	100
15MA	*Petrobacter* sp. Clone SEQ55_11FClone_AER	HM059778	98.2
16MA	Uncultured Bacteroidetes bacterium from clone QEDR2DB12	CU922568	99.6
	Uncultured *Anaerophaga* sp. Clone MDAF11	EU214540	90.6
17MA/19MB	Uncultured Aminanaerobia bacterium from clone QEDN10CG08	CU926332	99.8
	Synergistetes bacterium enrichment culture clone DhR^2/LM-F01	HQ012836	97.4
18MA	Uncultured Aminanaerobia bacteriumfrom clone QEEA1CC11.	CU918717	98.7
	Synergistaceae bacterium enrichment culture clone B31171	HQ133014	98.3
1MB/1MA	Uncultured Unclassified bacterium clone QEEB2BA10	CU918318	100
	Uncultured Bacteroidetes bacterium clone QEDQ	CU923255	99.8
	Uncultured Caldiserica bacterium clone NRB39	HM041956	94.7
3MB	Uncultured Unclassified bacteriumfrom clone QEEB2BA10	CU918318	99.6
	Uncultured Bacteroidetes bacterium from clone QEDQ2DA03	CU923255	99.4
5MB	Uncultured Bacteroidetes bacterium from clone QEDN11CG07	CU926077	99.2
6MB	Uncultured Bacteroidetes bacterium from clone QEDP1BC04	CU924115	97.6
3MA-4MA/8MB	*Clostridium butyricum* strain TM 9B	FR734080	99.0-100
11MB	Uncultured bacterium clone 382H08 .	HQ236882	97.5
	Uncultured Clostridium sp. Clone MS7r-11	HQ396565	97.3
16MB/MA	Uncultured OP8 bacterium clone QEDP1CE04	CU924634	99.4-99.8

Table 3. Phylogenetic affiliation of the bacterial 16S rRNA sequences from DGGE bands.

Moreover, is important to emphasize that the first one is characterized by a higher affinity to substrates but lower growth yield, while the latter has a lower affinity to the subbstrates and outcompete *Methanosaeta* at high concentrations of acetate (>70 mg/l).
Genetic profiles of bacterial community is shown in Figure 3b. Compared to genetic fingerprints obtained for *Archaea*, a more complex community was observed.
This is not surprising considering the high variability of the chemical characteristics of the substrates tested. Bacterial communities in all samples were characterized by a high diversity, including species belonging to phylogenetic groups of *Bacteroidetes*, *Deltaproteobacteria*, *Firmicutes* and *Aminoanaerobia*. High stability of the community was observed for the different substrates tested, as already seen for the archaeal community structure. The most intense bands (e.g. 1MB/1MA, 9MA/16MB, 14MA/20MB) were present in all the samples. The same profile was clearly visible for the inoculum bacterial community suggesting, as already seen for *Archaea*, the importance of the inoculum for the good performance of the AD process. Only few bands were observed only in the inoculum of the second trial (8MB, 11MB). However for the majority of the bands sequenced, it was not possible to obtain a specific identification. Most of the microorganisms identified were related to unknown *Bacteria*, mostly found in anaerobic digesters. Phylogenetic affiliation of the sequences is reported in Table 3. The most abundant bands were 1MA/1MB and 3MB strictly related to unknown *Bacteroidetes* (>99% similarity); bands 9MA/16MB were closely affiliated to a microorganism isolated from mesophilic anaerobic sludge; sequences of bands 14MA/20MB were identical to *Coprothermobacter sp.*, a microorganism belonging to *Thermoanaerobacteriaceae*; bands 17MA/19MB were found to be very similar to *Bacteria* belonging to *Aminoanaerobia*.
These results are in agreement with the data of recent studies which found the presence in anaerobic reactors of a core of bacterial phylotypes formed by microorganisms affiliated to Chloroflexi, Betaproteobacteria, Bacteroidetes e Synergistetes (Riviere et al., 2009) As observed for *Archaea*, sometimes some characteristic bacterial species were found only at the end of the process. *Clostridium butyricum* was observed in the digestate sample of whay in the first trial (bands 3MA and 4MA/8MB).

4. Conclusions

BMP trials showed that whey can be a extremely good substrate for anaerobic digestion alone and in co-digestion with other biooamsses (METTERE I VALORI DI PRODUZIONE MASSIMA).
Microbial characterization at the beginning and at the end of the AD trials showed a high microbial diversity both for *Archaea* and *Bacteria*. Samples of different substrates tested often had a similar microbial community. However some significant differences were observed in the genetic profiles between start and end of the same substrates, allowing to identify microorganisms with a main role in the digestion process. Archaeal DGGE fingerprints showed in almost all the samples the dominance of acetoclastic methanogen belonging to *Methanosaeta* and of hydrogenotrophic methanogens

affiliated to *Methanomicrobiales*. The genetic profiles of the bacterial communities suggested substantial stability and diversity in the structure, comprising species belonging to *Bacteroidetes*, *Deltaproteobacteria*, *Firmicutes* and *Aminoanaerobia*. Sometimes the presence of microbial characteristic species was observed at the end of the process, e.g *Clostridium butyricum* in digestate samples of whey.

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Chapter 4

Microbial community structure and dynamics of a two-stage anaerobic digestion process treating swine slurry and market bio-waste and comparison with a single-stage system[1]

1. Introduction

Anaerobic digestion process is considered an effective way to treat organic waste producing energy in the form of biogas of high calorific value (methane and hydrogen) (Angenent et al., 2004). This technology has been successfully performed to produce methane since 1970s, but recently its use has raised an revived and increased interest as an alternative to fossil fuel-derived energy, with the benefit of reducing environmental impact by providing a clean fuel and reducing carbon dioxide emissions to the atmosphere (Chynoweth et al., 2001; Nath and Das, 2004). Significant advantages compared to aerobic treatment include: reduced generation of sludge, lower energy requirement, lower space requirements, lower overall costs, stabilization of organic waste, reduction of the odor (Van Starkenburg, 1997; Chan et al., 2009). Anaerobic process has been applied on a variety of solid biomasses, such as organic fractions of municipal solid wastes, and high-strength organic wastewaters, such as agro-industrial wastewaters (Gunaseelan 1997; Chong et al., 2009; Nishio et al., 2004).
Anaerobic digestion process is a complex biological process requiring three major steps (hydrolysis/acidogenesis, acetogenesis and methanogenesis) and involving the participation of different functional groups which, interacting together in a delicate balance system, make the whole process of conversion of complex organic matter to methane. It has been commonly performed by using a single-phase anaerobic digestion process, however recently a two-phase process has been received significant attention Demirel and Yenigun, 2002; Demirel et al., 2010. Splitting the anaerobic biological process in two phases, hydrolysis-acidogenesis and methanogenesis, and using two reactors in series with production of hydrogen and methane, respectively, is considered a way to enhance the control and stability of the process and has been proposed recently also an approach to increase the efficiency of energy recovery (Liu et al., 2006; Luo et al, 2011; Tenca et al., under submission). Biological production of hydrogen entails bacteria fermenting organic compounds, generally carbohydrates, directly to hydrogen, carbon dioxide and organic acids and alcohols (Valdez-Vazquez and Poggi-Varaldo, 2009). Residual energy contained in the high-volatile fatty acids (VFAs) content effluent is subsequently converted to bio-methane by methanogens in a second-stage

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reactor. Early studies of bio-hydrogen production were focused on pure cultures of *Clostridia* (Karube et al., 1982; Taguchi et al., 1996) and *Enterobacteria* (Tanisho et al., 1989; Kumar and Das, 2000) fermenting simple soluble substrates, like starch, glucose or sucrose. However, pure carbohydrates are very expensive and the use of pure culture system is problematic as they are prone to contamination. In following studies, exploitation of mixed cultures proved to be successful because easy of control and permitting the use of low cost solid organic waste. Recently, a number of studies have been performed investigating the feasibility of use of different wastewaters. Investigations for production of hydrogen were carried out using diverse organic wastes, such as agro-industrial wastewaters (Hussy et al., 2003; Fang et al., 2006; O-Thong et al., 2008), organic municipal wastes, including food wastes (Shin et al., 2004; Ueno et al., 2006; Liu et al., 2006; Chu et al., 2010; Lee et al., 2010). The utilization of organic wastes as substrate for hydrogen production may also be useful for solving the problem of problematic waste disposal, as the case of substrates with very low pH or with high concentrations of ammonia (Demirel et al., 2010).

In general two categories of organic substrates particularly promising to be used for suistainable bio-hydrogen production, because of their abundance and cheapness, are livestock waste and food waste. Fruit and vegetable wastes are produced in large quantities, especially in the big cities, where constitutes about 40% of the total organic solid waste. Also animal manure is produced in large amounts, only in Europe 1500 million tons of livestock manure are produced yearly (Holm-Nielsen et al., 2009). Food waste, due to its high biodegradability it represents a nuisance in municipal landfills, manure a major risk of air and water pollution. Since livestock manure has been typically treated in conventional single-step biogas plants in co-digestion together with other biomasses, these farm biogas plants provide the necessary equipment to readily implement bio-hydrogen bioprocesses (Cantrell et al., 2008). Although research studies were performed examining the influence on the digester performance of operational conditions, such as temperature, pH, heat, chemical treatments and addition of nutrients, only a limited number of experiments were performed in continuous system (Zhu et al., 2007; Karlsson et al., 2008; Kotsopoulos et al., 2009; Wang et al., 2009; Xing et al., 2010), and characterization of microbial community was rarely carried out (Yokoyama et al., 2007a; Yokoyama et al., 2007b). Hence, further research is desirable, especially investigating the potential of the co-digestion of livestock manure and carbohydrate-rich feed, known to be ideal substrate for hydrogen production (Guo et al., 2010).

In the anaerobic digestion process molecular hydrogen is an important intermediate of the energy transfer system. In a two-phase anaerobic system, in the acidogenic phase for the production of hydrogen, the key issue is to enable the accumulation of hydrogen which in anaerobic environments is typically consumed very quickly by different microbial groups. In particular, due to energetic of biochemical reactions carried out by different microorganisms, three microbial groups appear to be most important: H_2 producers (fermenters), H_2-consuming methanogens and the H_2-consuming acetogens. In order to facilitate hydrogen producing bacteria, while preventing H_2-consuming microorganisms, pretreatments and biokinetic control of parameters, such as, pH and hydraulic retention time (HRT), have been applied. Heat-treatment of the inoculum,

selecting for spore-forming bacteria, is adopted to kill methanogens, while operating at low pH and high dilution rate is employed for inhibition of methanogens and possibly other H_2-consuming microorganisms (Valdez-Vazquez and Poggi-Varaldo, 2009). However, the knowledge on the microbial characterization of the anaerobic community in two-stage processes is still limited. Studies on hydrogen fermentation system have been mainly focused in investigating the structure of the microbial consortia; the dynamics of bacterial populations were often limited to qualitative evaluations (Fang et al., 2006; Xing et al., 2008; Huang et al., 2010), and quantitative analysis, when performed, were restricted to the monitoring of the hydrogen producing bacteria (Hung et al., 2008; Tolvanen et al., 2008; Wang M-Y et al., 2008). Quantitative analysis carried out by real time PCR were recently reported for single-phase anaerobic digestion (Hori et al., 2006; Yu et al., 2006), but, to our knowledge, no reports relating to quantitative dynamics of the key functional bacterial groups have yet been reported for two-phase anaerobic processes. Knowledge of evolution of microbial community structure during bioreactor operation is important in order to improve the anaerobic digestion process and increase process stability (McMahon et al., 2007)
The aim of this study was to characterize and compare the microbial community of a two-stage reactor and a conventional single-phase bioreactor both fed with a mixture of liquid swine manure and fruit and vegetable market waste. Energetic and chemical performance of these lab-scale intermittent-continuous stirred tank reactor (I-CSTR), operating under thermophilic conditions, was previously reported (Tenca et al., under submission). Here the structure and the dynamics of the microbial population were qualitatively and quantitatively analysed. Denaturing Gradient Gel Electrophoresis (DGGE) was used to investigate the structure and the dynamics of bacterial and archaeal communities and to identify the dominant hydrogen-producing bacteria and methanogens; Real-Time PCR was employed to monitor quantitatively the temporal changes of the major functional bacterial groups involved in the anaerobic process.

2. Materials and methods

2.1 Bioreactor set up and operation

Three anaerobic previously described (Tenca et al., under submission) completely stirred tank reactor (CSTR) were used as source of biomass samples. Briefly, the two-stage process consisted of a hydrogen-producing reactor with 2.3 Lworking volume (H) and a methane-producing reactor with 14.7 L working volume for (M). The single-stage process was a reactor with 14.7 Lworking volume (MM). The hydrogen-producing reactor was inoculated with heat-shocked (100 ℃ for 2 h) anaerobic seeding sludge collected from a full-scale anaerobic digester treating household source-separated bio-waste and agro-industrial by-products. The same sludge, without heat-shock, was used as inoculum for both M and MM. A mixture of swine manure and fruits and vegetables market waste (3:1 w/w ratio) were used as feeding substrate and added to H and MM twice a day. The feeding source to H was drastically reduced to about once a week

during the days 16-35 17-32 and the response of the community to starvation was evaluated. The characteristics of the feeding mixture are reported in Table 1. The operational hydraulic retention time (HRT) in H, M and MM were 3, 22 and 25 days, respectively. The organic loading rate (OLR), expressed as total solids (TS) were 13.3, 2.3 and 1.6, respectively.

Parameters	Values
Total solids (TS) (g kg^{-1})	40 ± 1
Volatile solids (VS) (g kg^{-1})	854 ± 26
Chemical Oxygen Demand (COD) (g kg^{-1})	40 ± 3
Total Kjeldahl Nitrogen (TKN) (g kg^{-1})	2.4 ± 1.2
Ammonium (mg kg^{-1})	1.5 ± 0.7
Acetate (mg kg^{-1})	689 ± 853
Propionate (mg kg^{-1})	182 ± 275
Butyrate	nd
pH	7.2 ± 0.1

Table 1: Physicochemical characteristics of the influent mixture “nd”, not detected.

Temperature was maintained at 55 ± 2°C by the temperature controller. pH was measured in continuous and was not actively controlled. Qualitative and quantitative biogas analysis were performed automatically in each reactor by gas flow-meters.

2.2 DNA Extraction

Samples were centrifuged (10000×*g*, 30 min, 4°C) and the pellet washed twice with 2 ml of sterile distilled water and centrifuged again in the same conditions. Variable volumes (2-3 ml) were used for centrifugation to obtain a final pellet of 100 mg. The pellets were stored at -20°C until DNA extraction. The DNA extraction was performed using the PowerSoil DNA Isolation kit (MoBio Laboratories, Inc., Milan, Italy) according to the manufacturer’s instructions. The purified DNA was eluted with 100 µl of elution buffer (10 mM Tris-HCl, pH 8.0) and stored frozen at -20°C until use. The quantity and quality of the DNA extracted was checked by agarose gel electrophoresis and by spectrophotometer measurement of the ratio A_{260}/A_{280}. All DNA were extracted in duplicate.

2.3 PCR-DGGE analysis

The primer sets targeting the 16S rRNA gene of *Bacteria* and *Archaea* were GC-357-F/907-R and GC-ARC787-F/ARC1059-R, respectively (Sass et al., 2001; Hwang et al., 2008). Primer pair GC-Bac357-F/Bac907-R generated a 550-585-bp fragment (Table 2), whereas primer pair GC-ARC787-F/ARC1059-R generated a 273-bp fragment. PCR reaction mix (50 µl) for GC-Bac357-F/Bac907-R primer pair contained the following concentrations: 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.3 µM of each primer (Primm, Milan, Italy), 1.5 U of Taq DNA polymerase. All reactions components, except primers, were from Invitrogen (Invitrogen, Milan, Italy). PCR amplification with

GC-ARC787-F/ARC1059-R primer set was carried out by using the "Fast Start High FastStart HiFi PCR System dNTPack" kit following the manufacturer's indications. The PCR program for primer sets GC-Bac357-F/Bac907-R and GC-ARC787-F/ARC1059-R, was performed as described by Sass et al. (2001) and Hwang et al. (2008), respectively.

Target group	Name	Target gene	Amplicon (bp)	Reference
Bacteria	Bac357-F Bac907-R	16S rRNA	550-585	Favia et al. 2007
Hydrogen-producing bacteria (HPB)	hydF1 hydH	*hyd*A	700	Xing et al. 2008
Acetogens	fhs1 FTHFS-r	*fhs*	250	Xu et al. 2009
Sulphate-reducing bacteria (SRB)	Drs1+-F Dsr-R	*dsr*A	221	Kondo et al. 2004
Archaea	Arch 931-F ArchM1100-R	16S rRNA	169	Einen et al. 2008
Methanosarcinales (MSL)	Msl812-F Msl1159-R	16S rRNA	354	Yu et al. 2005

Table 2. Real time PCR primer sets used in this study.

All PCR reactions were performed using as template dilutions corresponding to 5 µl of the DNA extracted from H and 1 µl of the DNA extracted from M and MM (100 ng). DGGE gels were prepared with a gradient maker (BioRad, Milan, Italy) according to the manufacturer's instructions. The PCR products (10 µl) were loaded onto 7% polyacrylamide gels containing a denaturant gradient of 30-70% or 40-60% for *Bacteria* and *Archaea*, respectively (100% denaturant contained 7 M urea and 40% [v/v] formamide). Electrophoresis was run at 90 V for 17 h in 1 × TAE buffer at a constant temperature of 60°C using a D-Code electrophoresis system (BioRad). Gels were stained with SYBR(R) Green I Nucleic A (Invitrogen) and documented with the GelDoc 2000 apparatus (BioRad) by using the Diversity Database software (BioRad). DNA bands of interest were excised from the gels, transferred to 50 µl of Tris-HCl 10 mM and incubated at 37°C for 4 hours. Eluted DNA (5 µl aliquots) were re-amplified by standard PCR conditions using DGGE primer set without GC clamp. The obtained sequences (Macrogen, Seoul, Korea) were compared with the sequences deposited in the National Center for Biotechnology Information (NCBI) database by using BLAST program. Sequence alignment and maximum likelihood phylogenetic tree construction were carried out using the MEGA software, version 5.0 (Tamura et al., 2011).

DGGE gel images were analyzed using the Quantity One software (Biorad). Lane background was subtracted by the "rolling disk" tool. Bands were detected automatically and matched manually. DGGE-based molecular parameters, namely dynamycs (Dy), richness (Rr) and community organization (Co), were calculated as previously described (Mertens et al., 2005, Marzorati et al., 2008). Briefly, Dy was calculated from the similarity matrix (100-%similarity); Rr was the total number of bands multiplied by the percentage of denaturing gradient used; Co was the percentage

of Gini coefficient, a value describing the degree of evenness within a community by measuring the normalized area between the Lorenz curve and the perfect evenness line.

2.4 Real-time PCR analysis

Quantitative PCR assays were performed using primer set reported in Table 2. Considering that in anaerobic reactor most *Archaea* are methanogens (Yu et al., 2005), an archaeal PCR real time assay was used to estimate quantitatively methanogens. PCR SYBR green reactions were prepared by using the "Brilliant SYBR Green QPCR Master Mix" kit (M-Medical) and carried out in 96-well plates on the I-Cycler (Biorad). The reaction mix (25 µl) contained: 1× Brilliant SYBR Green (2.5 mM $MgCl_2$), 0.12 µM of each primer, and 5 µl of the DNA extracted from H and 1 µl of the DNA extracted from M and MM. For each sample, a real time assay was carried out (one amplification per extracted DNA). In the case of primer set Msl812-F/ Msl1159-R extra $MgCl_2$ was added to a final concentration of 4.0 mM. The thermal cycling program consisted of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at X°C (X = 58°C for Bac357-F/Bac907-R, 49°C for hyd-F1/hyd-H, 55°C for fhs1-F/THFS-R, 59°C for Drs1+-F/Dsr-R, 64°C for Arch 931-F/ArchM1100-R, 60°C for Msl812-F/Msl1159-R) and 1 min at 72°C. Finally, a melting curve analysis was performed for verifying the specificity of PCR products. The program was as follows: denaturation of 1 min at 95°C, cooling of 1 min at 55°C and then 95°C again, at a rate of +0.5°C per cycle [81 cycles, 10 s/cycle]. At the end of the cycling, Cycle threshold (Ct) values were calculated using the Biorad real-time software (version 3.0a) according to the manufacturer's instructions. Standard curves were generated by tenfold diluting the standard plasmids to obtain a series of concentrations ranging from 10 to 10^8 copies of plasmid DNA per 5 µL volume. The standard plasmids were prepared by cloning into pGEM T-Easy vector (Promega, Milan, Italy) or pCRII-TOPO vector (Invitrogen) the amplicons obtained by PCR amplification of the target genes (Table 2) using as template genomic DNA from *Asaia* sp. (AM404260) in the case of *Bacteria* (Favia et al., 2007) or DNA isolated from an anaerobic batch digester in the case of other target groups. The cloned gene fragments were: *Methanobrevibacter sp.* (DQ402034) with 98% similarity for *Archaea*; *Methanosarcina mazeii LM5* (DQ987528) with 98% similarity for *Methanosarcinales*; *Desulfobacterium autotrophicum* (CP001087) with 98% similarity for sulfate-reducing bacteria; *Clostridium beijerinckii* (CP000721) with 76% similarity for acetogens; uncultured bacterium (EU828435) with 75% similarity for hydrogen-producing bacteria. Plasmid DNA was extracted and purified from *E. coli* using the QIAprep Spin Miniprep kit (Qiagen, Milan, Italy). DNA concentration was determined by UV_{260} absorption on a SmartSpec 3000 spectrophotometer (Biorad) and its purity checked on the basis of 260/280 nm absorption ratio. Conversion of 16S rRNA gene copy numbers, determined by real-time PCR analysis, to cell number was done considering the average 16S rRNA gene copy numbers of bacteria (4/cell) and methanogens (2.5 copies/cell) reported in the Ribosomal RNA Database (rrnDB, Lee et al, 2009). In the case of real-time PCR targeting functional genes, it was assumed that copy number was equivalent to cell number based on the premise that the majority of

known bacteria in the database have a single copy of the functional gene (Kondo et al, 2004, Xu et al, 2009).

3. Results

3.1 Operation performance of anaerobic bioreactors

The two-stage hydrogen-methane and the single-stage methane anaerobic digestion processes were monitored for 2 and 1 month, respectively. In the acidogenic reactor (H) the content of hydrogen in biogas had an average value of 46% (± 6.4) v/v, ranging from a minimum of 28% to a maximum of 55%; methane was detected from 25 day to 50 day of operation in proportion of 1-5% to the total biogas. Hydrogen production was discontinuous with a range of production rate of 0.27-3.09 L_{H2}/L d and an average hydrogen yield of 95 L_{H2}/KgVS added. Low hydrogen production rate was registered during the days of deficient feeding, whereas high yield, with the exception of day 46, was recorded afterwards with a maximum at day 38. In the methanogenic reactor of two-stage process (M), methane production rate varied from a minimum of 0.11 L/L d to a maximum of 0.64 L/L d, with an average rate of 0.48 L_{CH4}/L d. The methane content in biogas was 66.8 (± 4.3) v/v. In the methanogenic reactor of single-stage process (MM) the methane production rate was 0.53 L_{CH4}/L d with a methane content of 54.3 (± 3) v/v.

VS removal rate was 31% for H and 67% for the overall process, whereas the single-stage process showed a 69% VS removal. In H carboxylic acids represented approximately almost 50% of the total volatile organic compounds (VOCs) in liquid digestate phase. The major acidogenic byproduct was hexanoic acid, representing 61% of total VFAs, followed by acetate and secondarily butyrate and propionate. Total VFAs concentration was of about 4000 mg acetate/L and acetate accumulated with an average value of about 3400 mg acetate/L. In M the concentration of acetate decreased drastically – it was 1518 mg acetate/L in the feeding source - and remained at low level (< 300 mg acetate/L) indicating the activity of acetotrophic methanogens. Butyrate was completely degraded and, because it cannot be directly used by methanogens, the complete degradation suggested the presence of actively sintrophic bacteria in the microbial consortia. At day 59, a partial accumulation of VFA (1045 mg acetate/L) and consequently inhibition of the process occurred (1890 mg acetate/L at day 66). In MM the total VFAs were detected at concentrations (763-1056 mg acetate/L) about two/three-fold higher than M with acetate as the major product and butyrate under the limit of detection. For both processes organic nitrogen mineralization was over 70% with average ammonium concentrations (1.5 g/kg) not inhibitory to the methanogenic *Archaea*. pH attested at average values of 5.4 (± 0.2) for H and 7.5 (± 0.07) and 7.75 (± 0.15) for M and MM, respectively. Whereas in M and MM pH was always stable, in H a significant of decrease of pH from 5.0 to 5.5 was observed after approx. day 51.

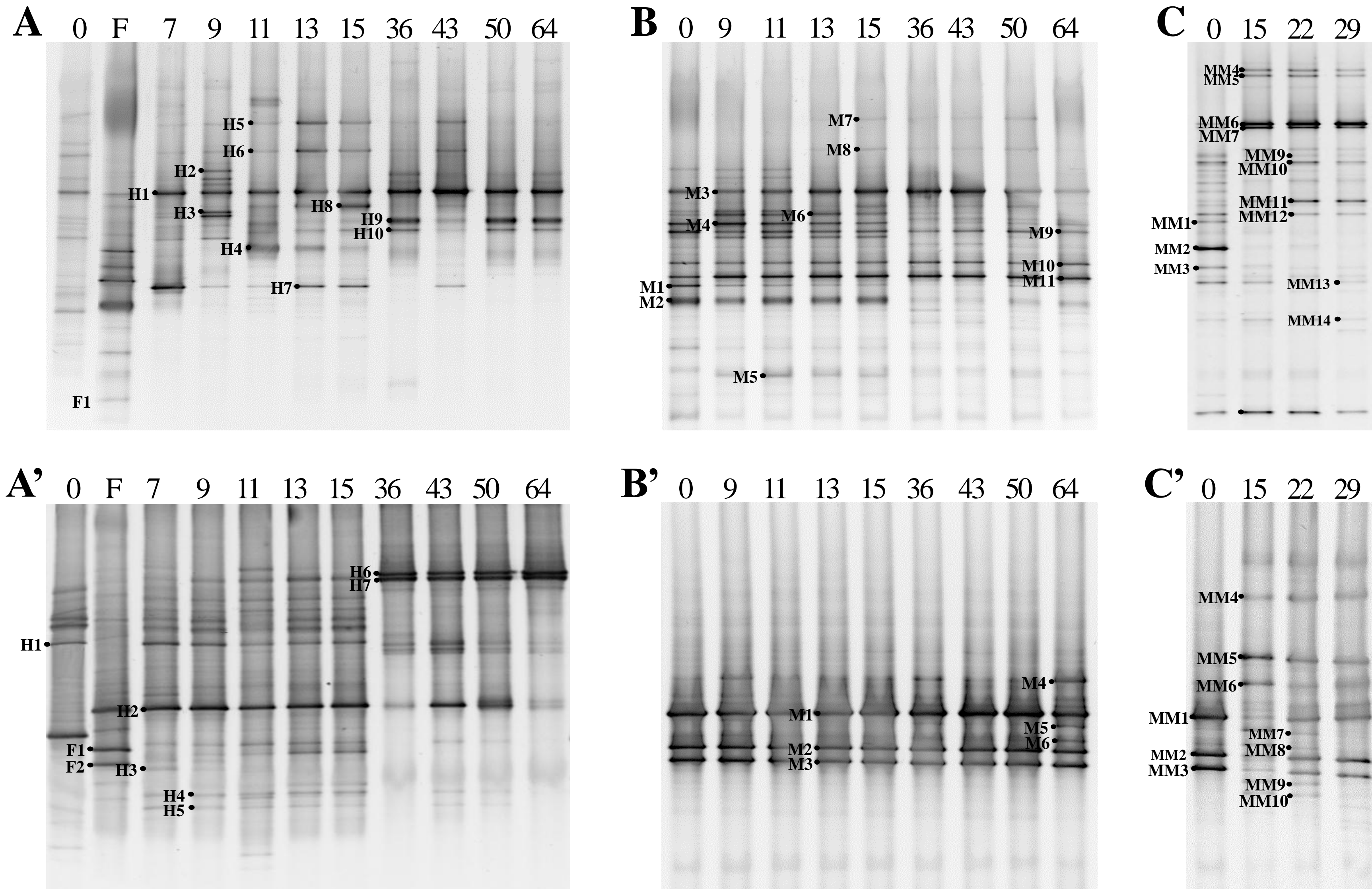

Figure 1. Bacterial (A, B, C) and archaeal DGGE (A', B', C') profiles of the 16S rRNA gene PCR products amplified from DNA extracted from samples obtained from acidogenic (A, A') and methanogenic (B, B') two-stage process reactors and from single-stage process reactor (C, C'). Lanes are labeled with the sampled time of the reactor (days), lane F indicate the DGGE profile of the feeding source. Dots and numbers indicate the bands sequenced.

3.2. Microbial community characterization of hydrogenogenic acidogenic process

3.2.1. Qualitative analysis

The dynamics of the bacterial community structure were studied by PCR-DGGE over a 65 days period (Fig. 1A). The variations were particularly marked during about the first month of the operation; after the starvation period, a quite stable, more even and low diversity community was established Fig. 4).

The major bands were retrieved for sequence identification. The sequences affiliations and the phylogenetic relationships of identified sequences with reference strains are shown in Table 3 and Figure 2. Most bands were not closely related to known species. All the 11 identified bands were assigned to the phylum of Firmicutes; 9 fell into the *Clostridiales* order (2 *Clostridium* sensu stricto) and 2 into the *Thermoanaerobacterales* order. Band H1, already identified at the steady-state (Tenca et al., under submission) as strictly related to an uncultured species from an hydrogen fermentor (Lee et al., 2010), was detected throughout the entire time-course process. According to the intensity of this band this bacterium had a high population density appearing as a predominant microorganism. Several other bands, namely H_2, H11, H14, H15, H39, mainly detected from day 9 to day 15, were also found to be closely related (99-100% similarity) to uncultured bacteria detected by the same authors.

Species relative to bands H1, H5, H6 and H8 were assigned (99% according to RDP classifier, confidence threshold of 80%) to the family of *Ruminococcaceae*, and showed 94% similarity to *Clostridium* sp. BS-1, a newly sludge isolate which ferments D-galactitol producing H_2, acetate, butyrate and hexanoic acid (Jeon et al., 2010), and 93% similarity to *Clostridium* sp. strain Z6, isolated from paper mill wastewater, and *Clostridium sporosphaeroides*, capable to produce hydrogen (and acetate) from glutamate. Bands H_2 and H3 were assigned to unclassified *Lachnospiraceae;* they were closely related to an uncultured bacterium clone found in the hydrogen digester of Lee et al. (2010) and showed >96% similarity to uncultured *Clostridium* clones involved in the cellulosic and lignocellulosic waste digestion (Shiratori et al., 2006; Yan et al, unpublished). Bands H9 and H10, present with high intensity mostly after 36 day of operation and at stable hydrogen production, felt into the *Clostridiaceae* cluster I and were identified with 100% similarity to many uncultured bacteria associated to swine and human feces, and anaerobic reactors treating swine manure (Perkins et al., unpublished; Talbot et al., unpublished). Band H7, present with only at the beginning of the process, matched (99.4%) with *Clostridium cellulosi*, a thermophilic cellulolytic bacterium previously detected in several hydrogen-producing microflora (Ueno et al., 2001a; Fang et al., 2002). Band H4, detected at days 11-15, fully matched with an uncultured *Thermoanaerobacterium* from an hydrogen reactor treating food waste (Wang Phd thesis, 2008). H4 was also closely related (99.1%) to *Thermoanaerobacterium thermosaccharolyticum*, a thermophilic saccharolytic microorganism involved in acetate/butyrate fermentation from complex carbohydrate with production of large hydrogen amount (Ueno et al., 2001a, b).

Band	Closest Relative	Accession number.	Identity (%)
F1	Uncultured bacterium clone B55-K-B-C04	DQ887963	99.8
	Clostridium sp. F-02	AB504377	96.7
H1, M3	Uncultured bacterium clone VKW-TB-3.3	GQ849504	99.8
	Uncultured *Clostridia* bacterium clone S44	EU887963	98.0
H2	Uncultured bacterium clone VKW-TB-7	GQ849508	99.4
	Uncultured bacterium clone EBR-02E-0436	AB221356	97.2
	Uncultured *Lachnospiraceae* bacterium clone 670	JN173113	95.4
H3	Uncultured bacterium clone VKW-TB-7	GQ849508	100
	Uncultured bacterium clone EBR-02E-0436	AB221356	97.8
	Clostridia bacterium enrichment culture clone 40D01	FJ796699	95.9
H4	Uncultured bacterium clone TKW-HPB-6	GQ505063	100
	Uncultured *Thermoanaerobacteriaceae* bacterium clone THPB-7	AM408569	99.7
	Thermoanaerobacterium thermosaccharolyticum strain D120-7	AF247003	99.1
H5, M7	Uncultured bacterium clone VKW-TB-3.3	GQ849504	100
	Uncultured *Clostridia* bacterium clone S44	EU887963	98.2
H6, M8	Uncultured bacterium clone VKW-TB-9	GQ849510	99.8
	Uncultured *Clostridia* bacterium clone S39	EU887964	97.9
H7, M1	*Clostridium cellulosi* strain D3	FJ465164	99.4
H8	Uncultured bacterium clone VKW-TB-9	GQ849510	99.4
	Uncultured *Clostridia* bacterium clone S39	EU887964	97.6
H9	Uncultured *Clostridium* sp. clone BBC516	GQ868438	100
H10	Uncultured bacterium clone GP_1aaa01g02	EU473328	100
	Uncultured bacterium clone 17s-53	HM036018	100
M2	Uncultured *Thermotogae* bacterium clone QEEA3DA10	CU918794	99.8
	Petrotoga mobilis strain SJ95	NR027612	90.4
M4	Uncultured bacterium clone CFB-1	AB274490	99.8
	Uncultured *Clostridiales* bacterium clone IAFpp8j31	GU214161	98.0
M5	Uncultured bacterium clone HAW-R60-B-727d-T	FN436062	99.8
	Clostridium sp. 6-31	FJ808611	93.2
M6	Uncultured *Firmicutes* bacterium clone TG-57	AB451852	99.4
M9	Uncultured bacterium clone MS14315-B003	FN994055	99.8
	Clostridium caenicola strain EBR596	AB221372	95.6
	Clostridium thermocellum ATCC 27705	NC009012	95.8
M10	Uncultured *Firmicutes* bacterium clone SHBZ858	EU639242	99.8
M11	Uncultured bacterium clone FB	AB494315	100
	Clostridium sp. 6-31 39	FJ808611	89.0
MM1	Uncultured bacterium clone SMQ16	AM930325	99.1
	Bacillus infernus strain TH-23	U20385	96.8
MM2	Uncultured bacterium clone SMQ9	AM930323	100.0
	Bacillus infernus strain TH-23	U20385	97.8
MM3	Uncultured bacterium clone SMQ9	AM930323	99.6
	Bacillus infernus strain TH-23	U20385	97.4
MM4	Uncultured *Bacteroidetes* bacterium clone QEDV3DE11	CU919667	99.6
	Uncultured *Porphyromonadaceae* bacterium clone TCB179x	DQ647169	90.4
MM5	Uncultured bacterium clone DC87	HM107074	100.0
	Uncultured *Porphyromonadaceae* bacterium clone TCB179x	DQ647169	90.7
MM6	Uncultured *Bacteroidetes* bacterium clone QEDV3DE11	CU919667	100.0
	Uncultured *Porphyromonadaceae* bacterium clone TCB179x	DQ647169	90.7
MM7	Uncultured bacterium clone DC87	HM107074	99.8
	Uncultured *Porphyromonadaceae* bacterium clone TCB179x	DQ647169	90.6
MM8	Uncultured *Anaerobaculum* sp. clone SHBZ995	EU639374	99.1
	Anaerobaculum mobile 16S rRNA gene, type strain NGA	AJ243189	97.0
MM9	Uncultured *Clostridium* sp. clone BBC516	GQ868438	100.0

MM10	Uncultured bacterium clone CFB-1	AB274490	100.0
	Clostridium sp. Irt-JG1-73	AJ295661	95.6
MM11	Uncultured bacterium clone thermophilic_alkaline-115	GU455355	99.8
	Uncultured bacterium clone 1-1B-02	JF417893	99.4
MM12	Uncultured bacterium clone 1-1B-28	JF417919	97.5
	Clostridia bacterium enrichment culture clone WSC-8	HM635205	90.5
MM13	Uncultured bacterium clone A55_D21_H_B_E12	EF559057	100.0
	Uncultured *Thermacetogenium* sp. clone De217	HQ183800	98.5
MM14	Uncultured bacterium clone 2-1B-14	JF417959	99.8
	Clostridium sp. F-02	AB504377	96.7
	Tepidanaerobacter syntrophicus	AB106354	96.7

Table 3. Closest relatives of bacterial 16S rRNA gene sequences of DGGE bands from feeding source (F) and thermophilic acidogenic (H), methanogenic two-stage (M) and single-stage (MM) anaerobic digestion processes.

Band	**Closest relative**	**Accession number.**	**Identity (%)**
F1	Uncultured archaeon clone F776O8Q02CA0E0	GU856162	100
	Methanoculleus sp. MAB1	AF107103	99.1
F2	*Methanoculleus* sp. 22	EF112188	98.6
	Methanoculleus palmolei strain DSM 4273	NR_028253	98.2
H1	Uncultured archaeon clone F776O8Q02B5DGG	GU840733	98,5
	Uncultured *Methanosaeta* sp. clone Pav-sed-103	GU135461	98,5
H2	Uncultured *Methanosarcinales* clone QEEG1BH021	CU916707	97,3
	Methanosaeta concilii strain Opfikon, DSM 2139	NR_028242	96.9
H3	*Methanothermobacter thermautotrophicus*	HM228400	97,7
	Methanothermobacter marburgensis strain Marburg	CP001710	97,7
H4	Uncultured archaeon clone F776O8Q02CKVTD	GU868593	98.0
	Methanosarcina siciliae strain C2J	U89773	97,0
H5	Uncultured *Methanosarcinaceae* archaeon	GU734641	97.0
	Methanosarcina mazeii strain LM5	DQ987528	96,5
H6	Uncultured archaeon clone F776O8Q02B98NU	GU880656	100
	Uncultured *Methanogenium* sp. isolate PSW32	EF043533	98.8
H7	Uncultured archaeon clone F776O8Q02B1HCR	GU837997	99.1
	Uncultured *Methanogenium* sp. isolate PSW32	EF043533	98.0
M4	Uncultured archaeon clone F776O8Q02CCQ51	GU861598	98.2
	Methnosarcina siciliae strain C2J	U89773	97.7
M1,	*Methanosarcina mazei* strain sk0808-2	FJ155845	98.6
MM1	*Methanosarcina siciliae* strain C2J	U89773	98.2
M2,	Uncultured archaeon clone K09-30-17	AB541940	100
MM2	*Methanosarcina mazei* strain sk0808-2	FJ155845	99.5
M3,	*Methanosarcina mazei* strain sk0808-2	FJ155845	99.1
MM3	*Methanosarcina siciliae* strain C2J	U89773	98.6
M5,	*Methanothermobacter wolfeii* strain KZ24a	DQ657904	99.4
MM7	*Methanothermobacter thermautotrophicus* strain KHT-2	AB020530	99.4
M6,	*Methanothermobacter thermautotrophicus* strain AM1	HM228400	98.9
MM8	*Methanothermobacter marburgensis* str. Marburg	CP001710	98.9
MM4	Uncultured *Methanosarcinaceae* archaeon band 028	GU734641	99.0
	Methanosarcina mazeii strain LM5	DQ987528	97.7
MM5	Uncultured *Methanosarcinales* archaeon clone A6	EU586063	100
	Uncultured *Methanosaetaceae* archaeon band 013	GU734639	99.4
MM6	Uncultured archaeon clone F776O8Q02CBY77	GU870909	98.3
	Methanosarcina siciliae strain C2J	U89773	97.7
MM9	*Methanosarcina mazeii* strain LM5	DQ987528	99.5
	Methanosarcina lacustris strain MS	AY260431	99.5
MM10	*Methanosarcina mazeii* strain LM5	DQ987528	97.3
	Methanosarcina lacustris strain MS	AY260431	97.3

Table 4. Closest relatives of archaeal 16S rRNA gene sequences of DGGE bands. Feeding source, F; acidogenic reactor, H; methanogenic reactor of two-stage process, M; single stage (MM).

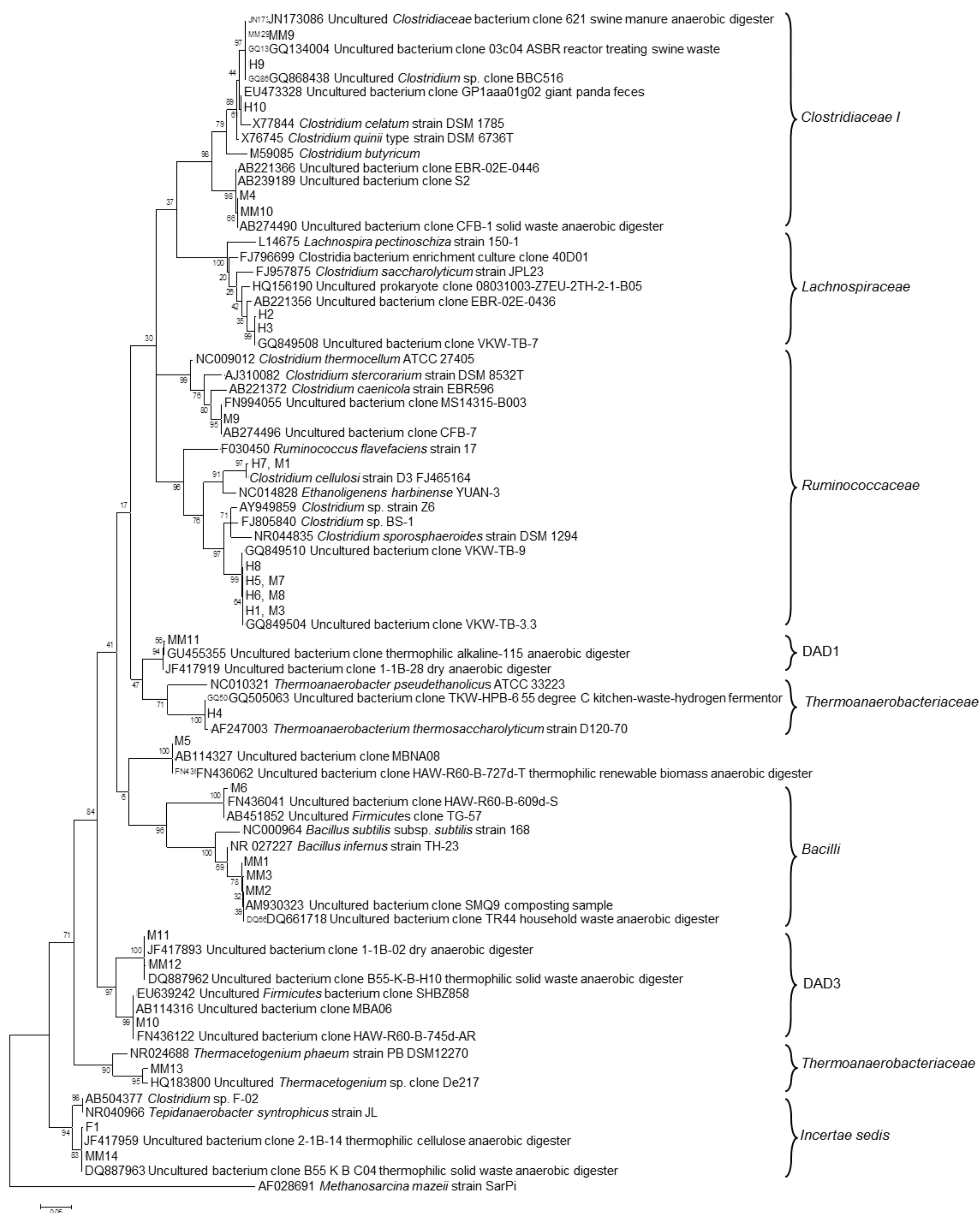

Figure 2. Phylogenetic tree showing the phylogenetic relationships of bacterial 16S rRNA sequences with reference sequences deposited at the GenBank database. The tree was construct using the Maximum Likelihood algorithm and the Tamura Nei parameter correction and was bootstrapped 2000 times.

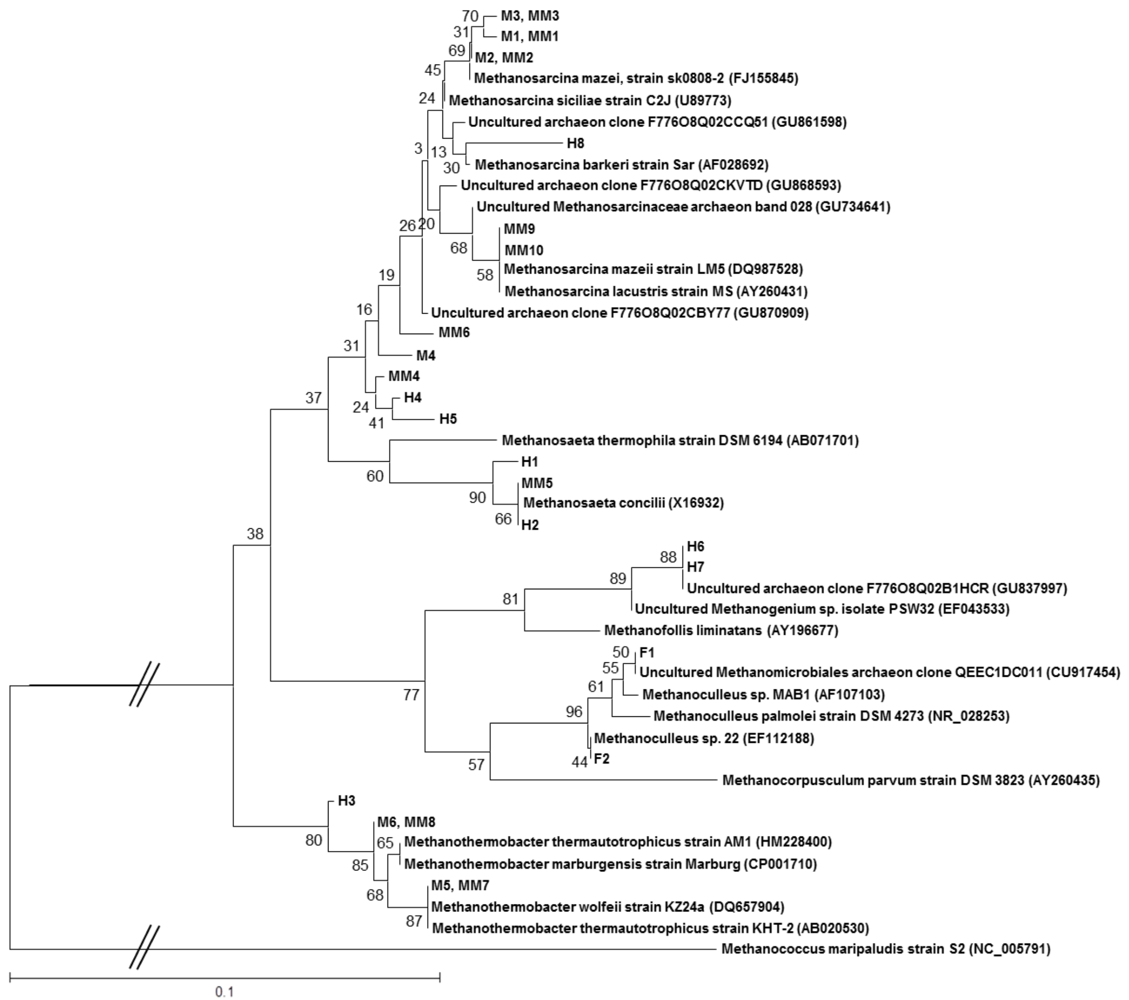

Figure 3. Phylogenetic tree showing the phylogenetic relationships of archaeal 16S rRNA sequences with reference sequences deposited at the GenBank database. The tree was construct using the Maximum Likelihood algorithm and the Tamura Nei parameter correction and was bootstrapped 2000 times.

Thermoanaerobacterium spp., mostly T. *thermosaccharolyticum* species, has been previously detected in other thermophylic acidogenic process fed with organic waste (Ueno et al., 2006; Shin et al., 2004; Lee et al., 2010; Chu et al., 2010). In order to account for the methane production (< 5% of produced biogas) recorded at days 25-50 of operation, a PCR-DGGE analysis was carried out on *Archaea*. Phylogenetic affiliation of sequences is reported in Table 4 and a phylogenetic tree is shown in Fig. 3. Results evidenced initially the presence of a dominant band (band 2) affiliated to *Methanosaeta*, and after approximately 1 operational month a strong change with the occurrence of uncultured archaeons strictly affiliated to *Metanogenium* sp. (band6 and band7), potential candidate accountable for the detected methane.

3.2.2. Quantitative analysis

Quantitative measurements used to monitor the abundance and variations of bacterial populations present in the acidogenic reactor are shown in Fig. 5. *Bacteria* were present at high concentrations, in the order of magnitude of 10^8 up to 10^9 bacteria per ml. Within *Bacteria*, the group of hydrogen-producing bacteria represented a minimal proportion to total *Bacteria*, varying within percentage ranges of 0.05-1.4% (average value of 0.4%), whereas the acetogens were determined at percentage ranges of 0.6-6.2% to total *Bacteria*. Number of hydrogen-producing bacteria remained almost constant during the process (10^6 bacteria per ml), though a decrease was observed from day 36 to day 50 with a ratio hydrogen-producing bacteria to total *Bacteria* of about 0.05%. The acetogens, present in the first ten days of hydrogen production at the same level of hydrogen-producing bacteria, in the following days of operation were enumerated one order magnitude higher than hydrogen-producing bacteria. The sulfate-reducing bacteria (SRB) were typically enumerated one order magnitude lower than hydrogen-producing bacteria (about 10^5 bacteria per ml); a slight increase was observed at day 43 in correspondence of a declining trend of hydrogen-producing bacteria and also of an increase of *Bacteria*. Quantitative PCR on *Archaea* showed a drastic abatement of this group after heat shock treatment (10^4 bacteria/ml). Their number, reasonably showing a lowering trend during the period of deficient feeding, was 2 or 3 order of magnitude lower than *Bacteria*. Within *Archaea*, *Methanosarcinales* represented approx. 1% of total *Archaea*, declining to 0.1% at day 64. In the influent, *Methanosarcinales* were detected at low concentration too (7%).

3.3. Microbial community characterization of methanogenic processes

3.3.1. Qualitative analysis

The bacterial and archaeal methanogenic species developed along the time in the methanogenic reactors from two-stage (M) were investigated and compared with those of single-stage process (MM) (Fig.1). DGGE analysis carried out on *Bacteria* evidenced in M, like in H, a bacterial community dominated by *Firmicutes* (Table 4 and Figure 3).

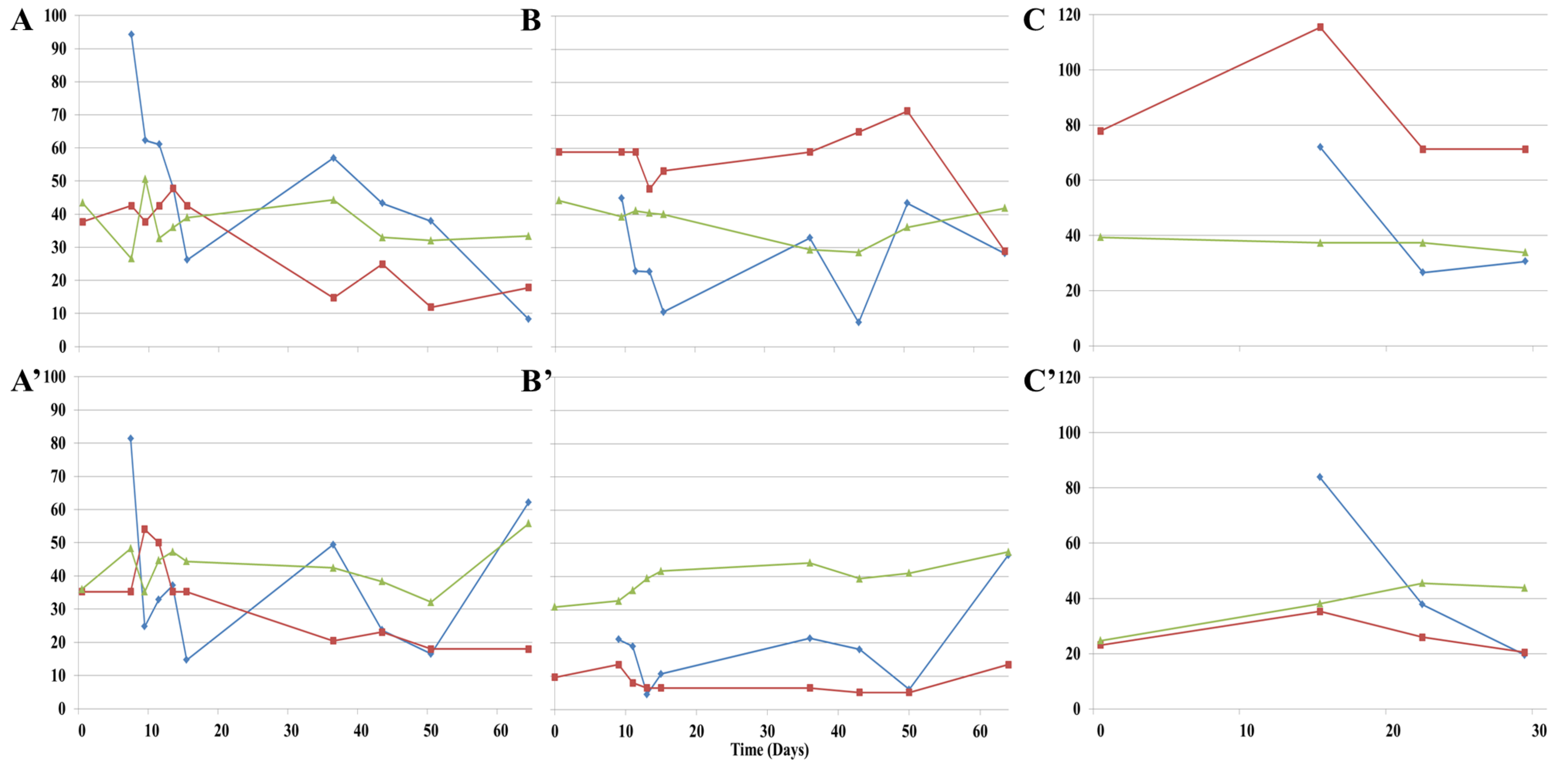

Figure 4. (a) Dynamics (Dy), (b) richness (Rr) and (c) community organization (Co) parameters from bacterial (A, B, C) and archaeal (A', B', C') DGGE profiles of acidogenic (A, A') and methanogenic (B, B') two-stage process reactors and from single-stage process reactor (C, C'). Dy (blue lines) is expressed as %, Rr (red lines) and Co (green lines) are dimensionless.

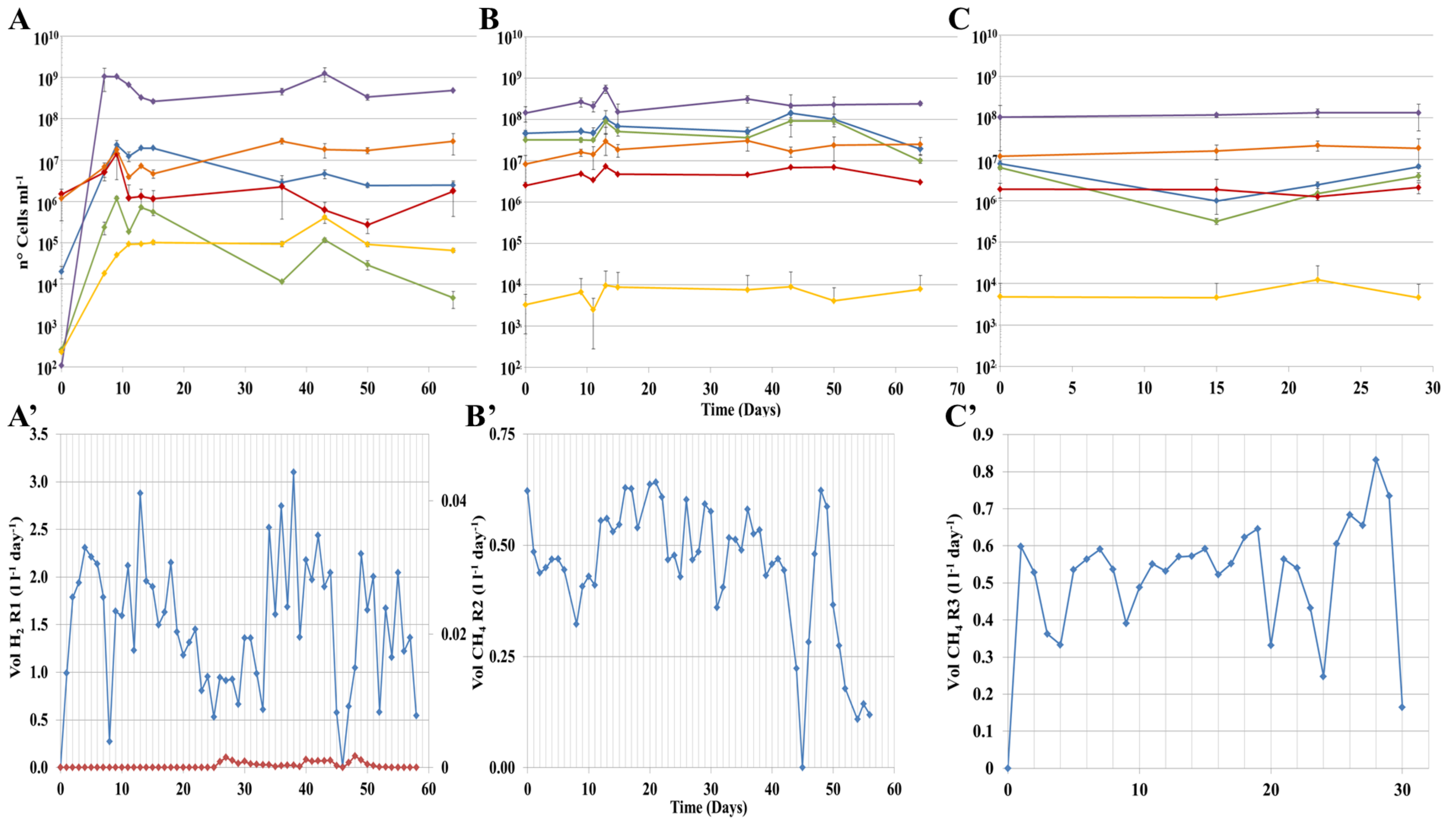

Figure 5. Concentrations of microorganisms (A, B, C) and biogas production (A', B', C') in acidogenic (A, A') and methanogenic (B, B') two-stage process reactors and in single-stage process reactor (C, C'). A,B,C: *Bacteria* (purple lines), hydrogen-producing bacteria (red lines), sulfate-reducing bacteria (yellow lines), acetogens (orange lines), *Archaea* (blue lines) and *Methanosarcinales* (green lines). A': hydrogen (blue line), methane (red line); B' and C': methane (blue line).

The bacterial community partially changed during the time course of the process, with only some bacteria present throughout the process (M11, M10, M9, M3, M2). The highest changes were observed at 36 day, after the starvation period, and at day 50, prior the partial accumulation of VFA (acetate and propionate) in the reactor (data not shown). The community was characterized by a higher richness (average value of 55%) than H (32), with several intense bands and a large number of bands of lesser intensity. In M was found *C. cellulosi* (M1) and several other microorganisms (bands M3, M7, M8) already identified in H (H5, H2, H14) and assigned to unclassified *Ruminococcaceae*. Also belonging to *Ruminococcaceae* family was the persistent band M9, present throughout the operation, and closely related (99.2-99.8%) to several uncultured bacteria from anaerobic solid waste digester (Sasaki et al., 2007; Sasaki et al., 2011; Goberna et al., 2009; Tang et al., 2011) and, more distantly (95-96%) to the cellulose/cellobiose-digesting bacteria *Clostridium thermocellum* (Ait et al, 1979) and *Clostridium caenicola* (Shiratori et al., 2009). Bands M11 and M10 belonged (99-100% similarity) to a unknown cluster named DAD cluster 3 (Tang et al., 2011) or MSW cluster 1 (Tang et al., 2004) which mainly consists of microorganisms isolated from thermophilic solid waste digesting reactors (Tang et al., 2004; Tang et al., 2011; Goberna et al., 2009; Sasaki et al., 2011). It might be speculated that could play potential roles in VFA degradation though further investigation is needed to explain the function of these bacteria. Similarly, band M5 was closely related (99.8% similarity) mainly to clones from thermophilic reactors (Goberna et al., 2009; Tang et al., 2004) and clustered close to the *Veillonellaceae* family. Band M2, observed only in the first 15 days of operation, was phylogenetically correlated (99.8% similarity) to an uncultured bacterium belonging to *Thermotogae* phylum. The single family of this phylum, the *Thermotogaceae*, is constituted by anaerobic hyperthermophilic bacteria capable of utilizing a great variety of carbohydrates, including xylan and cellulose, and generating hydrogen (Gupta and Bhandari, 2011; Eriksen et al., 2010). Also observed only the first days of operation were bands M19 and M33. Band M4 grouped into *Clostridiaceae* cluster I and was closely related (99.8-99.2%) to clones from thermophilic digesters degrading cellulose and starch (Sasaki et al., 2007; Shiratori 2006). Also Band M6 was related (>99% similarity) to several clones from thermophilic digesters (Kudo et al., unpublished; Satke, unpublished), though classification for this microorganism by RDP database was limited to the phylum level.

In MM the bacterial community changed deeply compared to the beginning (rate 72%) and was always characterized by a high (average value of 84) microbial diversity (Fig. 4). Initially, the community was dominated by *Firmicutes* bacteria belonging to both *Clostridia* and *Bacilli* classes. After the day 15, bands affiliated to *Bacilli* (MM1, MM2, MM3) disappeared or became less intense, whereas microorganisms related to *Bacteroidetes* appeared with high intensity (MM4, MM5, MM6, MM7). A microorganism stably detected throughout the process was *Anaerobaculum* (band MM8), fermenting organic acids and a limited number of carbohydrates to acetate, hydrogen and CO_2 and also peptide-fermenting (Menes and Muxì, 2002). Bands MM1, MM2 and MM3 were all affiliated with high similarity (99.1-100%) to uncultured bacteria and to the species *Bacillus infernus* (96.8-97.8%). *B. infernus* is the only

anaerobic species in the genus able to ferment glucose and utilize formate and lactate for growth (Boone et al., 1995).The spore-forming, facultative anaerobe *Bacillus* genus it has been often detected in mixed cultures in anaerobic reactors (Shin et al., 2004; Ueno et al., 2006; Kim et al., 2006). It is able to produce hydrogen (Nandi and Sengupta, 1998) with the maximum hydrogen production rate determined in 2,28 mol H_2/mol glucose for *Bacillus coagulans* (Kotay and Das, 2007). Bands MM4, MM5, MM6 and MM7 were assigned, according to the RDP Classifier, to unclassified *Porphyromonadaceae*. This family consists of acidogenic bacteria capable of producing various VFA from carbohydrates or proteins and its detection in anaerobic digesters is frequently reported (Ziganshin et al 2011, Li et al. 2009). Band MM10, as shown for M4 of M, grouped into the *Clostridiaceae* cluster I. Band MM11, present with high intensity, could not be assigned to any known family and showed more than 99% sequence similarity to clones from thermophilic anaerobic reactors (Zhang et al., 2010, Tang et al., 2011). Band MM12, as previously observed in M (bands M10 and M11), clustered with bacteria from thermophilic reactors. Band MM13 resulted strictly correlated (98.5% similarity) to *Thermacetogenium*, a thermophilic syntrophic acetate-oxidizing bacterium capable to form methane in association with hydrogenotrophic methanogens (Hattori et al., 2000). Band MM14 was closely related (99.8%) to uncultured bacterium from thermophilic bioreactor and (96.7%) to the syntrophic acetate-oxidizing *Tepidanaerobacter* genus (Sekiguchi et al., 2006).

DGGE images performed on *Archaea* are showed in Fig. 1. Phylogenetic affiliation of sequenced bands and the relative phylogenetic tree are reported in Table 4 and Fig. 3. In particular, the DGGE profiles from H showed a constant archaeal community throughout the process (dynamics rate < 10%) with a modest change observed only at 64 day in correspondence to a partial accumulation of VFAs (>1500 mg acetate/L). The community was characterized by three strong intensity bands (M1, M2, M3), closely related (98.6-100%) to each other and to *Methanosarcina mazeii* and a number of weak bands. Also M4, a band of less intensity, showing high similarity (98.2%) to an uncultured archaeon, grouped into the *Methanosarcinaceae* cluster. Bands M5 and M6 appeared the last day of sampling and were closely related (99.4% to 98.9%) to *Methanothermobacter* genus though it was not possible a precise affiliation of the sequences. The taxonomy of thermophilic *Methanothermobacter* genus is complex and has been varied several times. According to a recent revision, it comprises six species with more than ten *Methanothermobacter* strains identified from several environments, including anaerobic digesters (Boone, 2000). All *Methanothermobacter* species depend entirely on H_2/CO_2 as substrates for energy and carbon sources; their growth is unusually highly exothermic, with a low biomass yield (Schill et al., 1999).

In MM, on the contrary of M, the community structure changed with time. Initially, the archaeal community was identical to that of H because of the use of the same inoculum sludge for the starting up of the two processes. After two weeks, the community was drastically shifted (rate of 84%) (Fig. 4), whereas thereafter it changed moderately becoming at the last sampling day a merge between the new and the initial sludge communities (Fig.1). The community was richer than that determined in M and, like in M, became more uneven during the process. The establishment of the new community

clashed with a quite stable methane production. Bands belonged mainly to the *Methanosarcinales* family (Table 4 and Fiugure 3). Bands MM4 and MM6, which showed highest similarities with uncultured archaeon, were closely (>97.7 similarity) related to *Methanosarcina* spp. Bands MM10 and MM9 both matched with high similarity, 97.3% and 99.5% respectively, with two *Methanosarcina* species, *Methanosarcina mazeii* and *Methanosarcina lacustris*. Band M5, which appeared with strong intensity at 15 day, already identified (Tenca et al. under submission) as a *Methanosaeta*-like organism, was found to group with *Methanosaeta concilii*. Bands MM7 and MM8 were identified as belonging to *Methanothermobacter* genus with high similarity (>98.0).

3.3.2. Quantitative analysis

In M the variations among the bacterial populations levels was less marked than those observed for H and the abundance of the different populations remained almost constant during the process (Fig. 5). *Archaea* and *Bacteria* were present at not very dissimilar concentration levels. The number of *Archaea* methanogens was higher than the value measured in H, attesting at concentrations of 10^7-10^8 bacteria per ml, a range of values quantified in several anaerobic digesters (Yu et al, 2005, Lee et al, 2008). Among *Archaea* methanogens, the dominant group were the *Methanosarcinales* which constituted approx. the 50% of the total archaeal concentration, in agreement with DGGE data analysis indicating a high abundance of *Methanosarcina*. *Archaea* and *Methanosarcinales* showed the same trend along the operation. The number of *Methanosarcinales* decreased at day 64 of operation, in accordance with DGGE data detecting at this sampling time bands related to the hydrogenotrophic *Methanothermobacter*. The SRB, potential competitors of methanogens at high concentration of sulfate, were enumerated four order magnitude lower than *Archaea* (10^3 bacteria per ml), confirming the good performance of the anaerobic process. The acetogens and the hydrogen-producing bacteria remained relatively stable during all the operation (average values of 2×10^7 and 5×10^6 bacteria per ml, respectively); interestingly the slightly variations observed in bacterial concentrations were diametrically opposed underling the competitive relationship between these two functional groups.

Quantitative measurements of microbial populations resident in reactor MM were similar to those determined in M (Fig. 5) Total *Bacteria*, acetogens, SRB and HPB were present in the two reactors at concentration of the same order of magnitude. On the contrary, both total *Archaea* and *Methanosarcinales* showed a lower bacterial density than M, *Archaea* not getting up to 10^7 bacteria per ml. It is likely that the different metabolic configuration of the two reactors accounts for this diversity. Within *Archaea*, the number of *Methanosarcinales* decreased with the changing of the community structure, dropping to only 20% to total *Archaea* at day 15 and increasing again to 40% at day 29.

4. Discussion

In this this study the dynamics of microbial populations in a two-stage and a single-stage reactors have been investigated and compared during approximately two-month operation. As models were used I-CTSR reactors operating with the same working conditions (inoculums of methanogenic reactors, feeding source, T, pH not controlled), though specific parameters, which are known to favor the microbiology of the two diverse anaerobic process configurations (HRT, loading rate, heat-shock treatment), were designed. The diversity and structural shift in microbial community were monitored along with the changes in the hydrogen/methane production and the concentration of intermediate metabolites.

The data obtained in the acidogenic reactor showed that in this study most bacteria were related to *Clostridiales* order and, more specifically, to the *Clostridium* genus. This genus comprises a great number of species which can be isolated from nearly every environment. The genus do not form a monophyletic group and includes, besides species sharing a common ancestry to the type species *Clostridium butyricum* (*Clostridium* sensu stricto or *Clostridiaceae* cluster I), many species phylogenetically related to other taxonomic clusters into *Clostridiales* (Ludwig et al. 2009). *Clostridium* spp. are well known for evolving hydrogen during anaerobic fermentation (Kataoka et al. 1997; McTavish 1998). They are capable of fermenting various types of carbohydrates to acetate, butyrate, hydrogen, carbon dioxide and other fermentations products including, lactate, ethanol. Among *Clostridium* spp. known to produce hydrogen are included the mesophilic *pasteurianum*, *butyricum*, *butylicum*, *acetobutylicum*, *acidosoli*, *beijerinckii*, *roseum*, *kluyvery* and the thermophilic *thermocellum*, *thermosuccinogenes*. Many studies showed that the prevalence of *Clostridium* spp. in anaerobic process generating hydrogen correlated with a stable microflora and good hydrogen production, process failures were typically associated with shifts versus other specimen (Jo et al., 2007). In our study, however, the majority of identified microorganisms were not referable to known species, with the exception of *C. cellulosi*. In this respect, it appeared that heat-treatment of inoculum has been efficient in killing vegetative cells and activate the development of spore-forming microorganisms. Many of the identified microorganisms were however related to microorganisms from other anaerobic reactors. In particular, several microorganisms were strictly phylogenetically related to uncultured bacteria found in thermophilic acidogenic anaerobic reactors fed with vegetable kitchen waste (Lee et al., 2010, Chu et al., 2010) and, more distantly, to *Clostridium* species falling into the *Ruminococcaceae* cluster (*Clostridium* sp. BS-1, *Clostridium* sp. Z6). In particular, the presence in the digester of species with degrading ability similar to that of *Clostridium* sp. BS-1 may account for the high detection of hexanoic acid in the reactor (36% of VFAs in the liquid phase). Bioavailable D-galactitol, a reduced form of D-galactose, is in fact contained in many fruits and vegetables that were used as feeding source. The occurrence of uncultured bacteria affiliated to *Clostridium* sp. Z6 has been previously reported. Chu et al. (2010) showed the dominance of microorganisms related with 93% similarity to *Clostridium* sp. strain Z6 and representing the 70% of a 16s rRNA gene

clone library. Similarly, Lee et al. (2010) showed that bacterial clones affiliated to *Clostridium* sp. strain Z6 accounted for 35% of the total sequence retrieved. Since in both studies the digesters operated at 55°C treating food waste without heat treatment of inoculum, it may be possible that the selection and growth of these microorganisms might be favored by a combination of various operational parameters (T, feeding source, reactor type, TS, pH, HRT). Both studies also showed the prevalence of *Thermoanaerobacterium thermosaccharoliticum* (*Thermoanaerobacterales*). This microorganism is characterized by high versatility conditions for H_2 production (O-Thong et al., 2008) and shows a rate of hydrogen yield nearly equivalent to that of *C. butyricum*, for which hydrogen rate production was calculated in 2.4 mol-H_2/mol-hexose (Ueno et al. 2001b). In our study, *Thermoanaerobacterium,* as well as *Clostridium cellulosi,* and other bacteria affiliated to *Lachnospiraceae* and most likely involved in the cellulose degradation, were detected in the reactor discontinuously and in the initial days of operation.

Changes and high diversity of community were evident in the first operational month as a consequence of process start-up instability and then starvation. During the second month of operation, the established bacterial community showed low microbial diversity, being essentially composed by only three microorganisms, in accordance with previous investigations on acidogenic thermophilic bioreactors (Lapara et al., 2000, Shin et al., 2004). Together with a putative D-galactitol degrading bacterium dominant throughout the process, two *Clostridium,* originating from the pig intestinal microbiota and consequently with a relevant role in the degradation of organic matter, were detected. The establishment of such a simple community suggests that it is likely that exists a form of cooperation between these bacteria that might result in a more efficient degradation of organic matter, therefore promoting the hydrogen production. Jeon et al. (2010) reported that production of butyric and hexanoic acids by *Clostridium* sp. BS-1 was enhanced in coculture with other *Clostridium* producing acetate. Considering that acetate was the major by-product in the system, present at concentrations of approx. 2000 mg acetate/L, it cannot be excluded that a similar situation occurred. Such a combination, improving the utilization of D-galactitol, might have promoted the hydrogen production. In this respect, several studies showed that coculture of specific bacteria allowed to obtain high yields of hydrogen production in comparison with single cultivation (Hsiao et al., 2009; Zidan and Niel, 2009; Levin et al., 2009). For example, Hsiao et al. (2009), using molasses as substrate, reported a 12-220% increase in hydrogen yield when a coculture of *C. pasteurianum* or *C. tyrobutyricum* with *C. sporosphaeroides* was employed. An explanation for this improved hydrogen yield might be the utilization of glutamate for hydrogen production by *C. sporosphaeroides*.

The hydrogen production rate was characterized by a great discontinuity which could be explained by the changes and fluctuations of the microbial community along the time. In particular, the general starvation of the microbial community was responsible for the low hydrogen production (about 1 L_{H2}/Lday) recorded during this operational period (Fig. 5). Starvation showed a direct impact on the significant decrease of *Archaea* and *Methanosarcinales* no more fluxing in from the influent (Fig. 5). In addition, this declining trend of *Methanosarcinales,* evident throughout the process, together with the

results of DGGE analysis, suggest that the responsible for the recorded methane in the biogas were the hydrogenotrophic *Metanogenium* species. Another significant quantitative population variation measured in the process was the one order magnitude H_2-producing bacteria decrease observed from day 36 to day 50. This decrease, due most likely to a drop in temperature, may account for the fall in the hydrogen production detected at day 46. During this period also a qualitative fluctuation of community was detected. Interestingly, the occurrence of community fluctuation around the average community as a response to a disturbance, and hence, the resilience to perturbations, has been recently evidenced as a relevant characteristic of a functional community (Werner et al., 2011 PNAS).

The quantitative determinations of principal functional groups involved in the process suggest that the major competitors of hydrogen producing bacteria were acetogens. This vast group of microorganisms, phylogenetically and metabolically very diverse, is unified by the ability of catalyze the reductive synthesis of acetate from CO_2 by using the acetyl-CoA. Generally speaking, they compete with primary fermenters, such as *Clostridium* spp., for monomeric substrates and with secondary fermenters for typical fermentation products such as lactate, ethanol and H_2. On the other hand, they can cooperate with syntrophic acetogenic bacteria for H_2 or with acetoclastic methanogens for acetate pathway (Drake et al., 2002). At low nitrate and sulfate concentrations, methanogenesis and acetogenesis may compete with hydrogen production (Weijma et al., 2002). Though methanogenesis is more thermodynamically favored than acetogenesis, since affinity of methanogens for H_2 is 10-100 times higher than the affinity of the reductive homoacetogens (Liu and Whitman, 2008), acetogens outcompete with methanogens in particular conditions. Hence, their abundance in the reactor could have been favored by the low pH, the accumulation of H_2 and possibly by a favorable spatial position proximal to hydrogen-producing cells (Drake et al., 2002). Their number in the reactor, ranging from 10^7-10^8 FTHFS genes per gram dry weight, was in accordance with Xu et al. (2009) who reported that abundance of acetogens is at least 10^7 copies FTHFS (formyltetrahydrofolate synthetase) genes per gram dry weight sample in anoxic environments, increasing up to 10^8-10^9 copies genes per gram dry weight in enrichment conditions of H_2/CO_2. Thus, it is reasonable they contributed to acetate production in the system. The occurrence of acetogenesis during hydrogen production was previously observed in studies using mixed culture and operating in continuous (Hussy et al., 2003; Kim et al., 2006). In these studies typically, the increased acetate levels where not accompanied by increasing hydrogen yields, or increasing butyrate production. A similar situation with a low butyrate/acetate molar ratio and a slight decrease of pH was observed in our study especially at the end of the operation (B/A 0.15 and pH 5 at day 60). In this regard, whereas heat-treatment of seed is adequate for methanogen control, it is not useful to control spore-forming H_2-consuming bacteria and for this reason considered unnecessary by some authors (Kraemer and Bagley, 2007rev).

In both methanogenic reactors was observed, though at different titers, the dominance of *Methanosarcinales* (average value of 70% and 58% to total *Archaea* in M and MM, respectively), suggesting that acetoclastic methanogenesis was the major pathway of

methane production. In M, *Methanosarcinales* were up to 90% of methanogens and were represented by the only genus *Methanosarcina*. The community was unchanged for quite the entire sampling period, with slightly quantitative fluctuations during the process, in accordance with produced methane yields attesting at average value of 0.5 L/L d. The decreasing trend in methane production recorded from day 49 of operation agrees with the observed quantitative and qualitative changes in methanogen population detected in the last days of sampling. In particular, the one order magnitude methanogens decrease and the decreased proportion of acetotrophic methanogens (50%) may be explained by the accumulation of acetate and propionate (370 and 325 mg/L, respectively, at day 59) in the digester. This accumulation may also account for the appearance in the methanogen population of *Methanothermobacter* species, less sensitive than acetoclastic methanogens to an increase in VFAs concentration (Hori et al., 2006). However, the buffering capacity of system was maintained and the increase in VFAs did not led to a change in pH.

Methanosarcina were previously detected as dominant in other thermophilic methanogen systems from two-stage processes (Chu et al., 2010, Luo et al., 2011) and in general in digesters treating manure (Demirel and Scherer 2008). Unlike *Methanosaeta* spp., prevailing at low acetate (< 1mmol/L), *Methanosarcina* spp. are dominant at high acetate concentration (Jetten et al, 1992). Hence, their abundance in M is justified by the high levels of acetate, the major byproduct of acidogenic fermentation after hexanoic acid. Particularly, the identified *Methanosarcina*-like species were related to *M. mazei* and *M. siciliae* which are reported to have both acetoclastic and hydrogenotrophic activity (Liu et al. 2009; Lee et al., 2010) being able to utilize various substrates other than acetate (methanol, methylamines and also H_2/CO_2 in the case of *M. siciliae*). However, due to their low affinity for hydrogen, they are not likely to be competitive for hydrogen with respect to other hydrogenotrophic methanogens (Boone and Castenholz, 2001), another possible explanation for their decrease in the later operational period.

In comparison to M, in MM the archaeal community was more diverse and dynamic. The genus *Methanosarcina* and *Methanosaeta* were detected simultaneously, though the different intensity of the related bands suggest that *Methanosaeta* prevailed in the first operational period and *Methanosarcina* in the later one, in accordance with the acetate concentration detected (data not shown). In addition, similarly to M, the only hydrogenotrophic archaea detected was *Methanothermobacter.* The detection of this archaea can account for the finding either in MM (genera *Thermacetogenium Tepidanaerobacter*) and M (*Thermotoga*) of thermophilic acetate-oxidizing bacteria as these bacteria degrade substrate syntrophically with hydrogenotrophic methanogens (Hattori, 2008). Overall however, the bacterial community was strongly different in the two methanogen reactors as a consequence of the heat-shock treatment, performed only in the two-stage process, and of the different reactor configurations, which were determining in selecting and enriching of characteristic, substrate-specific species. The most evident difference was the dominance of *Firmicutes* in H-M and of *Firmicutes* and *Bacteroidetes* in MM, phyla dominant of swine and other mammalian fecal communities (Ley et al., 2008). The diversity in bacterial communities together with the

different concentration of methanogens in the two processes are most likely the main factors responsible for the different performance of the processes.

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Chapter 5

General discussion and conclusions

The energy needs of our society has always been based on the exploitation of fossil fuels reserves, from the industrial revolution until the present days. However, these energy sources are non-renewable and will be depleted in the next future. Thus, the need to establish new renewable energetic sources in order to replace fossil fuels is urgent and can't be delayed any more. In this context, anaerobic digestion (AD) has recently been pointed as an environmentally-friendly, economic valuable alternative. One of the most promising possibilities, in particular, is to produce energy through anaerobic degradation of wastewater and waste biomasses derived from industrial and agricultural productive chains.

In recent years, scientific research has been focused on the investigation of several technological solutions for the AD of a wide range of residues and biomasses. The studies were focused mainly on the engineering of the process, with the principal goals of (i) evaluating the methane potential of the biomasses tested and (ii) optimizing reactor design and operational parameters. Despite this, and the widespread application of AD worldwide, few information is available on microbial community diversity and dynamics in response to the different organic residues. Also little is known about the quantitative changes of key functional bacterial groups found in anaerobic digesters, particularly during bioreactor start-up. In this context, there is the need to implement the knowledge of the microbiology of the anaerobic process in order to improve the process stability, prevent imbalances and carry our corrective measures.

The recent development and application of molecular biological techniques targeting the 16S rDNA molecule in the field of microbial ecology have provided an additional and valuable tool to the existing culture-based methods for studying the microbial communities. The introduction of denaturing gradient gel electrophoresis (DGGE) to microbial ecology has established a valuable molecular fingerprinting technique for studying the microbial community structure. In addition, statistical tools have allowed to analyse molecular fingerprints data helping to better elucidate the structure-to-function relations inside the microbial consortium. The real time PCR, the most precise and sensitive technique to quantify specific nucleic acids, has allowed to quantitatively examine the community at order or family taxonomic levels.

The aim of this PhD thesis was to investigate the structure, the dynamics and the interactions of microbial populations, through the use of high-throughput molecular techniques, in batch and continuous anaerobic digestion processes.

The investigation on microbial community structure during anaerobic digestion of agro-industrial byproducts, described in chapter two and three, showed the important role of the seeding sludge in determining the basal microbial community, with some dominant archaeal and bacterial taxa detected by DGGE analysis throughout the course of the

process. However, definite changes in the microbial community were observed, suggesting that quantitative changes in the abundance of some key species occurred as response to microbial community adaptation to the different biomasses. In particular, compositional features of biomasses or processing (ensiling) seemed to play a role in the changes of archaeal microbial communities indicating *Archaea* as good indicators for monitoring AD microbial community dynamics. Finally, a correlation trend was identified between archaeal community and bio-methane potential (BMP) indicating that more even archaeal community were associated to higher BMP.

The study of temporal changes in microbial community structure in a two-stage anaerobic process, before and after a 15-days starvation period, and the comparison with a conventional single-stage process was reported in chapter four. It was shown that in the acidogenic reactor microorganisms were mostly related to heat-shock resistant, spore-forming bacteria affiliated to the *Clostridiales* order. In particular, bacteria belonging to the genus *Clostridium*, capable of fermenting various types of carbohydrates, were identified as the major responsible for the hydrogen production. On the contrary, a more diverse and complex community (*Firmicutes, Bacteroidetes, Synergistes*) was observed in the single-stage process where heat-treatment of inoculum had not been performed.

The changes and fluctuations of the bacterial community observed in the acidogenic reactor might account for the great discontinuity of hydrogen production rate over time. Changes and high diversity of community were evident in the first operational month as a consequence of process start-up instability and then starvation. During the second month of operation, the established bacterial community showed low microbial diversity, in accordance with previous investigations on acidogenic thermophilic bioreactors.

In both methanogenic reactors was observed, though at different titers, the dominance of *Methanosarcinales*, indicating that acetoclastic methanogenesis was the major pathway of methane production. However, in the single-stage process the archaeal community was more diverse and dynamic compared to the one of the methanogenic reactor of the two-stage process and genus *Methanosarcina* and *Methanosaeta* were detected simultaneously. In both the reactors, in general, the quantitative changes of the main phylogenetic and trophic microbial groups over time were rather limited, with slightly quantitative fluctuations during the process that well correlated with biogas data performances. In the methanogenic reactor of two-stage process, it was observed a decrease of methanogens at the end of the operation that could be explained by an accumulation of acetate and propionate in the digester. This accumulation accounted also for the appearance in the methanogen population of *Methanothermobacter* species, less sensitive than acetoclastic methanogens to an increase in VFAs concentration.

Taken together the data suggested that the diversity in bacterial communities, together with the different concentration of methanogens in the two processes, were significant factors determining the different performance of the two processes.

Appendices

Curriculum Vitae

Personal information

Giuseppe Merlino

Via Borsellino 10, 21049 Tradate (VA), Italy
Tel. +39 333 3366725
E-mail: giuseppe.merlino@unimi.it

Education

- Secondary school diploma in scientific studies obtained in 2001 in Tradate, Italy.
- Degree in Molecular Biotechnology at the University of Insubria.
  Title of the experimental thesis: "Production of *Triticum aestivum* marker-free plants by *Agrobacterium tumefaciens*-mediated transformation".
  Advisor Prof. Marcella Bracale.
- Degree in Industrial Biotechnology at the University of Milan, (score 110/110).
  Title of the experimental thesis: "In cis and in trans acting elements in the regulation of polinucleotide phosphorilase expression".
  Advisor Prof. Gianni Dehò.
- At present PhD student (3th year) in Innovazione per le Scienze Agro-Alimentari ed Ambientali at the Department of Food Science and Microbiology (DiSTAM) of the University of Milan.
  Title of the PhD thesis: "Structure and dynamics of microbial communities in anaerobic digestion processes".
  Tutor Prof. Daniele Daffonchio.

Fields of specialization

Industrial biotechnology, environmental microbiology, microbial biotechnology, molecular biology, microbial ecology of extreme environments.

Project participation

- 2009-2011 Participation to the research project PROBITEC: "Produzione di biogas da biomasse vegetali e reflui zootecnici: ottimizzazione del processo e innovazione

tecnologica”, funded by Ente Regionale per i Servizi all’Agricoltura e alle Foreste (ERSAF).
Research Unit PI Prof. Daniele Daffonchio.

- 2009-2011. Participation to the research project AGRIDEN: “Produzione di bio-idrogeno ed energia rinnovabile da residui ago-zootecnici”, funded by Regione Lombardia.
  Research Unit PI Prof. Daniele Daffonchio.
- 2010-2011 Participation to the research project MIERI “Miniaturizzazione e semplificazione di linee di trasformazione per piccole produzioni agroalimentari e impiego di energie rinnovabili”: funded by Ministero delle Politiche Agricole Alimentari.
  Research Unit PI Prof. Daniele Daffonchio.
- 2010-2011. Participation to the research project ULIXES “Unravelling and exploiting Mediterranean Sea microbial diversity and ecology for Xenobiotics’ and pollutants’ clean up”, funded by the European Union.
  Research Unit PI Prof. Daniele Daffonchio.

Publications in international peer-reviewed journals and book

- Carzaniga T., Briani F., Zangrossi S., Merlino G., Marchi, P. and Deho G. Autogenous regulation of *Escherichia coli* polynucleotide phosphorylase expression revisited. *Journal of bacteriology*. **191**, 1738-48 (2009).

Oral presentation

- G. Merlino. "Response of methanogenic archaea and sulphate reducing bacteria during the anaerobic digestion of vegetal biomasses". May 2011, Bertinoro Meeting di Microbiologia Ambientale, BMMA 2010, Bertinoro (Italy).

Abstract selected for oral presentation

- G. Merlino, Aurora Rizzi, F. Villa, C. Sorlini, M.Brambilla, P. Navarotto, B. Bertazzoni, M. Zagni, F. Araldi and D. Daffonchio. "Microbiological monitoring of anaerobic digestion processes treating agro-industrial energetic crops and byproducts". July 2011, Fifth European Bioremediation Conference , Chania (Greece).

Poster

- G. Merlino, A. Rizzi, A. Tenca, A. Schievano, R. Oberti, F. Adani and D. Daffonchio. “Qualitative and quantitative response of bacterial and archaeal communities to substrate-limited conditions in a two-stage anaerobic process treating swine slurry and market bio-waste”. October 2011, First International Conference on Microbial Diversity - Environmental Stress and Adaptation. Milan, Italy.

Participation to national and international congresses

- July 2009. "Utilizzo agronomico del digestato e tutela dell'ambiente". Milan, Italy.
- January 2010. "Il biogas, le matrici organiche e la gestione del digestato". Milan, Italy.
- March 2010. "La digestione anaerobica della frazione organica dei rifiuti urbani". Milan, Italy.
- April 2010. "Cortona Procarioti 2010". Cortona, Italy.
- May 2010. "Bertinoro Meeting di Microbiologia Ambientale" BMMA 2010. Bertinoro, Italy.
- February 2011. Kick-Off meeting of European ULIXES project. Milan, Italy.
- June 2011. Advanced course in Environmental Biotechnology, Delft. The Netherlands.
- October 2011. First International Conference on Microbial Diversity - Environmental Stress and Adaptation. Milan, Italy.

Participation to field expeditions

- April 2011. Participation to the oceanographic expedition M84/3 on the Research Vessel Meteor in the Mediterranean Sea in order to study the microbial community colonizing the deep and superficial waters of the eastern Mediterranean Sea.
- September 2011. Participation to the oceanographic expedition on the Research Vessel Urania in order to study the biodiversity of marine Mediterranean environment and to exploit and manage this microbial diversity for addressing bioremediation of polluted marine sites.

Tutor activity for laboratory course for students at University of Milan

- 2011: Course of "Microbiologia Generale", Prof. Claudia Sorlini.

Others

- Conference Secretariat for the First International Conference on Microbial Diversity - Environmental Stress and Adaptation. October 2011. Milan, Italy.

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