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Title

Zeolite favours propionate syntrophic degradation during anaerobic digestion of food waste under low ammonia stress

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Abstract

Zeolite addition has been widely suggested for its ability to overcome ammonia stress occurring during anaerobic digestion. However little is known regarding the underlying mechanisms of mitigation and especially how zeolite influences the microbial structuration. The aim of this study was to bring new contributions on the effect of zeolite on the microbial community arrangement under a low ammonia stress. Replicated batch experiments were conducted. The microbial population was characterised with 16S sequencing. Methanogenic pathways were identified with methane isotopic fractionation. In presence of ammonia, zeolite mitigated the decrease of biogas production rate. Zeolite induced the development of *Izimaplasmatales* order and preserved *Peptococcaceae* family members, known as propionate degraders. Moreover methane isotopic fractionation showed that hydrogenotrophic methanogenesis was maintained in presence of zeolite under ammonia low stress. Our results put forward the benefit of zeolite to improve the bacteria-archaea syntrophy needed for propionate degradation and methane production under a low ammonia stress.

Keywords

Microbial syntrophy; mineral support; methanogenesis; carbon-isotopic fractionation

1. Introduction

In a context of sustainable energy development, anaerobic digestion (AD) is recognised worldwide as a promising bioenergy technology. It is a multi-steps biodegradation process that has the double advantage to reduce the volume of organic waste and produce at the same time methane-rich biogas, usable in both heat and power generation. However, AD is managed by a complex microbial community that is very sensitive to the modifications of the environmental conditions or the presence of inhibiting molecules. Disruption of the microbial equilibrium inside the anaerobic digesters can have dramatic consequences on their degradation performances.

In particular, ammonia is recognized as one of the inhibitor influencing the most anaerobic digestion process. Ammonia is formed during the degradation of protein-rich substrates such as animal manure, slaughterhouse waste or grass (Mata-Alvarez et al., 2014; Munk et al., 2017). Even if ammonia is an essential nutrient for microbial growth, a concentration beyond 200 mg/L TAN (Total Ammonia Nitrogen) can be harmful for the microorganisms (Chen et al., 2008), and a wide range of ammonia concentrations, from 1.1 to 11.8 gTAN/L or 0.027 to 1.45 gFAN/L (FAN, Free Ammonia Nitrogen), has been reported to half-inhibit methanogenic activity (Capson-Tojo et al., 2020).

To counteract the effect of ammonia on AD performances, different strategies have been studied. Anaerobic co-digestion allows to balance the C/N ratio by mixing at least two substrates, one with a low protein content (Prabhu and Mutnuri, 2016), to reduce the concentration of ammonia formed in the digesters. Stripping allows to capture ammonia in gas bubbles to decrease its concentration in the digesters (Bousek et al., 2016). Bioaugmentation is used to increase the resistance of the microbial community by adding specific cultures (Tian et al., 2019; Yang et al., 2019). Addition of mineral supports such as natural zeolite has also been proven to mitigate the ammonia inhibition and improve digester performances (Montalvo et al., 2005; Poirier et al., 2017; Tada et al., 2005). However, optimizing these strategies requires a better understanding of the underlying mechanisms of mitigation.

Zeolite presents different properties useful in the mitigation of the inhibition: natural ion-exchange properties, absorptive capacity and could be a support for the biomass (Montalvo et al., 2012). In particular, zeolite naturally contains Na^+, K^+, Ca^{2+} and Mg^{2+} which could stimulate microbial growth or serve as antagonist to NH_4^+ (Chen et al., 2008; Krakat et al., 2017). These ion-exchange and absorptive capacities could be improved by modifying physically or chemically the zeolite (Ates and Hardacre, 2012; Zhang et al., 2019). For example, Zhang et al (2019) used lignite-modified zeolite that adsorbed ammonium and improved by 7 times the methane production in digesters inhibited by ammonia. They also demonstrated a higher biomass immobilisation using the modified zeolite, but provided no further information regarding the identity of these microbes. In general, the role of zeolite in the immobilisation of the biomass has been mainly evidenced using electronic microscopy (Fernández et al., 2007). Weiß et al showed that hydrolytic microorganisms such as *Ruminofilibacter xylanolyticum* and methanogenic archaea could grow on zeolite under non inhibiting conditions (Weiß et al., 2013, 2011).

However, only a few studies investigated the consequences of natural zeolite addition on microbial structuration during ammonia inhibition and related it to the performances of the digester. In digesters inhibited by 19 g-TAN/L, Poirier et al. observed that methane production could be increased by 39% and lag phase reduced by 50% when zeolite was added. This mitigation was associated to the preservation of archaea *Methanosarcina* and development of *Methanobacterium* (Poirier et al., 2017). However, clear role of the zeolite was not understood. For this reason, in the present work we focused on the identification of the microbes influenced by the presence of natural zeolite under ammonia inhibition and on the role of the zeolite on specific microbial degradation pathways.

Microbial community structure was evaluated with 16S metabarcoding and linked to the digester performances. Two series of lab digesters were set up: a control series without ammonia and a stressed series with ammonia. In the stressed series, 4 g-TAN/L were added in order to induce a low stress and simulate a bioprocess at an early stage of ammonia accumulation. If multiple studies have been carried out in order to determine the mitigation effect of the zeolite under medium to high ammonia inhibition (Poirier et al., 2017; Wang et al., 2011), a few studies also demonstrated that zeolite improves the methane production under low ammonia inhibition (Milán et al., 2001; Wijesinghe et al., 2019), but did not collect information regarding the microbial communities.

Different pre-treatments were applied to the zeolite to test different hypotheses on its role on the microbial community structuration. The influence of fine particles release was tested (Abadzic and Ryan, 2001). For that purpose the zeolite was boiled to release directly soluble particles into water. Effects of both the modified zeolite and the obtained solution were tested. The effect of an increase of the surface available was also evaluated by pre-treating thermically the zeolite to remove water and free sites. By removing the adsorbed water, the adsorptive capacity of the zeolite could be improve (Kesraoui-Ouki et al., 1994) as well as microbial growth by increasing the surface. In total, for each series, 5 zeolite conditions were tested.

2. Material and Methods

2.1. Feedstock preparation

The inoculum used in the experiment came from a mesophilic lab-scale anaerobic digester (60L) treating biowaste. In order to degrade the residual organic matter in excess it was stored at 35°C during two weeks in anaerobic condition without feeding before being used. The inoculum was centrifuged at 10,000g during 10 minutes before use.

The substrate used to feed the digesters came from an industrial food waste collector (Valdis Energie, Issé). The food waste came from different origin such as schools, markets and expired products. The food waste was crushed before use.

The physico-chemical characteristics of the inoculum and biowaste are presented in the supplementary material.

2.2. *Preparation of zeolite*

Zeolite was obtained from Somez society (France). The zeolite was sieved to obtain a homogeneous size between 0.48-0.50 mm. To release easily soluble ions a part of the zeolite was boiled at 100°C in water during 20 minutes. The liquid residue, containing the easily soluble ions, was filtered at 0.22 µm before use and residual solid part was dried before use. To increase the surface availability for microbial colonisation and modify the ion-exchange capacity another part of the zeolite was heated in the oven at 400°C during 4 hours.

2.3. *Experimental set-up*

Table 1 summarises the composition of the different digesters. In total 30 anaerobic batch bioreactors were set-up in 1 L glass bottles (700mL working volume). Each digester was inoculated with methanogenic sludge and fed with biowaste to reach a substrate/inoculum ratio of 12 g COD/1,2 g COD. Zeolites previously prepared were added at a concentration of 15g/L measured before pre-treatment. In parallel a control without zeolite was set-up. In total 5 conditions were tested in triplicate. Ammonium carbonate (Alfa Aesar) was added to reach a concentration of 4 g/L of TAN. In parallel to the series with ammonia (N series) a control series without addition of ammonium carbonate (C series) was set-up. All the digesters were complemented with a biochemical potential buffer (International Standard ISO 11734 (1995)) to reach a final working volume of 700 mL. To obtain an equal quantity of carbonate in the different digesters, sodium carbonate was added into the BMP for the control C series. The bioreactors were then sealed with a screw cap and a rubber septum. The headspaces were flushed with N_2 (purity >99.99%, Linde gas SA) and the bottles were incubated at 35°C in the dark and without agitation.

Weekly, 6mL of liquid phase were sampled through the septum using a syringe and centrifuged at 10,000 g for 10 minutes. Supernatant and pellet were snap frozen using liquid nitrogen and kept at -20°C and -80°C respectively.

2.4. *Gas production and chemical analyses*

The different parameters such as biogas composition, NH_4^+/NH_3, volatile fatty acids were measured as described in (Cardona et al., 2019; Chapleur et al., 2014).

Production parameters were calculated using R CRAN software and the Gompertz equation with R Grofit package.

$$y(t) = A.\exp[-\exp\left(\frac{\mu.e}{A}(\lambda - t) + 1\right)]$$

Where y(t) is the cumulative CH_4 production (mgC) at date t, A is the ultimate CH_4 production (mgC), μ is the maximum CH_4 production rate (mgC/day), and λ is the lag phase (days).

The link between Free Ammonia Nitrogen (FAN), Total Ammonia Nitrogen (TAN), pH and temperature can be summarized with the following equation (Anthonisen et al., 1976):

$$FAN = \frac{10^{pH}}{(\exp\left(\frac{6344}{T}\right) + 10^{pH})} x\, TAN$$

Where T is the temperature in Kelvin.

2.5. *Isotopic fractionation*

As indicator of the methanogenic pathway the isotopic fractionation of the CH_4 ($\delta^{13}C$-CH_4) was measured using a Trace Gas Chromatograph Ultra (Thermo Scientific) attached to a Delta V Plus isotope ratio mass spectrometer via a GC combustion III (Thermo Scientific). Periodically gas was sampled into 7-mL vacuumed serum tubes for the analysis. It is assumed that a value of $\delta^{13}C$-CH_4 inferior to -60‰ indicates a CH_4 production through the hydrogenotrophic pathway and a value of $\delta^{13}C$-CH_4 superior to -60‰ indicates a CH_4 production through the acetoclastic pathway (Whiticar et al., 1986).

2.6. *Analysis of the microbial community*

Total DNA was extracted from sample's pellet using DNeasy PowerSoil kit (QIAGEN) following the manufacturer instructions. The DNA quantity was measured by Qubit (dsDNA HS assay kit, Invitrogen).

Archaeal and bacterial hyper variable region V4-V5 of the 16S rRNA gene was amplified by PCR with fusion primers 515F (5′- Ion A adapter–Barcode–GTGYCAGCMGCCGCGGTA-3′) and 928R (5′-Ion trP1 adapter–CCCCGYCAATTCMTTTRAGT-3′), which include a barcode and sequencing adapters, and then sequenced according to the protocol described by Madigou et al. (2019).

2.7. *Data analyses*

2.7.1. Gas production

The variance between the biogas productions observed under the different conditions was estimated by comparing the 3 Gompertz parameters calculated for both CH_4 and CO_2 with analysis of variance (ANOVA). The function *granova.1w* from the R package *granova* (version 2.1) was used to compute the ANOVA. This package provides a graphical representation of the ANOVA results. For each parameter tested (6 in our case), one graphic represents on the y-axis the individual and mean value of the parameter for each condition (C0, C1, C2, C3, C4, N0, N1, N2, N3, and N4), classified on the x-axis according to the estimated contrast coefficient for each condition. The contrast coefficient is calculated as the difference between the group mean (mean of all the digesters of the condition) and the grand mean (mean of all the digesters). This representation enables to visualise at the same time the variation within and between the groups. In addition the graphic represents the mean square error between the conditions (MS-between) and the mean square error within the conditions (MS-within) in the form of squares. The comparison between the area of the MS-between and the area of the MS-within indicates if some conditions are significantly different form the others. The F-statistic value is also indicated on the graph. This value is calculated as the ratio of the area of the MS-between and the area of the MS-within. Combined to the p-value, the F-statistic value (F>1) allows to determine if there is a significant difference in the mean between the conditions for the different Gompertz parameters. Finally the residuals, portions of the variability unexplained by the ANOVA, are also represented. The repartition of the residuals indicates if the residuals are independent or not and confirm if the dataset meets the assumptions of the ANOVA (independence, normality, homogeneity).

2.7.2. Microbial dynamics

An OTU count matrix was designed using FROGS (Find Rapidly OTU with Galaxy Solution), a galaxy/CLI workflow (Escudié et al., 2018). The OTUs abundances were examined through statistical analyses using R CRAN software (version 3.5.1). Low abundant OTUs were filtered: OTUs present above 1% in at least one sample were kept. OTUs abundances in the samples were finally transformed with centered log ratio (CLR) (Lê Cao et al., 2016).

Multivariate analyses were computed using mixOmics R package (version 6.6.1) (Rohart et al., 2017). Firstly, Principal Component Analysis (PCA) was performed to highlight the relationship between samples from the different conditions (ammonia/zeolite). Secondly, sparse Partial Least Square Discriminant Analysis was carried out to compare the different conditions and evidence the microorganisms influenced by the presence of ammonia and/or zeolite. More precisely, three pairwise sPLS-DA were carried out respectively between the control C series and N series without zeolite (N0-N1); between N series without and with solid zeolite (N1-N3-N4) and between C series and N series with solid zeolite. The abundances of the most discriminant microorganisms selected by the three sPLS-DAs were summarised in a heatmap using Euclidean distance and ward clustering method.

3. Results and discussion

3.1. Modification of the performances of anaerobic digestion

Differences in CH_4 and CO_2 productions could be observed between the digesters from N and C series (supplementary material). To compare them accurately between the different experimental conditions, each gas production curve was modelled with the Gompertz equation (the estimated Gompertz parameters are summarised in the supplementary material). The variance of the estimated Gompertz parameters between each condition was evaluated using ANOVA. Results are summarized in Figure 1. We first observed that, for all the parameters, calculated residuals were randomly distributed. It showed that the assumptions of ANOVA were met and that ANOVA results could be trusted. For all the parameters the p-value and F-statistic values were significant ($p\text{-value}<0.05$ and $F>1$) which meant that at least one of the conditions was significantly different from the others. The different conditions were then compared by groups to evidence the effect of ammonia and zeolite on the production of biogas.

3.1.1. Effect of a low concentration of ammonia on biogas production

Regarding CO_2 production (figure 1-A-B-C), the presence of ammonia, independently of zeolite addition (N series), resulted in a decrease of the maximal production (from 499 to 270 mgC for the extreme values) and of the production rate (from 19.8 to 9.6 mgC/day for the extreme values) compared to C series. In addition the lag phase increased from 0 to 5.8 days between the digesters from the C and N series.

Regarding the CH_4 production (figure 1-D-E-F), the presence of ammonia resulted in an increase of the maximal production (from 895 to 1005 mgC for the extreme values). However the lag phase increased from 5.8 to 9 days for respectively the C series and the conditions N0 and N2 (without zeolite). No general trend was observed for CH_4 production rate.

The comparison of the CH_4 and CO_2 production values indicated that the presence of a low concentration of ammonia slowed down the process, even if no strong inhibition was observed. This was expected as the objective of the study was to investigate the effect of the zeolite during the early stage of ammonia accumulation. The operational performances were even improved as production of CH_4 increased in presence of ammonia. One hypothesis is that ammonia stressed the acetoclastic archaea in favour of the hydrogenotrophic ones, leading to an enrichment of the biogas into methane. This hypothesis is supported by different authors (Lv et al., 2018; Ruiz-Sánchez et al., 2019; Westerholm et al., 2011).

3.1.2. Effect of zeolite in presence of ammonia (N series)

Based on the lag phase and production rate of the CH_4 and CO_2 production, the digesters of N series could be separated in two groups. A first group was composed of N0 (no zeolite) and N2 (liquid residue). A second group was composed of the digesters with solid zeolite, no matter the treatment (N1-N3-N4).

The presence of solid zeolite (N1-N3-N4) allowed to decrease the lag phase by 2 days for both CO_2 and CH_4 and to increase the production rate by 1.2 times compared to N0-N2. Similarly, Poirier et al (2017) used a similar natural zeolite and observed a reduction of the lag phase by 20 days for CH_4 production and 39% improvement of the maximal production in digester inhibited with 19g/L of TAN. In that case effect of zeolite was greater, probably because the level of stress imposed was also greater.

Boiling the zeolite was done in order to release fine particles from the zeolite. The removed particles do not seem to be the mechanism behind the inhibition mitigation in the conditions of our experiment. Heating the zeolite was done in order to remove water and organic residues and release free sites to improve molecules adsorption or microbial colonisation. Increasing the surface on the zeolite did not improve the performances. This could be due to a limited growth of microorganisms in the duration of the experiment which did not saturate the surface of the zeolite in N1 and did not benefit from the increased surface in N4. In order to evidence the microbial colonisation of the zeolite, electronic microscopy could be applied as described in several studies (Fernández et al., 2007; Zhang et al., 2019).

In all cases solid zeolite mitigated ammonia stress. It may have acted as a support for the microbial growth and/or improved the microbial interaction by decreasing the spatial distance between microorganisms engaged for example in a syntrophy.

3.1.3. Effect of zeolite without ammonia (C series)

Regarding the digesters without ammonia addition (C series) biogas performances were not significantly different between the conditions. Indeed the CH_4 and CO_2 maximal production were respectively close to 925 ±27.8 and 487 ±8.9 mgC for all the digesters. Compared to the control (C0), the CH_4 production rate was slightly reduced in presence of solid zeolite (C1-C3-C4) and liquid residue (C2), from 47 mgC/day for the control to 42-46 mgC/day for solid zeolite and 38 mgC/day for liquid residue. However the lag phase was not modified between the reactors. This result is in accordance with previous study showing that the presence of the zeolite in absence of ammonia had little influence on the digesters performances (Poirier et al., 2017).

3.2. Effect of zeolite treatments on different chemical parameters

The Figure 2 summarises the evolution of the acetate, propionate and butyrate concentrations, pH, Free Ammonia Nitrogen (FAN) and Total Ammonia Nitrogen (TAN) concentration for the different conditions.

3.2.1. Ammonia evolution

In the digesters of the C series, the ammonia concentration remained under 200 mg-TAN/L (10 mg-NH_3/L). In the digesters of N series, the TAN concentration remained stable around 4000 mg/L all along the experiment. The concentration of ammonia did not decrease across the experiment in presence of zeolite. This confirmed that the adsorption of ammonia by the zeolite remained limited in our experiment.

The free ammonia nitrogen (FAN) concentration rapidly stabilised at 300 mg-NH_3/L in the digesters of the N series even if it was higher than 1000 mg-NH_3/L at the beginning of the experiment. This decrease was due to a drop of pH, consequence of the VFAs accumulation observed for all the digesters. It is usually assumed that the FAN is the most toxic form of ammonia for the methanogens community. Several studies reported an inhibitory threshold between 50 to 1400 mg-NH_3/L (Astals et al., 2018; Rajagopal et al., 2013). In our case, the first days at a high concentration of FAN was probably the most stressful for the microorganisms. This can explain the delay observed in the biogas production in presence of ammonia. However, after a few days, decrease of pH resulted in the expected low ammonia stress in the digesters of N series.

3.2.2. Volatile fatty acids evolution

All the digesters in absence of ammonia (C series) presented the same VFA evolution. The maximum accumulation was around 750 mgC/L, 350 mgC/L and 300 mgC/L for respectively acetate, propionate and butyrate. No effect of zeolite was observed in that case.

Under low ammonia stress, the VFA accumulations confirmed the grouping of the different conditions from the N series observed with the gas production (N0-N2 and N1-N3-N4).

In presence of ammonia and without solid zeolite (N0-N2) higher acetate (1050 mgC/L) coupled to lower propionate (200 mgC/L) and butyrate (150 mgC/L) accumulations than in digesters from C series was observed. The acetate can be degraded by acetoclastic archaea or bacteria in syntrophy with hydrogenotrophic archaea. The increase of the acetate accumulation observed in these digesters could be due to the stress of the acetoclastic archaea by the ammonia, as observed in previous studies (Wang et al., 2015). On the other hand, both propionate and butyrate require syntrophy for their degradation (Müller et al., 2010). Their lower accumulation in presence of ammonia suggested that syntrophic archaea were not inhibited. This is contradictory with several studies that evidenced that ammonia could inhibit the syntrophic propionate degrading bacteria (SPOB) and methanogens leading to an accumulation of propionate (Calli et al., 2005; Zhang et al., 2018). However in our study the stress was lower than in the cited studies (4 gTAN/L versus 7 gTAN/L in Zhang et al 2018) which could explain the difference in the propionate and butyrate degradation. The complete propionate degradation was achieved at day 40 such as in the C series. Consequently, as suggested by the biogas composition and the accumulation of acetate, we hypothesized that the acetoclastic methanogens were delayed in favour of the hydrogenotrophic methanogens in N0-N2. This succession of events may have favoured the syntrophic degradation of the butyrate and propionate in presence of ammonia.

In presence of ammonia and with solid zeolite (N1-N3-N4) the pattern of evolution of the butyrate was similar to the one of N0-N2, while acetate and propionate evolutions were similar to the one in C series, except that the propionate was fully consumed earlier. It can be hypothesized that the zeolite limited the perturbation of the acetoclastic archaea by NH_3. The preservation of the activity of the acetoclastic methanogens allowed to consume the acetate faster than in presence of ammonia. The high activity of the acetoclastic archaea may have limited the growth of the hydrogenotrophic archaea resulting in higher accumulation of propionate than in N0-N2. However, butyrate did not accumulate. We hypothesized that a competition for the syntrophy with the hydrogenotrophic archaea was won by the butyrate degraders, explaining this low accumulation. It would be in accordance with thermodynamic observation that the Gibbs free energy needed for the propionate degradation is higher than the one for the butyrate degradation, and makes it harder to degrade (Wu et al., 2016). However, due to the presence of the zeolite, the propionate degradation occurred faster than in absence of zeolite (N0-N2). This means that the presence of zeolite played a significant role on the propionate degraders activity and the syntrophic interactions needed for the VFAs degradation.

3.3. Effect of the ammonia and zeolite on the methanogenic pathways

The methanogenic pathways for the different conditions were evaluated by measuring isotopic fractionation of the CH_4 ($\delta^{13}C\text{-}CH_4$) (Figure 3). In absence of ammonia (C series) in all the experiments the $\delta^{13}C\text{-}CH_4$ increased from -55 ‰ to -35 ‰ during the degradation of acetate (day 0 to day 30). This progressive increase indicates that the acetate degradation was mainly due to acetoclastic archaea. During propionate degradation the value decreased to -55 ‰ and stabilised at this level until the end of the experiment (day 30 to day 100). It confirmed that propionate degradation implied hydrogenotrophic archaea. The CH_4 produced by their activity led to the dilution of CH_4 produced by acetoclastic archaea.

In presence of ammonia (N series) with or without zeolite the $\delta^{13}C-CH_4$ was lower at the beginning of the degradation than for the C series. The $\delta^{13}C-CH_4$ increased from -70 ‰ to -45 ‰ and -35 ‰ respectively for the digesters N1-N3-N4 (with zeolite) and N0-N2 (without solid zeolite) during acetate degradation. This indicates that, at the beginning of the experiment, the acetoclastic pathway was minored in favour of the hydrogenotrophic pathway. This result is in line with the lower CO_2 production observed, as CO_2 was used by the hydrogenotrophic archaea for CH_4 production. After 15 days two types of patterns were observed, grouping the digesters N0-N2 on one side and N1-N3-N4 on the other side, as observed with the previous chemical data.

In absence of zeolite when ammonia was present (N0-N2) the $\delta^{13}C-CH_4$ reached the same value than the control without ammonia (-35 ‰) at day 33. The propionate degradation then led to the decrease of the $\delta^{13}C-CH_4$ until -50 ‰. In contrast with the stability in the isotopic composition observed for C series once all the propionate degradation was achieved, a further decrease in the isotopic composition for the N0-N2 digesters was observed until -60 ‰. This result suggests that a mix of both methanogenic pathways seemed to occur under ammonia inhibition without zeolite. This result is in line with a previous study from Ruiz-Sanchez et al (2018). The authors observed a mix of the methanogenic pathways at an ammonia level of 1.9 g-TAN/L (Ruiz-Sánchez et al., 2018).

In presence of zeolite and ammonia (N1-N3-N4) the $\delta^{13}C-CH_4$ increased until -35 ‰ at day 25. The propionate degradation led to a rapid decrease of the $\delta^{13}C-CH_4$ to -60 ‰. The value continued to decrease to reach a final value of -65 ‰. The lower increase of the $\delta^{13}C-CH_4$ compared to the digesters without zeolite N0-N2 indicates that a more important part of the CH_4 was produced by the hydrogenotrophic pathway even if acetoclastic archaea were active.

These results indicate that the acetoclastic archaea were sensitive to the presence of ammonia even at the low concentration used in our experiment. Acetoclastic archaea are usually considered as the most sensitive methanogens to ammonia (Yenigün and Demirel, 2013) and a switch from acetoclastic to hydrogenotrophic methanogenesis is typically observed when ammonia exceeds a threshold of 0.14-0.28 gNH_3/L (3.0-3.3 gNH_4^+-N/L) (Westerholm et al., 2016). In addition ammonia can induce the production of CH_4 through the Syntrophic Acetate Oxidation (SAO) (Schnurer and Nordberg, 2008). Acetoclastic archaea were able to partly overcome this stress when NH_3 concentration decreased due to the VFAs accumulation. However, the presence of zeolite in the digesters enabled an important activity of the hydrogenotrophic archaea leading to a fast degradation of the VFAs and an enrichment of CH_4 in the biogas.

3.4. Identification of the key phylotypes of the different conditions

The microbial community was analysed with 16S RNA metabarcoding for each digester at one time point (day 22) corresponding to the peak of CH_4 production in all the digesters (supplementary material).

Microorganisms specifically influenced by the presence of ammonia and/or zeolite were characterised using sPLS-DA. This method allows the classification of the samples into groups and allows the identification of the key microorganisms discriminating these groups. To evidence respectively microbes discriminant of digesters inhibited by ammonia and not inhibited, of digester containing zeolite or not in presence of ammonia, of digesters without ammonia and digesters with ammonia but containing zeolite, three pairwise sPLS-DA were carried out between the following groups: A (samples from the C series), B (samples from N0-N2 conditions) and C (samples from N1-N3-N4 conditions). The samples could be classified into the different groups with a mean error rate inferior to 4%. Abundances of the key microorganisms characteristic of the different groups and selected through the different sPLS-DA are summarised in a heatmap (Figure 4). The corresponding taxonomic affiliation is reported in the supplementary Table A.3. The microorganisms could be grouped in 5 clusters according to the presence of the zeolite and ammonia.

3.4.1. Microorganisms inhibited by the presence of ammonia

The cluster 1 grouped together the microorganisms abundant in the group A (without ammonia) and less abundant in presence of ammonia no matter the presence of zeolite. Seven OTUs belonging to the families *Lachnospiraceae*, *Rikenellaceae, Bacillaceae, Clostridiaceae* and to the order *Bacteroidales* (GZKB124) were identified. *Lachnospiraceae* family is recognised as cellulolytic degrader and is known to produce large amounts of acetate and CO_2 (Florentino et al., 2019). *Rikenellaceae* family is known to use lactate fermentation where acetate and propionate are the end-products (Yi et al., 2014). *Clostridiaceae* and *Bacteroidales* are usually reported to be the main phyla in the anaerobic digesters and are known to be cellulolytic microbes (Hassa et al., 2018). In presence of ammonia, microorganisms of cluster 1 were probably replaced by microorganisms with similar functions.

3.4.2. Microorganisms preserved under ammonia low stress by the presence of zeolite

The cluster 2 grouped the microorganisms present in group A, inhibited by the ammonia (group B) but preserved thanks to the presence of zeolite (group C). Nine OTUs were found and belong to the families *Peptococcaceae, Spirochaetaceae, Lachnospiraceae, Cloacimonadaceae, Izimaplasmatales* order and the *Methanoculleus* genus.

Cloacimonadaceae are known to be acetogens (Lee et al., 2018). Members of the family *Peptococcaceae* are syntrophic obligate propionate oxidizers (Ziganshin et al., 2011). The presence at day 22 of this microorganism in group A and C was in accordance with the degradation pattern of the propionate. Indeed in digesters without ammonia and with ammonia and zeolite (group A and C) the propionate degradation occurred earlier than in digesters with ammonia and no zeolite (group B). OTU from genus *Treponema* (*Spirochaetaceae* family) was identified. Some members of this genus are known to degrade cellulose and some others to be homoacetogens and reduce H_2 and CO_2 into acetate (Lee et al., 2013). Finally *Izimaplasmatales* from the *Tenericutes* phylum was described recently by Skennerton et al (Skennerton et al., 2016). It was identified from methane seep sediment samples. The members of *Izimaplasmatales* order are able to ferment anaerobically simple sugars. To the best of our knowledge this study is the first one reporting the presence of *Izimaplasmatales* order in anaerobic digesters. Further analyses should be done to fully understand its role in anaerobic digesters.

3.4.3. Microorganisms whose development results from the presence of ammonia

The cluster 3 grouped the microorganisms which were absent in the group A but abundant in the groups B and C, meaning that their development was favoured by the presence of ammonia, independently of the presence of zeolite. The microorganisms from this cluster, represented by 9 OTUs, belonged to the same families than the one identified in the previously described clusters *Lachnospiraceae*, *Peptococcaceae*, *Clostridiaceae, Izimaplasmatales* and also to *Syntrophomonadaceae*. *Syntrophomonadaceae* family degrades long chain fatty acids into acetate and glycerol (Sousa et al., 2009) but members of the *Syntrophomonas* genus, identified in this system, are butyrate degraders (Narihiro et al., 2016; Zou et al., 2003). The development of this *Syntrophomonas* could explain the faster butyrate degradation observed in the groups with ammonia no matter the presence of zeolite (groups B and C). Moreover, *Syntrophomonas* was already shown to be resistant to the increase of ammonia in CSTR experiment (Bonk et al., 2018). In general, we hypothesized that microorganisms of cluster 3 had similar metabolisms than microorganisms of cluster 1. Switch from one cluster to the other could derive from the presence of ammonia. It could explain the relative similarity of the VFA patterns observed in the reactors no matter the condition.

3.4.4. Microorganisms slightly inhibited by the presence of ammonia

The cluster 4 grouped the microorganisms more abundant in group A than in groups B and C. Seven OTUs were identified in this cluster and belonged to the families *Dysgonomonadaceae, Synergistaceae* and *Syntrophomonadaceae. Dysgonomonadaceae* family is composed by three genera, *Fermentimonas*, *Petrimonas* and *Proteiniphilum*, which have fermentative activities and produce acetate (Hahnke et al., 2016). *Synergistaceae* family is composed of amino acid degraders (Rivière et al., 2009). All the *Syntrophomonadaceae* present in this cluster belonged to the genus *Syntrophomonas*. Members of this genus seemed to be differently influenced by the presence of ammonia as in cluster 3 another OTU of this genus was favoured by the presence of ammonia. This result is supported by a previous study where a reorganisation within the *Syntrophomonas* population was observed due to the presence of ammonia (Poirier et al., 2020).

3.4.5. Microorganisms whose development is enhanced by the presence of ammonia

The cluster 5 grouped microorganisms more abundant in groups C and B than in group A. Their relative abundance was increased by the presence of ammonia. Mostly archaea were identified in this cluster, with three bacteria. Identified archaea belonged to the genera *Methanoculleus, Methanobacterium* and *Methanosarcina.* If these genera seemed to be present in the different conditions, the OTUs *Methanobacterium_41, Methanoculleus_2* and *Methanosarcina_1* were specifically favoured by the presence of ammonia. These 3 archaea are known to be resistant to the presence of ammonia (Jarrell et al., 1987; Poirier et al., 2016; Tian et al., 2019). *Methanoculleus* and Methanobacterium are hydrogenotrophic archaea while *Methanosarcina* is a versatile archaea able to use both acetoclastic and hydrogenotrophic pathways. Unfortunately, no additional information at the species level was available from the sequencing results. The modification of archaea species, specifically for *Methanosarcina*, was already observed when ammonia concentration was increased (Poirier et al., 2016). Bacteria belonged to *Marinilabiliaceae* and *Paludibacteraceae* families and to the order *Clostridiales*. Members of *Paludibacteraceae* family use various sugars to produce acetate and propionate as major end-products (Ueki et al., 2006).

3.5. Biological pathway reconstruction

To evidence the effect of zeolite addition in presence of a low concentration of ammonia we summarized the characteristics of AD in the different situations observed (no ammonia, ammonia with solid zeolite, ammonia without solid zeolite). The figure 5 represents for each situation the patterns of VFA degradation and methane production evidenced from the performances analyses, as well as the microbes that could be responsible of these patterns.

In control conditions without ammonia and no matter the zeolite treatments (C series) different VFAs producers were identified such as *Lachnospiraceae*, *Cloacimonadales*, *Rikenellaceae or Spirochaetaceae*. The degradation of the VFA, especially propionate and butyrate, was mainly due to respectively the families *Peptococcaceae* and *Syntrophomonadaceae*. The biogas was composed of 66% of CH_4 and 34% of CO_2. The main pathway for the CH_4 production was the acetoclastic one. It was confirmed by the isotopic fractionation and the patterns of VFAs degradation.

In presence of ammonia and without zeolite (N0-N2) the VFA production was mainly due to *Paludibacteraceae* and *Lachnospiraceae.* The genus *Syntrophomonas*, responsible of the butyrate degradation, was observed indicating its ability to develop in presence of ammonia. The main pathway for methanogenesis before day 15 was the hydrogenotrophic one as the acetoclastic archaea were stressed by the presence of NH_3 (1000 mg-NH_3/L). After day 15, the NH_3 concentration decreased, the acetoclastic archaea overcame the stress and the CH_4 was produced through both hydrogenotrophic and acetoclastic pathways. The relative abundance of specific OTUs of *Methanosarcina*, *Methanoculleus* and *Methanobacterium*, was enhanced by the presence of ammonia. The presence of the bacteria *Marinilabiliaceae* was observed but no specific role could be attributed to this organism in our system.

In presence of ammonia and with zeolite (N1-N3-N4) the VFA production could mainly be attributed to the families *Spirochaetes* and *Cloacimonadaceae.* The hydrogenotrophic pathway seemed to be preserved more importantly than in absence of zeolite. The preservation of the hydrogenotrophic archaea led to a faster degradation of the VFAs in syntrophy with bacteria, such as *Peptococcaceae* and *Syntrophomonas*, respectively propionate and butyrate degraders. The same genera of archaea than the one observed in presence of ammonia only, *Methanosarcina*, *Methanoculleus* and *Methanobacterium,* were also observed in presence of ammonia only. *Izimaplasmatales,* identified only in presence of zeolite could play a role in the performance modification; however we could not put forward any hypothesis on its role due to the lack of knowledge on this microorganism. The development of the family *Marinilabiliaceae* was maintained in presence of zeolite.

In our study the presence of ammonia, even at a low concentration, led to an important dynamics of obligate syntrophic bacteria and hydrogenotrophic archaea. Zeolite could act as a support for the microbial growth which improved the proximity and exchanges between the microorganisms and protected them. Poirier et al (2017) showed that different supports mitigated the ammonia inhibition differently and the mitigation was associated to changes of the microbial community. The influence of the zeolite to facilitate electron transfer pathways should be further investigated, especially as *Peptococcaceae*, preserved by the zeolite, was recognised to be able to use DIET (Direct Electron Transfer) (Jing et al., 2017). The investigation of the functional pathways with metatranscriptomics analysis would provide further information on the microbial metabolism involved in presence of zeolite and ammonia.

4. Conclusion

This study evidenced the influence of the zeolite on the structuration of the anaerobic microbial population during a low stress caused by the presence of ammonia. Zeolite acted on the microbial distribution and allowed the growth of microorganisms like *Izimaplasmatales, Peptococcaceae* or *Methanobacterium*. The rearrangement of the community resulted in the modification of the equilibrium between the VFAs producers and degraders. Particularly, the syntrophic propionate and butyrate degradation were enhanced. The reasons for this rearrangement cannot be explained by the pre-treatments tested in our study as no strong modification between raw and modified zeolites could be observed. The effect seemed to be mostly due to the presence of a solid support. We hypothesize that it enabled the immobilisation of the biomass, inducing close interactions and protection, favourable in particular for the methanogens, and resulting in a limited deterioration of the bioprocess performances. Targeted analyses and the investigation of the microbial functions would allow to further understand the influence of zeolite on the syntrophic microbial metabolism.

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Figures legends:

Table 1. Experimental set-up.

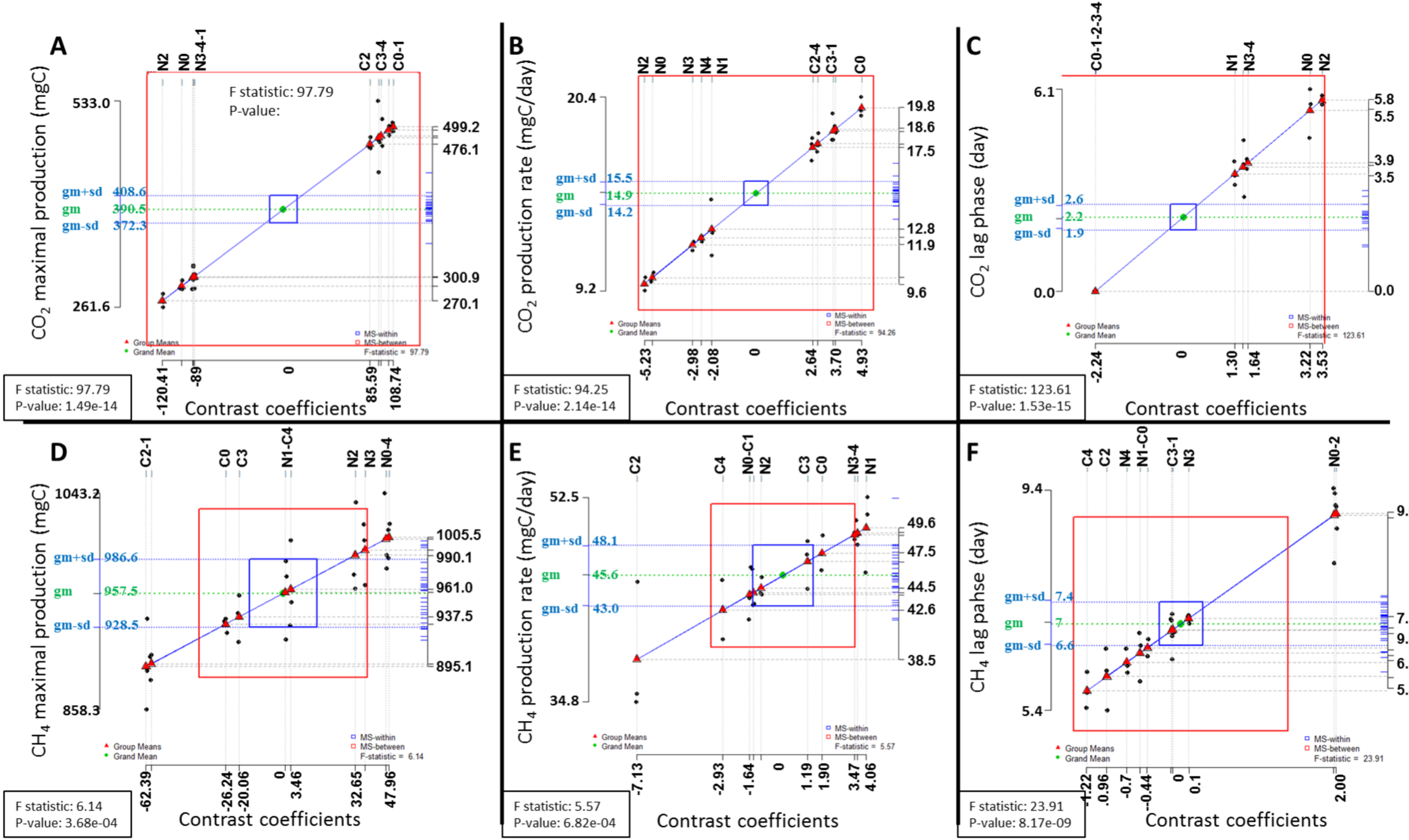
Figure 1. Comparison of the Gompertz parameters (maximal production, production rate and lag phase) for both CO_2 and CH_4 for the different conditions. Each black point represents one digester. Red triangles represent the mean for each condition. Green point represents the mean of all samples (Grand Mean). The different conditions are labelled on the top. Digesters with ammonia are named N and digesters without ammonia are named C**.** The number corresponds to the different conditions of zeolite 0: no zeolite, 1: raw zeolite, 2: liquid residue, 3: solid residue, 4: heated zeolite. Conditions are ordered according to the contrast coefficient represented on the x-axis. The range of the values of the parameter is represented on the left in addition to the value of the Grand Mean and its standard deviation. The values of the group means are represented on the right. The blue square represents the area of the mean square root error within the conditions. The red square represents the area of the mean square root error between the conditions. The F-statistic and p-value are indicated. F-statistic value is the ratio of the area MS-within and of the MS-between. The residuals are represented as a blue line reported on the right of the y-axis. A- ANOVA of the CO_2 maximal production. B- ANOVA of the CO_2 production rate. C- ANOVA of the CO_2 lag phase. D- ANOVA of the CH_4 maximal production. E- ANOVA of the CH_4 production rate. F- ANOVA of the CH_4 lag phase.

Figure 2. Evolution of the chemical parameters. The data are the mean values for the triplicate bioreactors, standard deviations are indicated

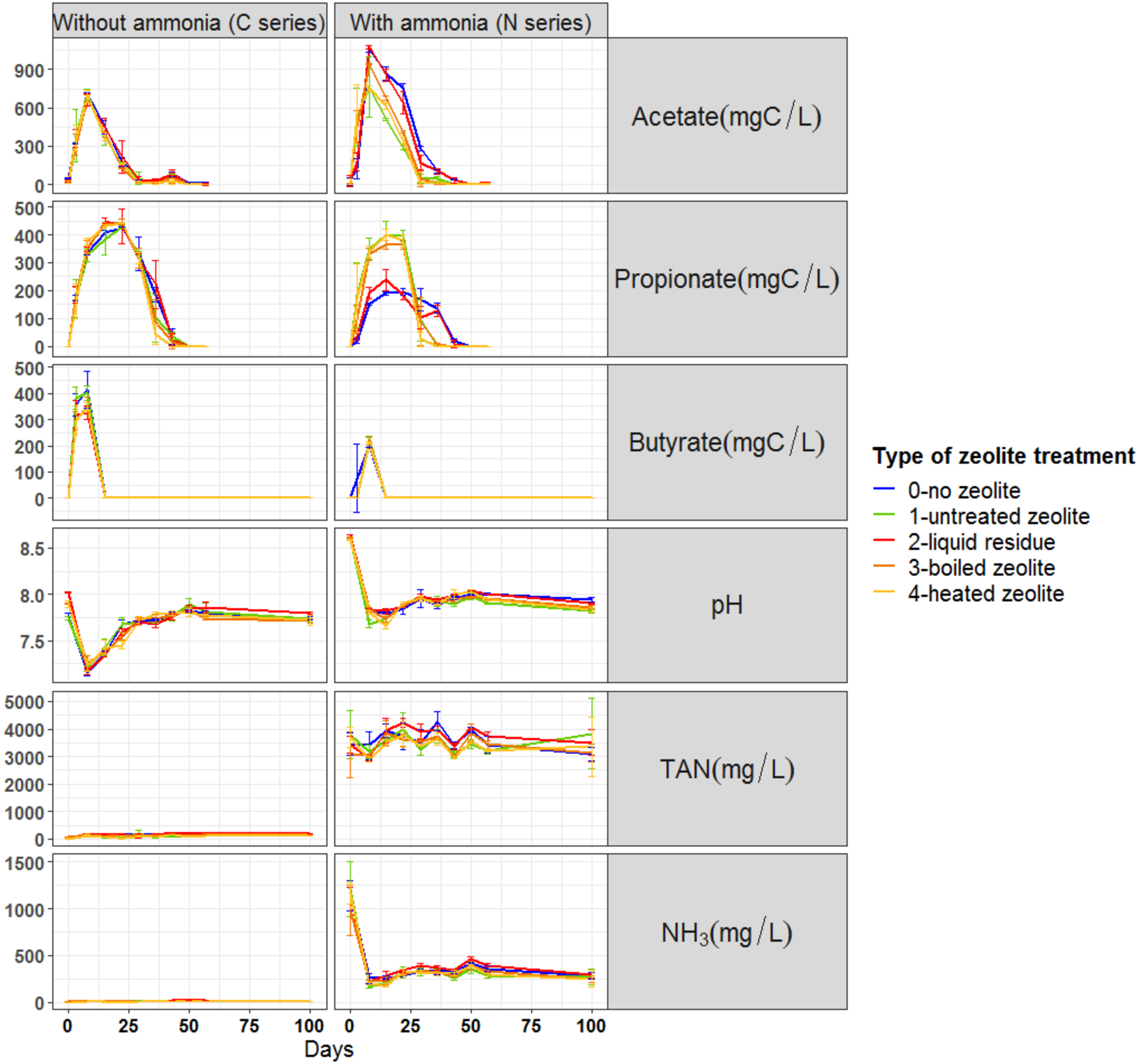
Figure 3. Methanogenic pathway measured through the isotopic fractionation of the CH4 (δ13C-CH4). The data are the mean values for the triplicate bioreactors, standard deviations are indicated.

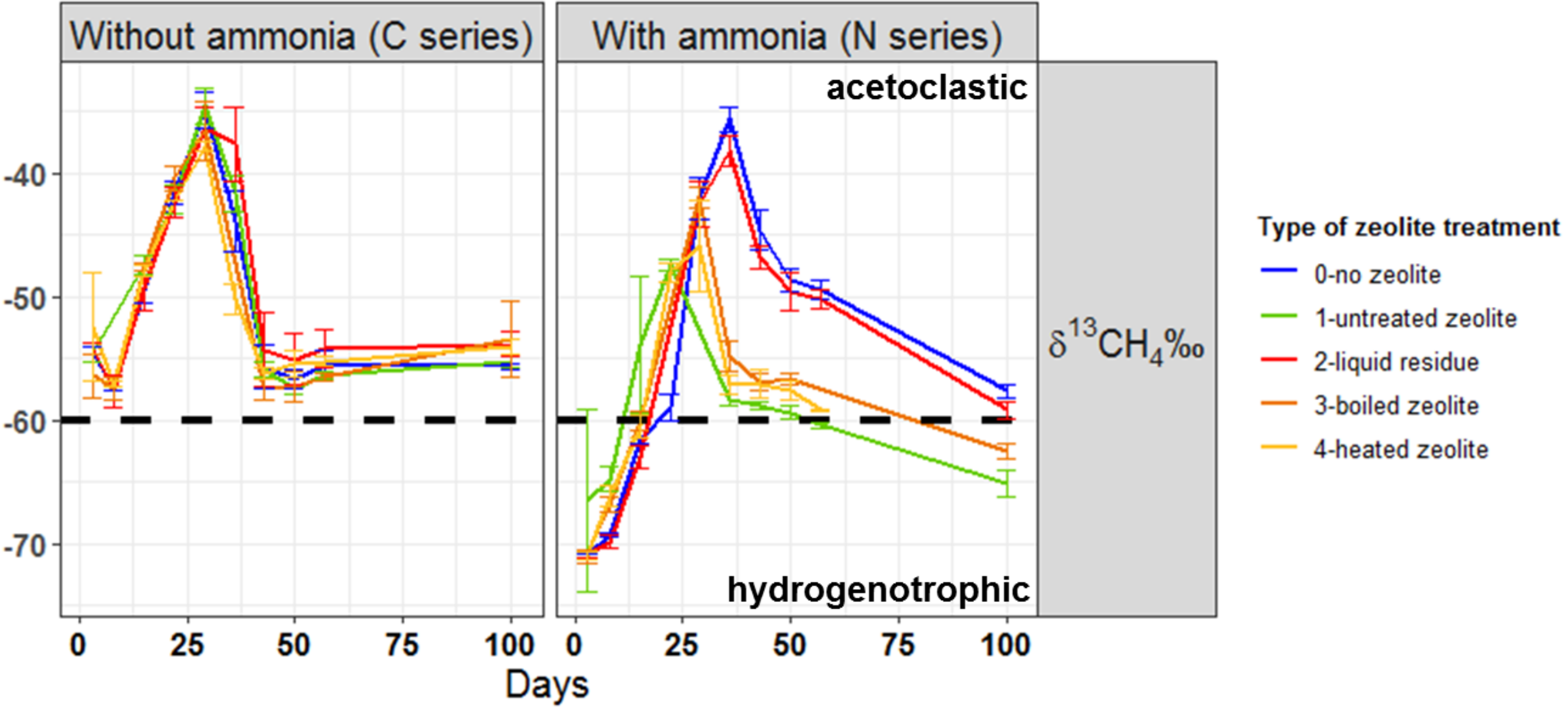
Figure 4. Abundances of the most discriminant microorganisms in the digesters under ammonia inhibition. This heatmap represents the OTUs (column) selected after 3 pair-wise sparse Partial Least Square Discriminant Analysis (sPLS-DA) on the samples (row) to discriminate the different types of digesters. The colour of the heatmap represents the abundances of the OTUs after centered log ratio transformation. Taxonomic affiliation is presented at the family level for the bacteria and genus level for the archaea. Arrows indicate remarkable OTUs.

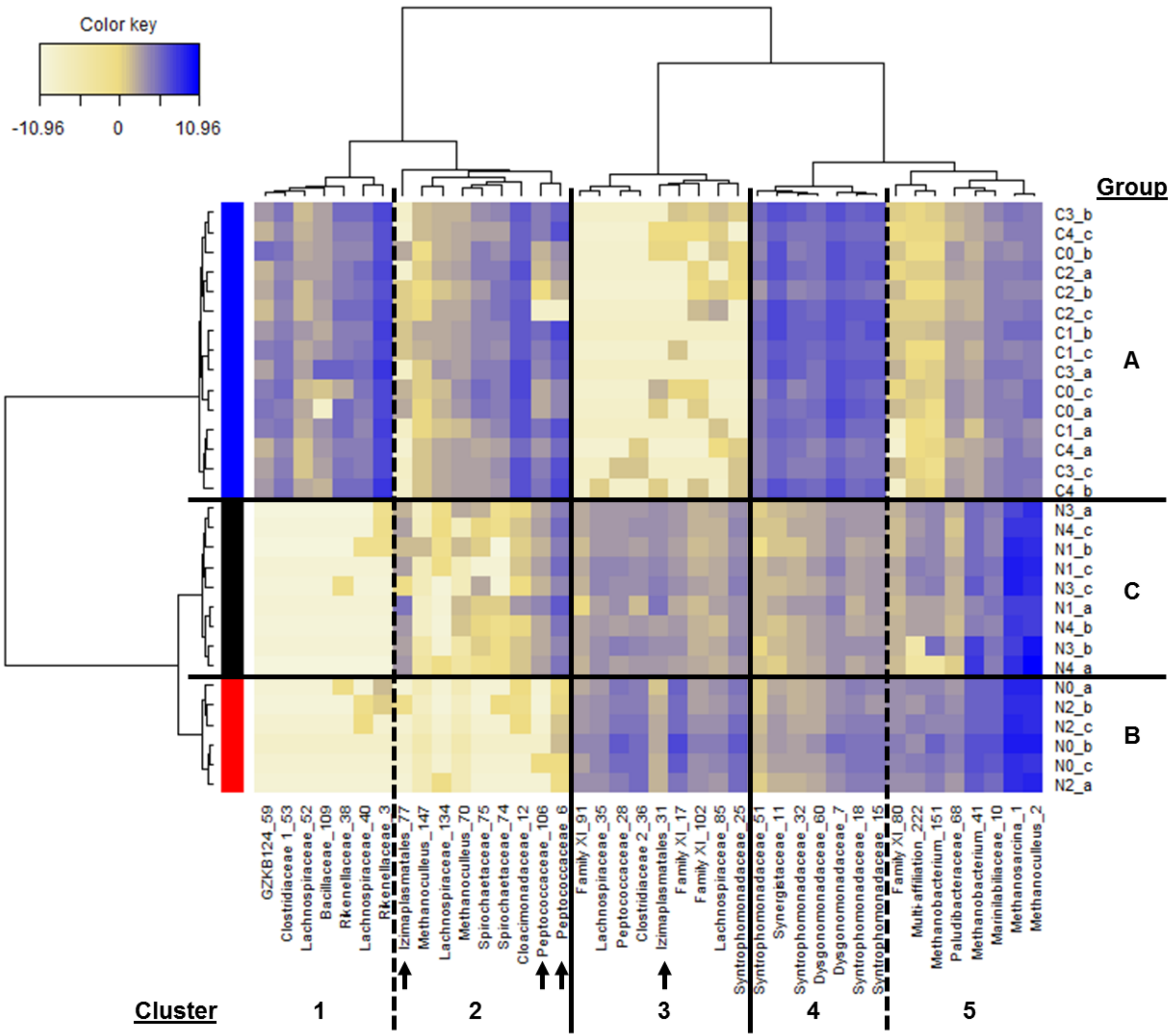
Figure 5. Summary of the hypothetical pathways of degradation for the different conditions. Molecules which accumulated and methanogenesis pathways involved during the degradation are highlighted in blue. Specific microorganisms identified with the sparse PLS-DA are represented inside a rectangle and situated in the pathway where we supposed they were active. The role of the microorganisms between question marks is unknown. Genus Mbac: *Methanobacterium; Msar: Methanosarcina; Mc: Methanoculleus;* Orders Lac: *Lachnospiraceae*; Spi: *Spirochaetaceae*; Cloa: *Cloacimonadaceae*; Rik: *Rikenellaceae*; Pal: *Paludibacteraceae*; Pep: *Peptococcaceae; Syn: Syntrophomonadaceae*; Mari: *Marinilabiliaceae*; Family Izi: *Izimaplasmatales*

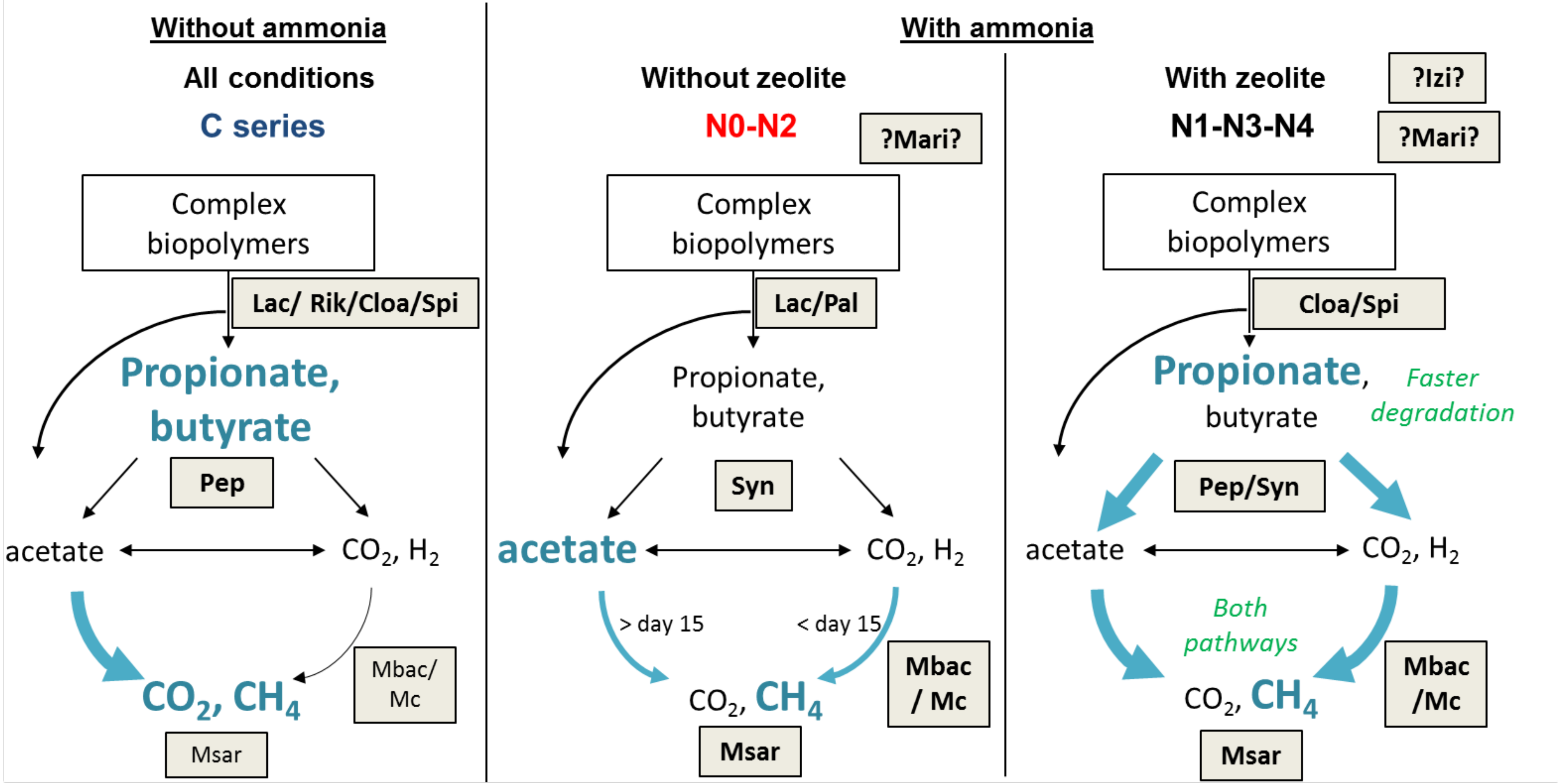


A
CO2 maximal production (mgC)
N2
N0
N3-4-1
C2
C3-4
C0-1
F statistic: 97.79
P-value:
533.0
261.6
499.2
476.1
300.9
270.1
gm+sd 408.6
gm 390.5
gm-sd 372.3
Group Means
Grand Mean
MS-within
MS-between
F-statistic = 97.79
-120.41
-89
0
85.59
108.74
Contrast coefficients
F statistic: 97.79
P-value: 1.49e-14
B
CO2 production rate (mgC/day)
N2
N0
N3
N4
N1
C2-4
C3-1
C0
20.4
9.2
19.8
18.6
17.5
12.8
11.9
9.6
gm+sd 15.5
gm 14.9
gm-sd 14.2
Group Means
Grand Mean
MS-within
MS-between
F-statistic = 94.26
-5.23
-2.98
-2.08
0
2.64
3.70
4.93
Contrast coefficients
F statistic: 94.25
P-value: 2.14e-14
C
CO2 lag phase (day)
C0-1-2-3-4
N1
N3-4
N0
N2
6.1
0.0
5.8
5.5
3.9
3.5
0.0
gm+sd 2.6
gm 2.2
gm-sd 1.9
Group Means
Grand Mean
MS-within
MS-between
F-statistic = 123.61
-2.24
0
1.30
1.64
3.22
3.53
Contrast coefficients
F statistic: 123.61
P-value: 1.53e-15
D
CH4 maximal production (mgC)
C2-1
C0
C3
N1-C4
N2
N3
N0-4
1043.2
858.3
1005.5
990.1
961.0
937.5
895.1
gm+sd 986.6
gm 957.5
gm-sd 928.5
Group Means
Grand Mean
MS-within
MS-between
F-statistic = 6.14
-62.39
-26.24
-20.06
0
3.46
32.65
47.96
Contrast coefficients
F statistic: 6.14
P-value: 3.68e-04
E
CH4 production rate (mgC/day)
C2
C4
N0-C1
N2
C3
C0
N3-4
N1
52.5
34.8
49.6
47.5
44.5
42.6
38.5
gm+sd 48.1
gm 45.6
gm-sd 43.0
Group Means
Grand Mean
MS-within
MS-between
F-statistic = 5.57
-7.13
-2.93
-1.64
0
1.19
1.90
3.47
4.06
Contrast coefficients
F statistic: 5.57
P-value: 6.82e-04
F
CH4 lag pahse (day)
C4
C2
N4
N1-C0
C3-1
N3
N0-2
9.4
5.4
9.0
7.1
9.8
6.3
5.8
gm+sd 7.4
gm 7
gm-sd 6.6
Group Means
Grand Mean
MS-within
MS-between
F-statistic = 23.91
-1.22
-0.96
-0.7
-0.44
0
0.1
2.00
Contrast coefficients
F statistic: 23.91
P-value: 8.17e-09


Without ammonia (C series)
With ammonia (N series)
Acetate(mgC/L)
900
600
300
0
Propionate(mgC/L)
500
400
300
200
100
0
Butyrate(mgC/L)
500
400
300
200
100
0
pH
8.5
8.0
7.5
TAN(mg/L)
5000
4000
3000
2000
1000
0
NH3(mg/L)
1500
1000
500
0
0
25
50
75
100
0
25
50
75
100
Days
Type of zeolite treatment
0-no zeolite
1-untreated zeolite
2-liquid residue
3-boiled zeolite
4-heated zeolite


Without ammonia (C series)
With ammonia (N series)
acetoclastic
hydrogenotrophic
$\delta^{13}CH_4$‰
-40
-50
-60
-70
0
25
50
75
100
Days
Type of zeolite treatment
0-no zeolite
1-untreated zeolite
2-liquid residue
3-boiled zeolite
4-heated zeolite


Color key
-10.96
0
10.96
Group
C3_b
C4_c
C0_b
C2_a
C2_b
C2_c
C1_b
C1_c
C3_a
C0_c
C0_a
C1_a
C4_a
C3_c
C4_b
A
N3_a
N4_c
N1_b
N1_c
N3_c
N1_a
N4_b
N3_b
N4_a
C
N0_a
N2_b
N2_c
N0_b
N0_c
N2_a
B
GZKB124_59
Clostridiaceae 1_53
Lachnospiraceae_52
Bacillaceae_109
Rikenellaceae_38
Lachnospiraceae_40
Rikenellaceae_3
Izimaplasmatales_77
Methanoculleus_147
Lachnospiraceae_134
Methanoculleus_70
Spirochaetaceae_75
Spirochaetaceae_74
Cloacimonadaceae_12
Peptococcaceae_106
Peptococcaceae_6
Family XI_91
Lachnospiraceae_35
Peptococcaceae_28
Clostridiaceae 2_36
Izimaplasmatales_31
Family XI_17
Family XI_102
Lachnospiraceae_85
Syntrophomonadaceae_25
Syntrophomonadaceae_51
Synergistaceae_11
Syntrophomonadaceae_32
Dysgonomonadaceae_60
Dysgonomonadaceae_7
Syntrophomonadaceae_18
Syntrophomonadaceae_15
Family XI_80
Multi-affiliation_222
Methanobacterium_151
Paludibacteraceae_68
Methanobacterium_41
Marinilabiliaceae_10
Methanosarcina_1
Methanoculleus_2
Cluster
1
2
3
4
5


Without ammonia
All conditions
C series
Complex biopolymers
Lac/ Rik/Cloa/Spi
Propionate, butyrate
Pep
acetate
CO_2, H_2
CO_2, CH_4
Mbac/ Mc
Msar
With ammonia
Without zeolite
N0-N2
?Mari?
Complex biopolymers
Lac/Pal
Propionate, butyrate
Syn
acetate
CO_2, H_2
> day 15
< day 15
CO_2, CH_4
Mbac / Mc
Msar
With zeolite
?Izi?
N1-N3-N4
?Mari?
Complex biopolymers
Cloa/Spi
Propionate, butyrate
Faster degradation
Pep/Syn
acetate
CO_2, H_2
Both pathways
CO_2, CH_4
Mbac /Mc
Msar

Ammonia	**Reactor**	**Zeolite type**	**Sludge (g)**	**Biowaste (g)**	**$(NH_4)_2CO_3$ (g)**	**Zeolite (g)**
4 g/L NH_4^+	N0	No zeolite	4	29	11	0
	N1	Untreated zeolite	4	29	11	11
	N2	Liquid residue after boiling zeolite at 100°C, filtered 0.22µm	4	29	11	11
	N3	Solid residue after boiling zeolite at 100°C	4	29	11	11
	N4	Heated 400°C	4	29	11	11
0 g/L NH_4^+	C0	No zeolite	4	29	0	0
	C1	Untreated zeolite	4	29	0	11
	C2	Liquid residue after boiling zeolite at 100°C, filtered 0.22µm	4	29	0	11
	C3	Solid residue after boiling zeolite at 100°C	4	29	0	11
	C4	Heated 400°C	4	29	0	11

Batch experiments

Methanogenic pathways and microbial diversity

Pathway reconstruction