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Research article

The effect of bacterial and archaeal populations on anaerobic process fed with mozzarella cheese whey and buttermilk

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ABSTRACT

Dairy wastes can be conveniently processed and valorized in a biorefinery value chain since they are abundant, zero-cost and all year round available. For a comprehensive knowledge of the microbial species involved in producing biofuels and valuable intermediates from dairy wastes, the changes in bacterial and archaeal population were evaluated when H_2, CH_4 and chemical intermediates were produced. Batch anaerobic tests were conducted with a mixture of mozzarella cheese whey and buttermilk as organic substrate, inoculated with 1% and 3% *w/v* industrial animal manure pellets. The archaeal methanogens concentration increased in the test inoculated at 3% (*w/v*) when H_2 and CH_4 production occurred, being 1 log higher than that achieved in the test inoculated at 1% (*w/v*). Many archaeal species, mostly involved in the production of CH_4, were identified by sequencing denaturing gradient gel electrophoresis (DGGE) bands. *Methanoculleus*, *Methanocorpusculum* and *Methanobrevibacter* genera were dominant archaea involved in the anaerobic process for bioenergy production from mozzarella cheese whey and buttermilk mixture.

1. Introduction

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

This new approach to waste management is eco-friendly, easy to be conducted and economical advantageous, mostly for undeveloped and developing countries that have an economic gap with more industrialized countries. This gap is often due to the lack of an available energy source and technological and infrastructural backwardness (Ragazzi et al., 2017), and the efforts to reduce it frequently can lead to an uncontrolled release of solid and liquid pollutants as well as gaseous emissions into the environment (Riahi et al., 2017). Furthermore, the biotechnological development contributes to replace fossil fuels with biomass (organic waste and/or energy crops) as source of energy and biomaterials, thus preventing the increase of CO_2 in the atmosphere and indirectly taking part to mitigate the global warming (Bauer et al., 2010). For instance, the organic fraction of the municipal solid waste (OFMSW) is successfully and worldwide used for producing enzymes (Clanet et al., 1988), biohythane (Escamilla-Alvarado et al., 2017) and ethanol (Ballesteros et al., 2010); agricultural biomass including corn, woods, sugar, rice and wheat straw, has found a wide use in generating bioalcohols, bio-oil, biogas and biohydrogen (Poggi-Varaldo et al., 2014; Mancini et al., 2016, 2018); and even not readily biodegradable C-based wastes, such as polystyrene (Goff et al., 2007) and polyethylene terephthalate (PET) (Kenny et al., 2012), have resulted to be suitable for polyhydroxyalkanoates (PHA) production. Potential substrates for bioenergy production are cheese whey and buttermilk, by-products of cheese, yogurt, milk and butter processing in dairy factories. Cheese whey represents approximately 80–90% of the total waste volume from dairy factories (Lee et al., 1997) and is the major by-product of mozzarella cheese production. Buttermilk is the liquid left after churning mozzarella cheese. These milk-based wastes have high concentrations of soluble organic matter and are biodegradable, thus suitable for being treated by an anaerobic process that converts them into

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ethanol, lactic acid, volatile fatty acids (VFAs), H_2 and CH_4. All of them are complementary products of the biological metabolism (Khan et al., 2016) and their production can be associated to one or more bacterial and/or archaeal strains in the system. Although the anaerobic bacteria belonging to the families *Streptococcaceae* and *Enterobacteriaceae* as well as the genera *Clostridium* and *Eubacterium* are the most frequently involved in the anaerobic digestion process (Novaes, 1986; Pagliano et al., 2017), the microflora present in anaerobic digesters is extremely various in species, highly specialized, selected on the base of substrates and inoculum as well as the operating conditions used. When the biological process is fed with dairy wastes, it is expected that: (i) *Lactobacillus* spp. and *E. coli* are the most common hydrolytic bacteria; (ii) *Acetobacterium* spp., which converts lactate to acetate, are the most common homoacetogenic bacteria (Schug et al., 1987); (iii) archaea are responsible for CH_4 production (Gonzalez-Martinez et al., 2016) from acidogenesis products following the acetoclastic pathway, typical of *Methanosaeta*, *Methanosarcina*, and *Methanothrix* genera, and/or the hydrogenotrophic pathway, typical of *Methanobacterium*, *Methanococcus*, *Methanospirillum*, or *Methanomassiliicoccus* (Gonzalez-Martinez et al., 2016). The main biochemical reactions involved in the anaerobic degradation of dairy wastes resulting in the production of H_2 and CH_4 are listed in Table 1 with the relative standard value of Gibbs free energy (ΔG°).

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

2. Materials and methods

2.1. Physico-chemical analysis of dairy wastes

Cheese whey and buttermilk were collected from a buffalo mozzarella cheese factory located in Casoria (latitude: 40° 54′ 32.62″ N and longitude: 14° 17′ 37.07″ E), Campania region (Italy). Dairy wastes were mixed maintaining a ratio of 2:1 (*v*/*v*) between cheese whey and buttermilk, in order to simulate the standard characteristics of a real dairy waste stream produced from a mozzarella cheese factory. Such mixture was used to conduct the tests. Cheese whey, buttermilk and their mixture were physically and chemically characterized as follows: pH was measured using a HI 221 pH meter (Hanna Instruments Inc., Woonsocket, RI, USA); total titratable acidity (TTA) was calculated as the mL of 0.1 N NaOH/10 mL of sample (AACC, 1975); total solids (TS) and volatile solids (VS) were evaluated as described in the standard methods (APHA, 2005); COD was measured with an ECO08 thermoreactor (VELP Scientifica, Usmate, Italy) and a PF-3 photometer (VELP Scientifica, Usmate, Italy) using kit NANOCOLOR®.

2.2. Microbiological analysis of dairy wastes

Serially diluted cheese whey, buttermilk and their mixture were enumerated by spread plate method using different solid media. Total aerobic and anaerobic bacteria were cultured on Plate Count Agar (PCA; Oxoid, Milan, Italy) and incubated for 48 h at 30 °C under either aerobic or anaerobic conditions (Oxoid Anaerogen™

Table 1
Main biochemical reactions involved in the anaerobic digestion of dairy wastes with the relative value of standard Gibbs free energy (ΔG°).

Reaction	ΔG° (kJ/mol)	Reference
Glucose to H_2/ethanol/acetate $C_6H_{12}O_6+3H_2O \rightarrow 2H_2 + 2\ CH_3CH_2OH + CH_3COO^- + 2\ HCO_3^- + 3H^+$	−182	Azbar and Levin, 2012
Glucose to H_2/ethanol/formate/acetate $C_6H_{12}O_6+2H_2O \rightarrow H_2+CH_3CH_2OH + HCOO^- + CH_3COO^- + HCO_3^- + 3H^+$	−183	Azbar and Levin, 2012
Glucose to ethanol $C_6H_{12}O_6+2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+$	−196	Azbar and Levin, 2012
Glucose to H_2/acetate $C_6H_{12}O_6+4H_2O \rightarrow 4H_2+2CH_3COO^- + 2HCO_3^- + 4H^+$	−168	Azbar and Levin, 2012
Glucose to H_2/butyrate $C_6H_{12}O_6+2H_2O \rightarrow 2H_2+2CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+$	−229	Azbar and Levin, 2012
Glucose to H_2 $C_6H_{12}O_6+12H_2O \rightarrow 12H_2+6HCO_3^- + 6H^+$	+64	Azbar and Levin, 2012
Glucose to H_2/acetate/formate $C_6H_{12}O_6+2H_2O \rightarrow 2H_2+2CH_3COO^- + 2HCOO^- + 4H^+$	−170	Azbar and Levin, 2012
Glucose to lactate $C_6H_{12}O_6 \rightarrow 2CH_3CH(OH)COO^- + 2H^+$	−172	Azbar and Levin, 2012
Acetate to H_2 $CH_3COO^- + 4H_2O \rightarrow 4H_2 + 2HCO_3^- + H^+$	+116	Azbar and Levin, 2012
H_2 to acetate $4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$	+48.3	Thauer et al., 1977
Butyrate to acetate/H_2 $CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	+88.2	Westermann, 1994
Propionate to acetate/H_2 $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+116.4	Westermann, 1994
Acetic acid to methane $CH_3COOH \rightarrow CH_4 + CO_2$	−36	Schlegel et al., 2012
H_2 to methane $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	−135.6	Voolapalli and Stuckey, 1999