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Keywords: cheese whey, dark fermentation, biohydrogen, organic acids, metabolic pathways modelling

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Abstract: Batch dark fermentation tests were performed on sheep cheese whey, without inoculum addition at different operating pHs, relating the type and production yields of the observed gaseous and liquid by-products to the evolution of fermentation. Cheese whey fermentation evolved over time in two steps, involving an initial carbohydrates conversion into lactic acid, followed by the degradation of this into soluble and gaseous products including short-chain fatty acids (mainly acetic, butyric and propionic acids) and hydrogen. The operating pH affected the production kinetics and yields, as well as the fermentation pathways. By varying the duration of the fermentation process, different cheese whey exploitation strategies may be applied that may be oriented to the main production of lactic acid or hydrogen or other organic acids.

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Dear Editor,

Please find enclosed the manuscript entitled "Control of fermentation duration and pH to orient biochemicals and biofuels production from cheese whey".

In the present work dark fermentation (DF) was applied for cheese whey (CW) valorisation, adopting different operating pHs and relating type and production yields of the observed gaseous and liquid by-products to the fermentation duration.

DF of organic substrates has been studied extensively during recent years since it is closely related to the transition towards an innovative approach for biowaste valorisation, the so-called waste biorefinery concept. Nevertheless, the process has been investigated with particular emphasis on biohydrogen production, whilst less attention has been paid to the possibility of recovering, through proper optimization of the operating parameters, other valuable products along with hydrogen according to a fermentation-centered biorefinery approach. Operating pH and fermentation time are known to govern the production yields of liquid and gaseous bioproducts by influencing the activity of enzymes, the degree of substrate hydrolysis, the metabolic pathways; for these reasons their optimization appears to be worth studying in order to adjust the type and yield of biochemicals and/or biofuels produced from CW. Furthermore, as the experimental tests were performed on raw sheep CW without any addition of biomass inoculum nor any pre-treatment of the substrate, the full-scale implementation of a CW DF treatment based on the indigenous mixed microbial cultures found in CW could lead to various advantages, i.e. no need for substrate sterilization, no added costs for dedicated inoculum, no energy consumption for inoculum/substrate pre-treatments, making, in turn, CW an even more attractive substrate and the process relatively more straightforward to implement.

To the best of the authors' knowledge, fermentation of raw CW making exclusive use of its indigenous biomass is not common in the literature, and the peculiar features of the process when operated under such conditions have never been pointed out before. An additional novel contribution of the present work to the knowledge in the field is also provided by modelling of the biochemical pathways governing the fermentation process in order to identify the relevant degradation reactions. This is essential to orient the fermentation process in the desired direction. Given the limited number of studies documented in the scientific literature on the combined biochemical and biofuel production from sheep CW, the present study is believed to open up the path to further research aimed at exploring innovative management and valorisation strategies.

Being the paper related to a topic of valid and relevant scientific and technical interest, it is appealing to both a scientific and a technical audience.

The manuscript's Subject Classification is "50.090 Optimization of bioprocess". All the authors mutually agree that the manuscript should be submitted to Bioresource Technology (BITE). The manuscript is the original work of the authors and was not previously submitted to BITE.

Thank you for your consideration of this manuscript. Authors hope that this work will be appreciated by your readers.

Best regards

Fabiano Asunis

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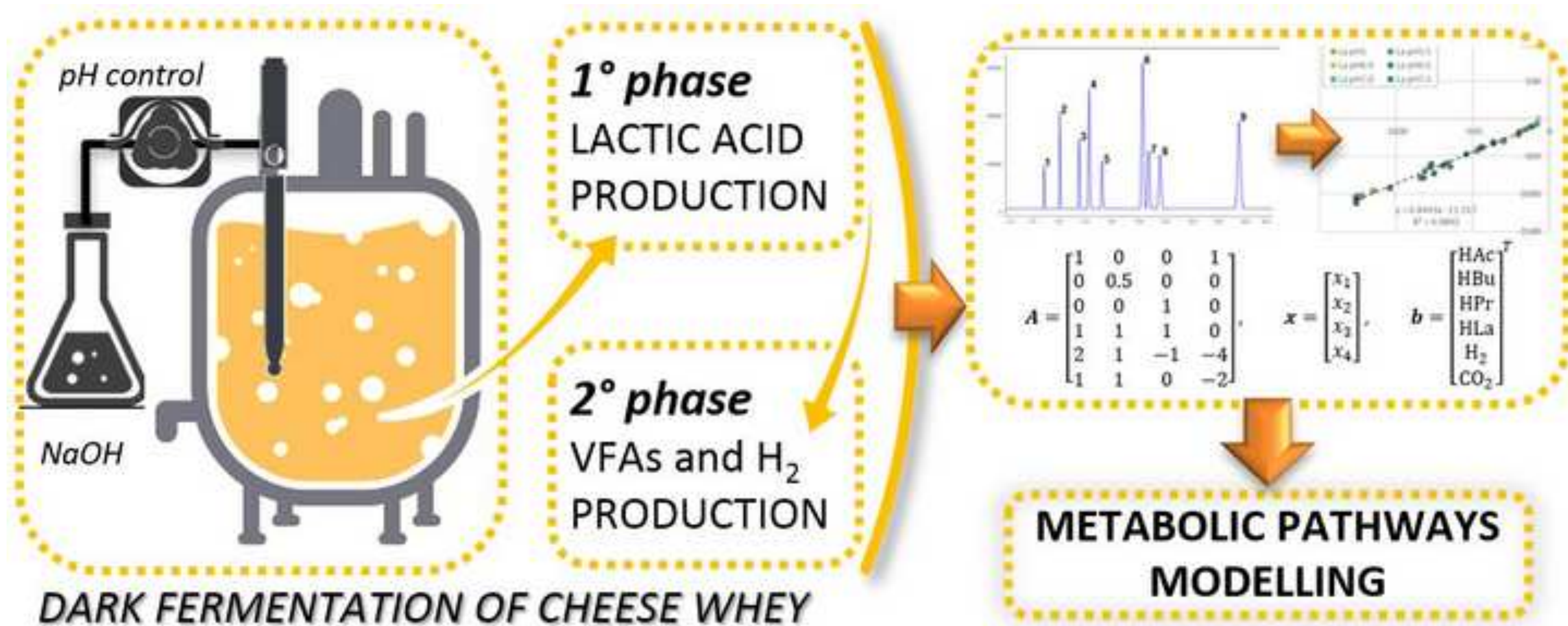
Graphical Abstract (for review)



*Highlights (for review)

CONTROL OF FERMENTATION DURATION AND pH TO ORIENT BIOCHEMICALS AND BIOFUELS PRODUCTION FROM CHEESE WHEY

F. Asunis[1*], G. De Gioannis[1,2], M. Isipato[1], A. Muntoni[1,2], A. Polettini[3], R. Pomi[3], A. Rossi[3], D. Spiga[1]

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HIGHLIGHTS

1. Fermentation of sheep cheese whey performed using exclusively indigenous biomass
2. Carbohydrates were converted to lactate that was then degraded to VFAs and H_2
3. Different metabolites were produced depending on pH and fermentation time
4. Maximum lactate yield (23 mmol/g TOCi) was attained at pH 6.0 after 45 h
5. Maximum H_2 yield (162 L/kg TOC_i) was attained at pH 6.0 after 168 h

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ABSTRACT

Batch dark fermentation tests were performed on sheep cheese whey, without inoculum addition at different operating pHs, relating the type and production yields of the observed gaseous and liquid by-products to the evolution of fermentation. Cheese whey fermentation evolved over time in two steps, involving an initial conversion of carbohydrates to lactic acid, followed by the degradation of this to soluble and gaseous products including short-chain fatty acids (mainly acetic, butyric and propionic acids) and hydrogen. The operating pH affected the production kinetics and yields, as well as the fermentation pathways. By varying the duration of the fermentation process, different cheese whey exploitation strategies may be applied that may be oriented to the main production of lactic acid or hydrogen or other organic acids.

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Keywords: cheese whey, dark fermentation, biohydrogen, organic acids, metabolic pathways modelling.

1. INTRODUCTION

Dairy manufacturing is one of the largest industrial activities in the agri-food sector in the European Union (EU): 170 million tons of milk from different origins were produced in 2017 and transformed into a wide range of dairy products whose characteristics largely depend on the nature of the raw milk used (Eurostat, 2018). Cheese whey (CW) is the main by-product of the cheese making process; the specific production ranges between 0.8 and 0.9 L per L of processed milk, or between 8 and 9 L per kg of produced cheese, depending on the cheese yield and type of processed milk (Carvalho et al., 2013). The main components of CW are lactose, proteins, lipids and mineral salts (Carvalho et al., 2013; Prazeres et al., 2012). Most of the milk lactose, around 40 - 60 g L^{-1}, remains in the CW and makes up the main fraction (90%) of its organic load (Prazeres et al., 2012).

Despite the fact that only 3% of total milk produced in EU is of ovine origin, sheep dairy industry plays a pivotal role in the agrarian economy of Mediterranean countries (especially Greece, Spain, Italy and France) (Balthazar et al., 2017). Due to the milk composition, compared to cow CW, sheep cheese whey (SCW) is characterised by higher levels of total solids, lipids (5.9% vs. 3.3% dried extract) and proteins (5.5% vs. 3.4% dried extract) (Balthazar et al., 2017; Pereira et al., 2015). These characteristics may offer alternative and promising options for SCW valorisation/management compared to the traditional ones.

In the past, agricultural land spreading and/or direct use for animal feeding have been the most widely applied solutions for CW management. Nowadays, these practices are no longer considered sustainable due to concerns about the potential adverse effects on the environment and animals health conditions (Akhlaghi et al., 2017), so that appropriate alternatives need to be explored.

In a circular economy perspective, efforts at looking for efficient reuse or recovery of materials/energy from any valuable waste stream originated by the production cycles need to be boosted (Asquer et al., 2017). To this respect, ambitious valorisation options aiming at producing either biofuels or biochemicals from organic waste streams are fully included in the definition of biorefinery given by the International Energy Agency Bioenergy Task 42 (IEA Bioenergy Task42, 2012). The valorisation processes encompassed by the biorefinery concept could be biochemical (e.g. fermentation, enzymatic conversion), thermochemical (e.g. gasification, pyrolysis), chemical (e.g. acid hydrolysis, synthesis, esterification), mechanical (e.g. fractionation, pressing, size reduction) or an adequate combination of these. Among the biochemical processes, and looking beyond traditional anaerobic digestion, dark fermentation (DF) may be a promising approach for CW valorisation (Akhlaghi et al., 2017; De Gioannis et al., 2014). Though the issue has been already addressed by several studies with particular emphasis on biohydrogen production, less attention has been paid to the possibility of recovering, through proper optimization of the operating parameters, other valuable products along with hydrogen (De Gioannis et al., 2014) according to an integrated, fermentation-centered biorefinery approach. Indeed, during fermentation only 30 - 40% of the organic substrate is utilized for biogas production, while the remaining 60 - 70% is converted into a range of soluble metabolites, the nature of which depends on the

specific metabolic pathways prevailing (Sarma et al., 2015). The exploitation of such metabolites may involve direct separation and commercialization of e.g. lactic acid or specific short- or medium-chain fatty acids, or further processing of the fermentation effluent. DF could be coupled with a range of different processes, aimed e.g. at biopolymer production (Colombo et al., 2016), electricity or further hydrogen production in microbial electrochemical systems (Moreno et al., 2015), methanogenesis (Fernandez et al., 2015), or others. In such an integrated system, the recoverable gaseous phase may well include, upon separation of the CO_2, either biomethane or biohydrogen, which could be exploited separately or as a mixture (biohythane) or be utilised to biologically reduce CO_2 to further biomethane.

It is acknowledged that DF is a complex process strongly depending on numerous and interconnected factors such as substrate composition, concentration and pre-treatment methods, presence/type of inoculum and inoculum pre-treatment, inoculum-to-substrate ratio, reactor type and operation regime, applied operating conditions (e.g. pH, hydraulic and cell residence time, temperature, organic loading rate, etc.) (De Gioannis et al., 2013). Operating pH and fermentation time, in particular, are known to govern the production yields of liquid and gaseous bioproducts by influencing the activity of enzymes, the degree of substrate hydrolysis, and the prevailing metabolic pathways (Akhlaghi et al., 2017); for these reasons, optimizing the operating pH and process duration appears to be worth studying in order to adjust the type and yield of biochemicals and/or biofuels produced from CW. To this aim, in the present study batch fermentation tests were performed on raw SCW without any addition of biomass inoculum nor any pre-treatment of the substrate. Full-scale implementation of a CW DF treatment based on the indigenous mixed microbial cultures (MMC) and/or lactic acid

bacteria (LAB) which are found in CW could lead to various advantages, i.e. no need for substrate sterilization, no added costs for dedicated inoculum, no energy consumption for inoculum/substrate pre-treatments, making, in turn, CW an even more attractive substrate and the process relatively more straightforward to implement.

To the best of the authors' knowledge, fermentation of raw CW making exclusive use of its indigenous biomass is not common in the literature, and (as shown later in the manuscript) the peculiar features of the process when operated under such conditions have never been pointed out before. An additional novel contribution of the present work to the knowledge in the field is also provided by modelling of the biochemical pathways governing the fermentation process in order to identify the relevant degradation reactions. This is essential to orient the fermentation process in the desired direction. Given the limited number of studies documented in the scientific literature on the combined biochemical and biofuel production from SCW, the present study is believed to open up the path to further research aimed at exploring innovative SCW management and valorisation strategies.

2. MATERIALS AND METHODS

2.1 Substrate

Samples of fresh raw SCW were collected at a medium-size dairy industry located in Sardinia (Italy) which processes ovine milk producing pecorino cheese. All samples were stored at -15°C until use to prevent biological degradation. The main characterisation parameters for the SCW samples are reported in Table 1.

2.2 Experimental setup

The batch fermentation tests were carried out at 39 ± 1 °C using a 2-L glass reactor (BIOFLO 110 - New Brunswick Scientific; BioCommand Lite software; working volume = 1.8 L). The reactor was supplied with a mechanical stirring device (stirring rate = 150 rpm) and an automatic pH control software continuously controlling the addition of a 5 M NaOH solution. Gas production was measured by means of a eudiometer adopting the volume displacement principle. The measured gas volume was converted to standard temperature and pressure conditions (T = 273.15 K, P = 10^5 Pa). The reactor was covered with a black plastic film to prevent photofermentative reactions and initially flushed with N_2 gas to drive off air from the headspace. Six operating pHs (5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) were adopted during the tests. An additional test was performed without continuous control of the operating pH (UCpH). All the fermentative tests were run in duplicate and the results will be reported as average values. Each test was stopped once any variation in metabolite concentration and/or appreciable gas production could be no longer detected.

2.3 Analytical methods

The concentration of total solids (TS), volatile solids (VS), total organic carbon (TOC), total (tCarb) and soluble carbohydrates (sCarb, on 0.45-μm filtered samples) were measured according to the analytical methods reported in previous paper (De Gioannis et al., 2014). The soluble protein (sProt) content was determined spectrophotometrically at 750 nm by the alkaline copper method as described by Lowry et al. (1951), using bovine serum albumin (BSA) as the standard. All the spectrophotometric analyses were performed with a HITACHI U-200 spectrophotometer. The concentration of Fe, Mg, K, Na, Ca was determined on 0.45-μm filtered samples using an inductively coupled

plasma-optical emission spectrometer (ICP-OES, Optima 7000DV, Perkin Elmer, MA, USA). The concentration of lactic acid (HLa) was analysed using a Dionex high-pressure liquid chromatography System UVD170U equipped with an Acclaim Organic Acid column. All analyses were conducted with isocratic elution (H_2PO_4 0.2% + sodium sulphate 100 mM at 0.9 mL min^{-1}). The concentration of VFAs (acetic [HAc], propionic [HPr], butyric + iso-butyric [HBu], valeric + iso-valeric [HVa], hexanoic + iso-hexanoic [HHex], heptanoic [HHep]) and ethanol [EtOH]) was determined using a gas chromatograph with flame-ionization detection (model 7890B, Agilent Technology) equipped with a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology). The samples were filtered using a 0.45 µm membrane and then acidified with concentrated H_3PO_4 (pH < 3). The injection volume was 0.6 µL. The temperatures of the injector and the detector were 230 °C and 300 °C, respectively. The oven temperature was initially set at 60 °C (3-min holding time), followed by a ramp of 10 °C min^{-1} up to 160 °C. He (1.6 mL min^{-1}, splitless) was used as the carrier gas.

The biogas was sampled periodically from the reactor headspace with a 1-mL gastight syringe and injected through a valve in a gas chromatograph (model 7890B, Agilent Technology) equipped with a thermal conductivity detector (TCD) and two stainless columns packed with HayeSep N (80/100 mesh) and Shincarbon ST (50/80 mesh) connected in series. The operating temperatures of the valve and the TCD were 90 °C and 200 °C, respectively, and He was the carrier gas at a constant pressure of 8 psi in the HayeSep N column and 25 psi in the Shincarbon ST column (at 70 °C). The oven temperature was set initially at 70 °C (3-min holding time), followed by a ramp of 10 °C min^{-1} up to 160 °C (3-min holding time).

All analyses were run in triplicate and results are presented as average values of the

replicates and the associated standard deviation.

2.4 Kinetic models

A first-order kinetic model (see Eq. 1) was used to describe the time evolution of the carbohydrates degradation process.

$$\frac{C}{C_0} = a + b * exp(-k * t) \tag{1}$$

where C_0 and C are the carbohydrates concentration at time 0 and t, while a, b and k are the kinetic constants. Specifically, k is the rate constant and $a + b = 1$.

The modified Gompertz equation was used to calculate the kinetic parameters for the H_2 production process, according to Eq. 2 (Lay et al., 1999):

$$HPY(t) = HPY_{max} * exp\left\{-exp\left[\frac{R_{max} \cdot e}{HPY_{max}}(\lambda - t) + 1\right]\right\} \tag{2}$$

where HPY is the cumulative H_2 production yield at time t, HPY_{max} is the maximum theoretical H_2 production yield, R_{max} is the maximum H_2 production rate, λ is the lag phase duration, t is the time and "e" is the Neperian number.

The experimental data were fitted through Eq. 1 and 2 using the TableCurve 2D® software (v. 5.01, Systat Software Inc.) through least-squares non-linear regression. The coefficient of determination R^2 was used to evaluate the quality of data fitting for each experimental dataset. The time required for H_2 production to attain 95% of the maximum production yield, referred to as $t_{95(H2)}$, was derived from the Gompertz equation as follows (Eq. 3).

$$t_{95(H2)} = \frac{HPY_{max}}{R_{max} \cdot e}(1 - \ln(-\ln 0.95)) + \lambda \tag{3}$$

3. RESULTS AND DISCUSSION

The main characteristics of the SCW reported in Table 1 indicate that the organic content was largely associated to carbohydrates, with a concentration of 58 g L^{-1}, which corresponds to 76% of total TOC assuming that carbohydrates were only present as lactose ($C_{12}H_{24}O_{11}$). Soluble proteins were measured at a concentration of 11 g BSA L^{-1}, accounting for 15% of total TOC assuming an average C content of 0.46 g (g BSA)$^{-1}$ (Rouwenhorst et al., 1991). This value is significantly higher than usually observed for cow CW (Carvalho et al., 2013).

3.1 Organic matter degradation and lactate production stage

Substrate degradation during the fermentation tests was evaluated by observing the evolution of the normalized concentration (C/C_0) of soluble carbohydrates over time. The results are depicted in Figure 1, where the solid lines represent the first-order model curves derived from Eq. 1. For all the experiments run at controlled pH conditions, the C/C_0 values decreased rapidly over time and the degradation kinetics was described with a high goodness of fit ($R^2 > 0.97$) by Eq. 1, as also observed by Akhlaghi et al. (2019), Akhlaghi et al. (2017) and De Gioannis et al. (2014). The uncontrolled test (UCpH) was also found to be described by a first-order-type kinetics (although with a slightly lower correlation – $R^2 = 0.90$), but the carbohydrates consumption rate and final consumption yield were considerably lower than for the other tests. In particular, the occurrence of inhibitory effects on carbohydrates degradation for the UCpH run was evident after 30 hours of fermentation, with the consumption yield levelling off after ~60 h and reaching a final value of 45%. Similar inhibition conditions of carbohydrates degradation in uncontrolled pH experiments were also observed in Tang et al. (2016) and most likely result from acid accumulation in the fermentation broth with an associated strong pH

decrease. In our experiments performed under uncontrolled pH conditions, pH dropped down significantly over time attaining a final value of 3.78.

In the controlled-pH tests, the operating pH was not found to affect the final carbohydrates removal, which was always rather high; conversely, it significantly influenced the degradation rate. The carbohydrates concentration was always reduced by more than 93% (up to 99%) of the original value, indicating a virtually complete removal of such species during the fermentation process.

The carbohydrates degradation kinetics was observed to be strongly dependent on pH, as clearly indicated by the trends of k and $t_{95(carb)}$ (see Figure 2). More specifically, both parameters were exponentially correlated with pH, with an almost tenfold increase in k from 0.015 h^{-1} at pH 5.0 to 0.176 h^{-1} at pH 7.5, and a decrease in $t_{95(carb)}$ from 395 h at pH 5.0 to 74 h at pH 7.5. Other authors showed similar effects of pH on the carbohydrates degradation rate (Infantes et al., 2011; Tang et al., 2016). Possible causes for the observed influence of pH on carbohydrates consumption kinetics are well known in the literature and include: 1) the increased enzymatic activity of biomass at higher pH conditions (Tang et al., 2016); 2) the decreased energy utilization yield by the biomass at low pHs, caused by undissociated acids crossing the cell membrane causing the need of an excess of metabolic energy to excrete the excess of protons released inside the cell (Infantes et al., 2011; Rodríguez et al., 2006); 3) the changes in the degree of nutrient transport to the microbial cells (Panesar et al., 2007). Although it would not be possible, from the characterization performed in the present study, to single out the individual contribution of the above mentioned mechanisms, the experimental results clearly show that pH had a well defined and univocal effect on the substrate degradation rate.

The time evolution of the metabolic products as a function of the operating pH is

presented in Figure 3, which shows some distinguishing features of the fermentation process. For all the controlled-pH experiments, the process was clearly governed by two consecutive substrate degradation stages, involving carbohydrates conversion into lactic acid followed by lactic acid transformation into VFAs (mainly, acetic, propionic and butyric acids). Compared to such metabolic products, other species including either higher-molecular-weight VFAs or ethanol were always detected at negligible concentrations. The UCpH test showed some initial HLa production, although at a much lower level than for the other experiments. In this case HLa production also displayed very slow kinetics, with a plateau of 5.4 mmol HLa (g $TOC_i)^{-1}$attained after approximately 60 h from the beginning of the process, mirroring the trend observed for carbohydrates degradation and confirming the occurrence of inhibitory effects on fermentation likely caused by the adverse pH environment (Panesar et al., 2007).

The maximum HLa concentration for controlled-pH tests was found to range from 15 to 24 mmol (g $TOC_i)^{-1}$ depending on the operating pH, while the peak production was attained after 12–96 h from the beginning of the experiments. The HLa production observed in the first stage of the fermentation process is related to the presence of lactic acid bacteria (LAB) in SCW, as they are added as starter cultures during the cheese making process (Sikora et al., 2013). LAB catabolize sugars (both mono- and di-saccharides) according to different metabolic pathways. Homolactic fermentation produces lactate as a single end product via the Emden-Meyerhoff-Parnas pathway, according to which 2 moles of pyruvate are produced from glycolysis of glucose and then reduced to lactate (Castillo Martinez et al., 2013; Sikora et al., 2013), as represented by the overall reaction in Eq. 4:

$$\text{glucose} \rightarrow 2\ \text{lactate} \qquad (4)$$

In heterolactic fermentation, 1 mole of pyruvate is converted to lactate while the other mole is converted to ethanol (or acetate) and carbon dioxide via the phosphoketolase pathway (Castillo Martinez et al., 2013; Sikora et al., 2013) according to Eq. 5 and 6:

$$\text{glucose} \rightarrow \text{lactate} + CO_2 + \text{ethanol} \qquad (5)$$

$$\text{glucose} \rightarrow \text{lactate} + CO_2 + \text{acetate} \qquad (6)$$

Where lactose is the initial substrate, the degradation process is known to involve a first hydrolysis stage during which lactose is hydrolysed to hexose (glucose and galactose) according to Eq. 7 (Fu and Mathews, 1999):

$$\text{lactose} + H_2O \rightarrow \text{glucose} + \text{galactose} \qquad (7)$$

and hexose is then fermented to lactate upon either homolactic or heterolactic transformations (Eqs. 4−6). This would imply for the homolactic and heterolactic pathways an expected lactate production yield of 4 and 2 moles per mol of lactose consumed, respectively.

If the fermentation process is oriented towards HLa production (very attractive for the biotechnology industry, the production of which is expected to grow from 0.7 Mt in 2013 to 1.9 Mt in 2020 (https://www.grandviewresearch.com/press-release/global-lactic-acid-and-poly-lactic-acid-market)), the heterolactic fermentation would obviously be less favourable over the homolactic pathway in terms of lactate recovery yields due

to the lower lactate productivity and the need for HLa separation and purification from the other metabolites (Mazzoli et al., 2014). The onset of either type of fermentation is governed by the nature of LAB present (Panesar et al., 2007), substrate or nutrient limitation factors (Bernárdez et al., 2008), as well as key operating parameters such as temperature and pH (Panesar et al., 2007). In the present study, the analysis of the fermentation system showed that the first stage involving lactate production was mainly associated to the homolactic pathway, on account of the fact that the other analysed metabolites were either undetectable or present at extremely low concentrations and no appreciable amounts of biogas were produced. As a further confirmation of this statement, the calculated yield of lactose conversion to HLa was found to be around 4 mol HLa (mol lactose consumed)$^{-1}$ for all tests, with the exception of the run at pH 7.0 which displayed a yield of 3.2. This is believed to be a very distinguishing feature of the fermentation process tested, considering that no specific effort was made in the selection of the microbial community of the fermentation system. The formation of HLa as the main metabolic product of CW has been documented by several literature studies, which mainly involved the use of whey powder or whey permeate inoculated with pure cultures (mostly, *Lactobacilli*) (Büyükkileci and Harsa, 2004; Göksungur et al., 2005; Kim et al., 2006; Prasad et al., 2014; Tang et al., 2016). Conversely, when whey powder or whey permeate was inoculated with different types of residual biomass previously acclimated under anaerobic conditions, multiple metabolic pathways were observed to overlap, likely due to the concomitant presence of different microbial species, and other products (VFAs and alcohols) turned out to form together with lactate at comparable concentrations (Gomes et al., 2015; Vasmara and Marchetti, 2017). However, it has been suggested that drying/osmotic pre-treatments of CW cause stress factors that may

lead to damages to the cell membrane and inactivation of most of the LAB strains (Gomes et al., 2015). To this regard, fermentation tests conducted on non-pretreated CW (Pagliano et al., 2018) indicated a more relevant role of the autochthonous LAB in the system, with a prevalence of lactate production over other metabolic routes. The fact that in the present study no preliminary treatment was applied to CW and no external inoculum was added, caused the fermentation process to be initially governed by the indigenous biomass in CW, which arguably comprised a significant portion of homolactic species. The absence of metabolic pathways overlapping with homolactic fermentation may have also resulted from the antimicrobial activity displayed by LAB that has been widely reported in the literature (Cabrol et al., 2017). While there are multiple mechanisms through which LAB can exert antimicrobial activity, it is likely that under the fermentation conditions tested in our experiments the excretion of bacteriocins by LAB may have inhibited the activity of other microorganisms (including hydrogen-producing bacteria) during this stage (Jo et al., 2007; Noike et al., 2002). The experimental results also indicate that the microbial community tended to change over the fermentation time. At some point, the depletion of the carbohydrates converted by LAB into HLa became a limiting factor for their metabolism, so that different microbial species took over during the second fermentation stage, and a range of metabolic products was found to appear (see Section 3.2 for further details).

3.2 Hydrogen and organic acids production

The second stage of the fermentation process started when HLa production peaked (see Fig. 3) and was dominated by lactate-consuming pathways with an accompanied production of VFAs, H_2 and CO_2. The soluble metabolic products detected mainly

included short-chain fatty acids (acetic, propionic and butyric acids), while medium-chain fatty acids including valeric, hexanoic and heptanoic acids were below the analytical detection limit (10 ppm).

Different microbial pathways involving the transformation of lactate into a range of metabolic products are known from the literature and include the elementary reactions reported in Eqs. 8-10 or their combinations (García-Depraect et al., 2019; McInerney and Bryant, 1981; Thauer et al., 1977):

$$\text{lactate} + H_2O \rightarrow \text{acetate} + CO_2 + 2\ H_2 \qquad (8)$$

$$\text{lactate} + H_2O \rightarrow 0.5\ \text{butyrate} + CO_2 + H_2 \qquad (9)$$

$$\text{lactate} + H_2 \rightarrow \text{propionate} + 2\ H_2O \qquad (10)$$

Given the fact that the main soluble metabolites were found to be present in the fermentation system in different proportions depending on the operating pH adopted, a specific investigation of the prevalent metabolic pathways was conducted by taking into account the possible biochemical reactions involving the species of concern. In addition to Eqs. 8-10, autotrophic homoacetogenesis (as described by Eq. 11 (Saady, 2013)) was also accounted for, since in our previous experiments on CW (Akhlaghi et al., 2017; De Gioannis et al., 2014) this was identified as a possible candidate to explain H_2 consumption during fermentation:

$$4\ H_2 + 2\ CO_2 \rightarrow \text{acetate} + 2\ H_2O \qquad (11)$$

A system of 6 linear equations, expressing the mass balance conditions for HLa, HAc,

HPr, HBu, H_2 and CO_2 in 4 unknowns (x_i) representing the relative contribution of reactions (8)–(11) to the fermentation process, was set up as $\boldsymbol{A} \cdot \boldsymbol{x} = \boldsymbol{b}$, where:

$$\boldsymbol{A} = \begin{bmatrix} 1 & 0 & 0 & 1 \\ 0 & 0.5 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 1 & 1 & 1 & 0 \\ 2 & 1 & -1 & -4 \\ 1 & 1 & 0 & -2 \end{bmatrix}, \quad \boldsymbol{x} = \begin{bmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \end{bmatrix}, \quad \boldsymbol{b} = \begin{bmatrix} \mathrm{HAc} \\ \mathrm{HBu} \\ \mathrm{HPr} \\ \mathrm{HLa} \\ \mathrm{H_2} \\ \mathrm{CO_2} \end{bmatrix}^T$$

For equations systems such as the one above that are overdetermined (containing more independent equations than unknowns), the solution is commonly found through a least-squares approach accepting the approximate form $\boldsymbol{A} \cdot \boldsymbol{x} = \boldsymbol{b} + \varepsilon$ and deriving the $\boldsymbol{x}$ vector that satisfies the condition $\min_x \|\boldsymbol{A} \cdot \boldsymbol{x} - \boldsymbol{b}\|^2$ under the inequalities $x_i \geq 0 \ \forall \ i$. The numerical solution to the problem was derived using the *limSolve* package (Soetaert et al., 2009) developed for application with the R software (www.r-project.org). The results are reported in Figure 4 in terms of values of the coefficients x_i as a function of pH and fermentation time. The degree of fitting of the mathematical model developed was evaluated through the predicted-versus-fitted plots for the six metabolic products of concern, yielding R^2 values in the range 0.74–0.99, proving that the fitting procedure was capable of adequately describing the experimental results (see Supplementary Information document). It can be noted from Figure 4 that changes in the operating pH caused a shift from one fermentation pathway to another, as indicated by different metabolic products becoming prevalent at different pH conditions. In general terms, homoacetogenesis (reaction (11)) turned out to provide a negligible contribution to the fermentation process when compared to the other metabolic pathways, which may be considered as a positive feature when the target metabolic product is H_2.

As for the other reactions, more acidic pHs (up to 6.0) were found to favour lactate conversion into butyrate, with reaction (9) yielding by far the most relevant contribution to the degradation process, and propionic fermentation (reaction (10)) overlapping with the former yet at remarkably lower levels (in the order of 30–40%). No appreciable acetate production was detected at pH values of up to 6.0. As the operating pH increased, the fermentation process became governed by a larger set of metabolic pathways overlapping with each other, so that all three metabolites acetate, propionate and butyrate were present at detectable concentrations in the fermentation liquid. Figure 4 also indicates that, while acetate production did not vary significantly as pH increased from 6.5 to 7.5, propionate production gradually tended to increase and overcome butyrate fermentation.

As expressed by reactions (8) and (9), the conversion of lactate into acetate and butyrate was also accompanied by H_2 and CO_2 production, with no traces of methane in any test. The H_2 content in biogas was found to be always higher than 45% vol., and to increase with the operating pH up to 65% vol. (pH = 7.5) as a consequence of the increased CO_2 solubility in the liquid phase.

The HPYs measured in the experiments, expressed per unit of initial TOC, are shown in Figure 5 along with the Gompertz curves derived by fitting the experimental data points with Eq. (2). The values of the kinetic parameters of the Gompertz equation are reported in Table 2. The data for the UCpH test are not reported, since no appreciable biogas production was observed during the fermentation process, due to the above mentioned biomass inhibition effect. The estimated HPY_{max} proved to be a non-monotonic function of pH, with a maximum of 162.7 L H_2 (kg TOC_i)$^{-1}$ at pH 6.0 and a minimum of 68.1 L H_2 (kg TOC_i)$^{-1}$ at pH 7.5. The observed HPY was clearly a combined effect of the

nature of the metabolic pathways governing the fermentation process in the hydrogenogenic stage and the conversion yield of the original substrate into lactate. The pH 6.0 condition combined the highest lactate production in the first fermentation stage with favourable metabolic pathways for H_2 generation (with a prevalence of butyrate fermentation along with some detectable contribution of acetate fermentation – see Fig. 4). For the tests at higher operating pHs, despite the high observed substrate conversion into HLa (max production of 17–21 mmol HLa (kg TOC_i)$^{-1}$), propionic fermentation became relevant over the other reactions implying lower net HPYs due to the fact that in reaction (10) 1 mole of H_2 is consumed for each mole of propionate generated. On the other hand, the experiments at pHs 5.0 and 5.5, although displaying no relevant H_2-consuming pathways, showed a lower carbohydrates conversion into lactate during the first degradation stage.

In order to compare the results obtained in the present study with those reported in the literature, the measured HPY values were expressed per unit mass of lactose or hexose consumed, under the assumption that carbohydrates in CW were present in the form of lactose only and assuming a 2:1 carbon equivalence between lactose and glucose on a molar basis. The minimum yield (0.66 mol H_2 (mol lactose)$^{-1}$, or 0.33 mol H_2 (mol hexose)$^{-1}$) was attained at pH 7.5, while the maximum value (1.54 mol H_2 (mol lactose)$^{-1}$, or 0.77 mol H_2 (mol hexose)$^{-1}$) was displayed at pH 6.0. This figure is comparable to what observed by Ferreira Rosa et al. (2014) who worked on inoculated CW, and higher than what reported by Akhlaghi et al. (2017) who estimated a specific HPY of around 110 L H_2 (kg TOC_i)$^{-1}$ for CW fermentation tests performed at pH 5.5 without inoculum addition.

It is also interesting to point out that the rate of H_2 production was apparently not

directly related to the achieved yield, as indicated by the values of $t_{95\text{-}H2}$ reported in Table 2.

It is tempting to hypothesise that the sharp shift from the first to the second fermentation stage, governed by, respectively, homolactic fermentation and a combination of the butyric, propionic and acetic pathways was caused by the intrinsic characteristics and composition of the original substrate as well as the existence of fermentation conditions favouring the growth of specialized biomass. As indicated in the previous section, the indigenous biomass present in CW was believed to be responsible for the onset of the homolactic pathway observed during the first stage. The interaction between hydrogen producing bacteria (HPB) and LAB has been widely reported in the literature, with controversial effects of the two having been identified by different authors. According to the evidence from our experiments, the detrimental effect of LAB on HPB reported in the literature (Noike et al., 2002) was likely to have occurred in the first fermentation stage due to the inhibitory effect exerted by the former. On the other hand, it may also be confirmed that, as reported by other investigations (Blanco et al., 2019; Baghchehsaraee et al., 2009; Cabrol et al., 2017; Chojnacka et al., 2011), some form of symbiosis exists between LAB and HPB. Blanco et al. (2019) proposed a trophic interaction between LAB and HPB as being capable of fermenting lactate and acetate (referred to as lactate cross-feeding). Based on the present study, this should be interpreted in the sense that the carbohydrates, once degraded during homolactic fermentation, become limiting for the LAB, while the lactate they produce is made available for use by HPB for H_2 production. To this regard, other authors (Fuess et al., 2018) have suggested that lactate can be utilized as the carbon source by a number of acidogenic biomass types, including both HPB and non-HPB. It should be mentioned

that no evidence could be derived from our study of the existence of a threshold in lactate concentration identified by some authors (Baghchehsaraee et al., 2009; Kim et al., 2012) as being capable of fostering hydrogenogenesis by causing a shift in the metabolic reactions. Nor could we confirm the finding that H_2 production is suppressed in the presence of lactate as the only carbon source for HPB (Baghchehsaraee et al., 2009). In summary, the conclusion we can derive from our experiments is that the fermentation process involved a sequence of lactate production and lactate utilization in a syntrophic system where the product of a given phase was the substrate for the subsequent stage. A similar finding has been previously documented by other authors (García-Depraect and León-Becerril, 2018; Ohnishi et al., 2010). We believe that the coexistence of LAB and HPB as well as the simultaneous presence of lactate and H_2 often observed in continuous fermentation systems (Chojnacka et al., 2011) should be interpreted in light of these considerations. It should also be mentioned that, as no specific microbial analysis of the digestate was performed, it was not possible to identify the biomass strains acting in either phase of the fermentation process, so that the postulated hydrogenogenic capability of some LAB strains (Cabrol et al., 2017) could not be assessed nor excluded either.

The evolution of the process according to two separate stages giving specific fermentation products may give rise to different CW exploitation strategies to be implemented by arranging the fermentation conditions. More precisely, if the focus of the process was on HLa production, the experiments performed suggest that ~23 mmol HLa (g $TOC_i)^{-1}$ could be obtained at pH = 6.0 by stopping the fermentation process after 45 hours. Increasing the operating pH to 6.5−7.5 would reduce HLa production by some 10% (18−21 mmol HLa (g $TOC_i)^{-1}$) while allowing for the reduction of the fermentation

time to 12–30 hours.

On the other hand, if the process was intended to optimizing H_2 production, the fermentation would need to be oriented to attain completion of the second stage in order to provide a maximum HPY of 162.1 L H_2 (kg $TOC_i)^{-1}$ at pH = 6.0 over a fermentation time of 168 hours; these operating conditions would also imply the concomitant production of HBu (4.9 mmol (g $TOC_i)^{-1}$) and HPr (2.8 mmol (g $TOC_i)^{-1}$). Increasing the operating pH to 6.5 would reduce HPY by some 30% (111.6 L H_2 (kg $TOC_i)^{-1}$), but would also entail shortening the fermentation time within ~35 hours.

Lastly, if the main target of the process was to involve the production of short-chain fatty acids, a maximum recovery of 6.5 mmol HAc (g $TOC_i)^{-1}$ and 5.8 mmol HPr (g $TOC_i)^{-1}$) could be attained at pH 7.0 in 168 hours, accompanied by the production of ~100 L H_2 (kg $TOC_i)^{-1}$.

4. CONCLUSIONS

- The indigenous biomass in CW was suitable to sustain the fermentation process, yielding a range of potentially valuable metabolic products.
- Two distinguished stages were involved, including conversion of carbohydrates to lactate followed by lactate degradation to soluble and gaseous products.
- Careful pH control proved essential either to foster lactate production or to prevent the inhibitory effects caused by pH drop due to lactate accumulation.
- Operating pH largely affected the substrate degradation yield and the kinetics of conversion into the final products.
- Different CW exploitation strategies may be arranged by adjusting the operating pH and controlling the fermentation time.

E-supplementary data of this work can be found in online version of the paper.

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Figure captions

Figure 1. Time evolution of soluble carbohydrates (normalized concentration) as a function of pH.

Figure 2. Carbohydrates degradation kinetics: dependence of a) k and b) $t_{95(carb)}$ on the operating pH.

Figure 3. Time evolution of metabolic products as a function of pH.

Figure 4. Values of the coefficients x_i as a function of pH and fermentation time.

Figure 5. Cumulative H_2 production yield as a function of pH.

Table 1. Main characterisation parameters of SCW (average value ± standard deviation).

Parameter	Unit of measure	Value
pH	-	6.16 ± 0.60
Total solids (TS)	%	7.62 ± 0.30
Volatile solids (VS)	%	7.05 ± 0.30
Total carbohydrates (tCarb)*	$g\ L^{-1}$	57.71 ± 4.90
Soluble carbohydrates (sCarb)*	$g\ L^{-1}$	46.53 ± 4.40
Total organic carbon (TOC)	$g\ L^{-1}$	32.06 ± 1.60
Soluble organic carbon (DOC)	$g\ L^{-1}$	26.82 ± 2.20
Soluble proteins (sProt)**	$g\ L^{-1}$	10.76 ± 1.50
Fe	$mg\ L^{-1}$	0.59 ± 0.06
Mg	$mg\ L^{-1}$	87.53 ± 16.72
K	$mg\ L^{-1}$	1149.73 ± 168.51
Na	$mg\ L^{-1}$	578.56 ± 80.58
Ca	$mg\ L^{-1}$	335.38 ± 58.07

* expressed as lactose

** expressed as bovine serum albumin (BSA)

Table 2. Hydrogen production kinetic parameters.

Parameters	**Unit of measure**	**pH 5**	**pH 5.5**	**pH 6**	**pH 6.5**	**pH 7**	**pH 7.5**
HPY_{max}	L H_2 kg TOC_i^{-1}	87.4	140.4	162.7	111.6	105.6	68.1
R_{max}	L H_2 kg TOC_i^{-1} h^{-1}	3.6	3.6	2.4	10.3	2.5	3.8
λ	h	124.1	54.6	37.3	18.8	10.9	19.1
$t_{95\text{-}H2}$	h	159.9	112.2	135.1	34.6	75.3	45.1
R^2	-	0.996	0.999	0.998	0.998	0.979	0.999

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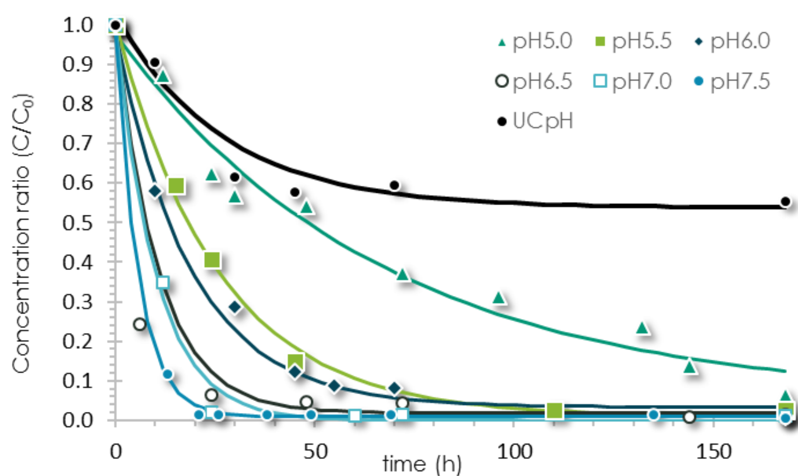

Figure 1

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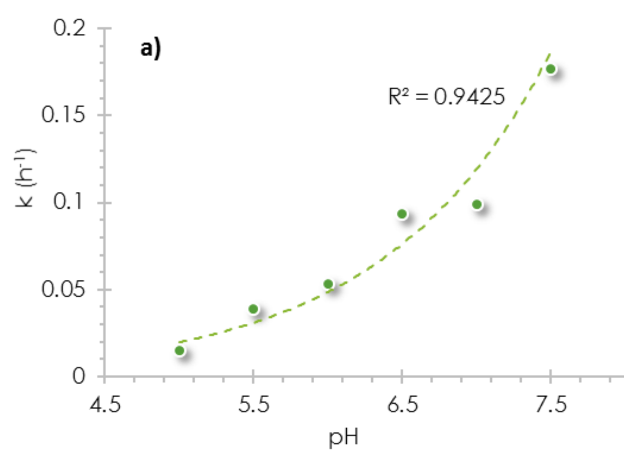

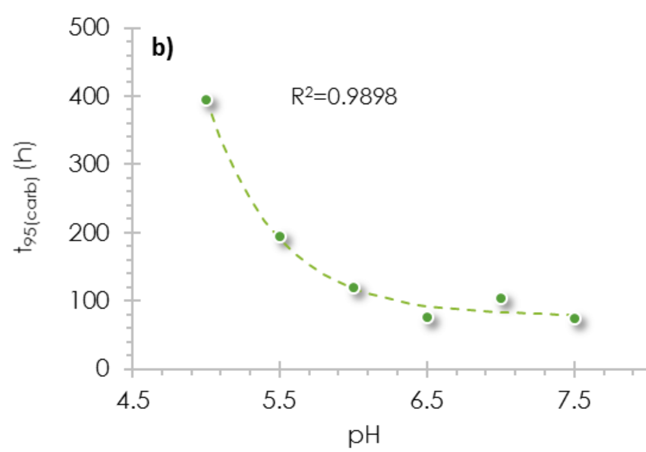

Figure 2

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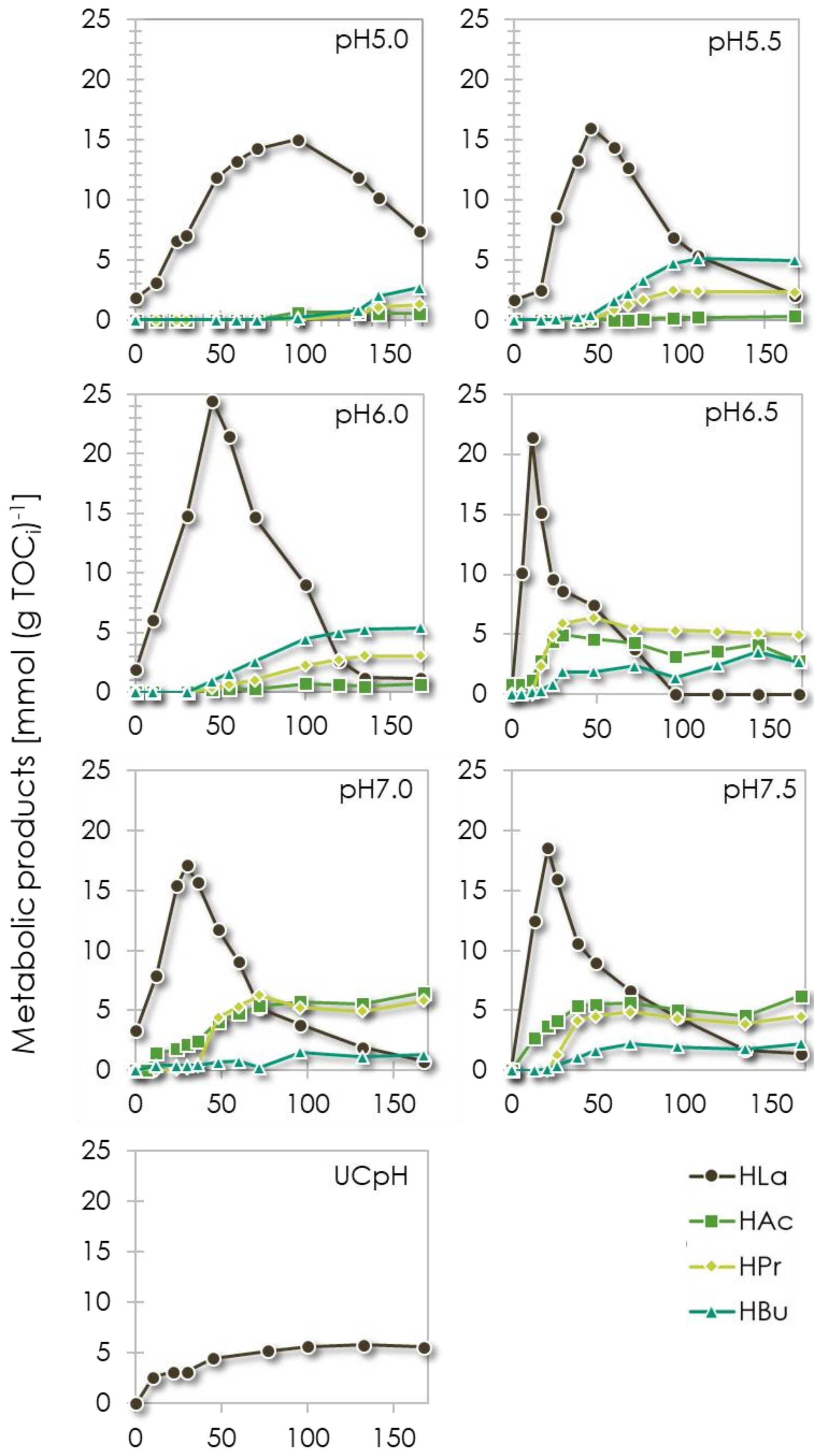

58 Figure 3
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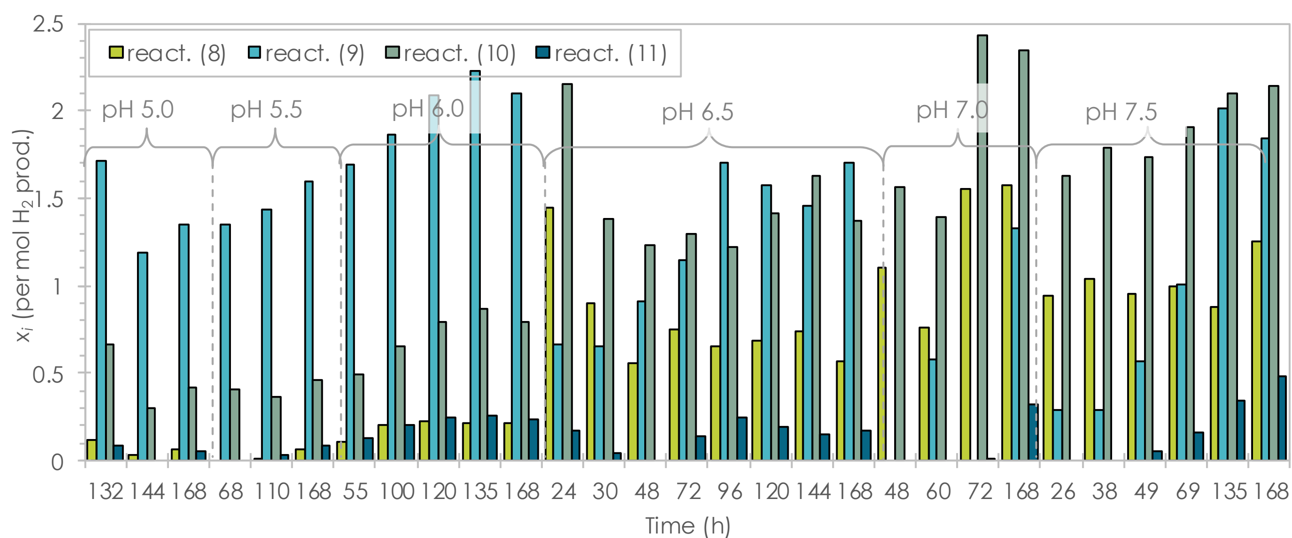

Figure 4

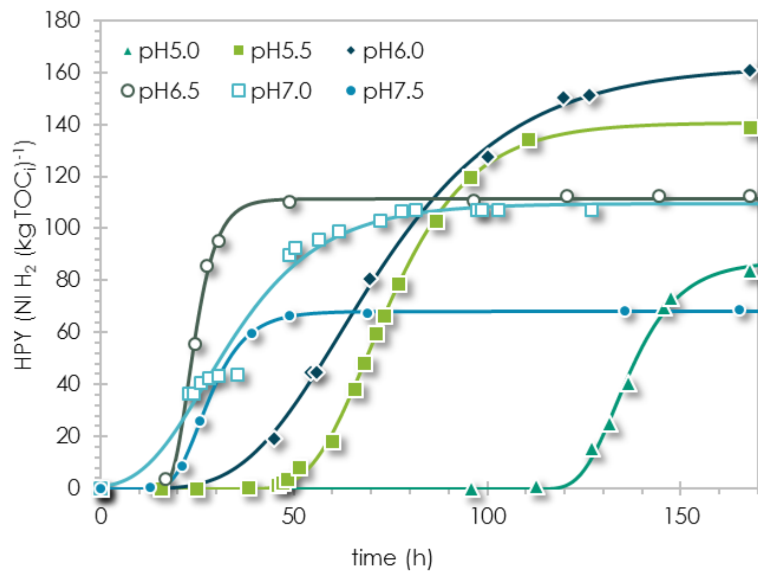

Figure 5

Electronic Annex
Click here to download Electronic Annex: Asunis et al 2019_Suppl Information.docx