Analysis of Sulfur and Nitrogen in Anaerobic Digestion

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Abstract

This study investigates the impact of nitrogen and sulfur on anaerobic digestion (AD) systems, using keratin (high sulfur, high nitrogen) and casein (low sulfur, high nitrogen) as feedstocks. With the global food processing industry generating vast quantities of poultry feathers and casein whey annually, efficient disposal methods are imperative. Whilst previous studies have focussed on the inhibitory aspects of sulfur and nitrogen, this study aims to understand the potential synergy between the two by adding concentrations at a reportedly beneficial level. A hexanoate feedstock was used as a control due to containing no nitrogen or sulfur. The feedstocks were added to separate vials containing sewage sludge and sampled regularly across a 192 hour time period. The monitoring of pH revealed that nitrogen in the form of ammonia buffered the pH between 7.5 and 8, slightly above the optimal range for methanogens. VFA concentrations, measured using GC-FID, showed isopentanoate accumulation for the keratin-treated sludge, an indication of system instability. An iterative inverse model was used to calculate the degradation rates of acetate as an indicator for methane production rate. The model revealed that casein has a slighlty faster degradation rate of k = 0.63, compared to keratins at k = 0.69. However, the discrepancy between the GC-FID and model VFA data, due to the extensive list of assumptions made, makes the k–values provided unreliable.

Introduction

The worldwide food processing industry generates 40 million tons of poultry feathers annually (Tesfaye et al., 2017). As the demand for feathers is relatively low, most are disposed in landfills (Gao et al., 2014; Wang et al., 2020), and initally undergo aerobic digestion. Once anaerobic conditions form, typically taking less than a year for landfills, microorganisms break down the metabolic byproducts of aerobic digestion, producing methane gas (Patinvoh et al., 2016). However, the use of landfills can lead to the spread of diseases, such as fowl cholera and avian mycoplasmosis (Agrahari and Wadhwa, 2010). Incineration and chemical decomposition are other common methods of feather disposal, however these can be expensive and hazardous (Gupta and Ramnani, 2006).

Anaerobic digestion (AD) has become the most used technique for the treatment of mixed food waste due to its dependability as a renewable source of methane (Kannan et al., 2024). In AD, proteins are hydrolysed into amino acids, which are then converted to volatile fatty acids (VFAs) during acidogenesis (Czatzkowska et al., 2020). Acetogens convert VFAs, such as hexanoate ($C_6H_{12}O_2$) and butyrate ($C_4H_8O_2$), into acetate ($C_2H_3O_2$), CO_2, and H_2

(Czatzkowska et al., 2020). Methanogens then convert acetate to methane (Czatzkowska et al., 2020). Using this technique on poultry feathers would be an ideal solution to combat the inefficient disposal of feathers.

However, the high degree of disulfide bonds in feathers make pretreatment a requirement for decomposition in AD (Friedrich and Antranikian, 1996; Kunert, 1972). This is due keratin, a protein making up >90% of feathers, containing a high proportion of cysteine (16.3%), a sulfur-containing amino acid (Block et al., 1939; Leon, 1972). This makes keratin a sulfur-rich (5%) protein (Leon, 1972), as well as nitrogen-rich (15.9%) (Block et al., 1939; Leon, 1972).

Casein whey, a protein produced by the dairy industry, is also nitrogen-rich (13.4%) (Wagner et al., 2012). Approximately half of whey produced globally is disposed of as waste effluent, posing a major environmental problem due to its high pollution load (Gelegenis et al., 2007). The high biological oxygen demand (BOD) of whey makes anaerobic digestion the only viable solution for treating this wastewater (Gelegenis et al., 2007). Unlike keratin, casein is a sulfur-poor protein (0.78%) (Kassel and Brand, 1938).

Ammonia and sulfate are produced from feedstocks containing nitrogen and sulfur, and in high enough concentrations, can lead to problems within the AD system (Hansen et al., 1998; Zhang et al., 2022). The most common upset is over-acidification, caused by the imbalance in VFA production and consumption rate (Babel et al., 2004; Siegert and Banks, 2005). Fischer et al. (1983) report that the accumulation of isomatic VFAs are a good indicator of process instability. VFA accumulation can have severe consequences on methanogens due to having a narrow optimal pH range of 6.5 to 7.5 (Liu et al., 2008; Zhang et al., 2015).

Very few studies have reported the direct relationship between sulfur and pH. Tian et al. (2020) reported a causal relationship between the two due to an inverse relationship between HS^- + S^{2-} and OH^-, however no studies have reported similar findings since. On the other hand, ammonia is widely known to act as a buffer in AD systems (Fricke et al., 2007; McCarty, 1964; Zhang et al., 2016). This is due to ammonia's ability to bind to H^+ ions by shifting its ammonia-ammonium equilibrium when the system becomes too acidic (McCarty, 1964).

High levels of sulfate and acidity create the ideal environment for sulfate-reducing bacteria, which compete with microbes for organic substrates (Liu et al., 2015). They further inhibit AD through the production of hydrogen sulfide (H_2S) during sulfate reduction, which can easily diffuse through the cell membrane of microbes and denature their primary proteins (Tursman and Cork, 1989). Multiple studies report that the inhibitory effects of sulfide begin at 50 – 60mg S/l (Cheung, 2004; Parkin et al., 1990). Studies also report that high ammonia concentrations can reduce the presence of both H_2S and sulfate-reducing bacteria (Dai et al., 2017; Yan et al., 2018). This is likely due to the prevention of an acidic environment, as well as the inhibitory effects of ammonia toxicity on sulfate-reducing bacteria (Dai et al., 2017). Multiple studies have reported that the inhibitory effects of ammonia begin at 1000mg/l (Duan et al., 2012; Hejnfelt and Angelidaki, 2009; Kotsyurbenko et al., 2004; McCarty, 1964), with higher concentrations leading to a greater reduction in methane production.

Sulfide is an especially important compound for methanogens, containing the highest concentration of sulfur than any other microbe in AD (Speece, 1983). Balch and Wolfe (1979) reported that all methanogens exhibit high levels of co-enzyme M (2-mercaptoethanesulfonic acid), a sulfur-rich cofactor that had been reported on a few years

before to be critical for methane production in methanogens (Balch and Wolfe, 1976). Sulfide concentrations of 1 to 25mg S/l is optimal for methanogenesis in anaerobic digestion (Scherer and Sahm, 1981). The benefits of sulfur on methanogens are still not completely understood, as most studies focus on its inhibitory impact and removal from AD systems (Sürmeli et al., 2019; Vu et al., 2022). However, some sources have reported increased methane production and methanogen growth when sulfur or cysteine is added to AD systems (Rönnow and Gunnarsson, 1982; Yekta et al., 2022; Zan et al., 2019; Zhang and Maekawa, 1996). One study looking into the effect of high and low ammonia and sulfide concentrations on methanogenesis found that an increase in ammonia doubled methanogen growth, whereas a higher sulfide concentration increased methane production (Rönnow and Gunnarsson, 1982). This gives insight into the role these two compounds play in methanogenesis.

The use of animal waste feedstock in anaerobic digestion can be a solution for both inefficient animal waste disposal, and the demand for renewable energy. At the right concentration, nitrogen and sulfur appear to be beneficial for AD systems. However, they can be inhibitory if the concentration is too high. To analyse the effect of sulfur- and nitrogen-rich proteins on the AD system, this study will use keratin and casein as separate feedstocks in sewage sludge under AD, and compare their pH, VFA concentration. Methane production rate will also be calculated using a first-order kinetics model, called iterative inverse modelling. A hexanoate feedstock will be used as a control due to being nitrogen- and sulfur-free.

This study hypothesises that keratin, due to being sulfur- and nitrogen-rich, will be beneficial to methanogens the most, resultint in a faster methane production rate than casein. It is also hypothesised that both proteins will maintain a neutral pH due to the ammonia buffer, whereas hexanoate will be more acidic. This study aims to contribute to our understanding of the synergistic relationship between sulfur and nitrogen, particularly in the context of using animal/food waste as feedstocks in anaerobic digestion.

Methods

Sampling

Sewage sludge samples were gifted from Naburn wastewater treatment facility. Samples were screened, aerated, and held within an industrial-scale AD reactor before collection. Samples were mixed, then 30mls were placed into 120ml serum vials. Vials were capped using Viton stoppers and incubated at 40°C for 2 weeks before feeding.

1ml of sodium hexanoate (0.0124 mol) was added to each vial, coming to a concentration of 4mM. A sample was taken immediately before and after the first hexanoate feeding. Four more samples were taken at 3h, 6h, 24h, and 48h after feeding. At 48h, a second feeding of hexanoate occured and was immediately sampled. Four more samples were taken at 51h, 54h, 72h, and 78h after the first feeding. Each timepoint had three replicates.

Keratin (Hydrolysed Keratin, Vedaoils; 2200da) was diluted to a concentration of 1.5g/100ml in distilled water at a pH of 8. Casein (Bovine Milk, Sigma-Aldrich; 20,000da) was diluted to a concentration of 0.775g/100.5ml in 97.5ml of distilled water, 2ml 1M NaOH, and 0.5ml 1M HCL, to aid in dissolving the casein. The pH of the solution was 9. Both keratin and casein solutions were added at 1ml in four separate vials. A sample was taken immediately

before and after the first feeding. Nine more samples were taken at 3h, 6h, 24h, 48h, 54h, 72h, 96h, 168h, and 192h after feeding.

The concentration of keratin in each vial was 483.87mg/l, resulting in 76.9mg/l of nitrogen, and 24.19mg/l of sulfur. The concentration of casein in each vial was 248.75mg/l, resulting in 33.33mg/l of nitrogen, and 1.94mg/l of sulfur.

Analysis

To measure pH, 10ml of each sample was taken into a separate vial, after which the sampling vial was sacrificed. The Mettler Toledo FEP20-TRISS Kit FiveEasy Plus pH Meter was used to measure pH, accurate to two decimal places.

To collect samples for VFA analysis, two 2ml Eppendorf tubes were filled with sludge and centrifuged at 10,000rpm for two minutes. The supernatant was pipetted off and 1ml was passed through a 0.2µm filter into a 2ml GC-FID vial with 0.1um of orthophosphoric acid and stored at 2-8°C to stabilise the liquid.

VFAs were detected using a Gas Chromatograph (GC) with Flame Ionization Detection (FID) (HP 5890 series II) fitted with a Nukol capillary column (30m × 0.25 mm, df 0.25; 24107, Sigma). Helium carrier gas flowed through at 5ml/min. Detectors and injectors were set at 200°C. 1ml of each sample was heated in a sequence: 100 to 150°C at a rate of 10°C/min, then 150 to 200°C at 20°C/min and held at 200°C for 10 minutes. The GC-FID was calibrated using a Volatile Free Acid Mix (CRM46975, Sigma-Aldrich, Dorset, UK) providing C_2-C_6 reference values from 0.1 to 10 mM. The calibration results showed a satisfactory correlation (R2>98%).

Model development

Raw data from the GC-FID was imported into Microsoft Excel (Microsoft Corporation, 2018) for data modelling, found in File S1. A first-order kinetic model, known as an iterative inverse model, was used to calculate the degradation rate of different VFAs under anaerobic digestion. Only hexanoate and butyrate were modelled alongside acetate in the study due to being the easiest to model for all three treatments. To find the degradation rate, the general equation used was:

$$[X]_{t+1} = X_k \cdot [X]_t$$

where X_t is the concentration of X (mM) at time t (hours), while X_k is the first-order degradation rate of X. This equation changes when considering hexanoate additions from octanoate (O). Calculating the k-value of butyrate and acetate becomes more complex, due to the addition of butyrate from hexanoate metabolism, and addition of acetate from hexanoate, butyrate, and octanoate metabolism. The equations, for each VFA, are as follows:

For hexanoate (H):

$$[H]_{t+1} = H_k \cdot [H]_t + O$$

For butyrate (B):

$$[B]_{t+1} = (B_k \cdot [B]_t) + ((1 - H_k) \cdot \frac{[H]_{t+1} + [H]_t}{2})$$

For acetate (A):

$$[A]_{t+1} = (A_k \cdot [A]_t) + ((1 - H_k) \cdot \frac{[H]_{t+1} + [H]_t}{2}) + ((1 - B_k) \cdot (B_{t+1} + B_t))$$

From this, we can calculate methane (M) production per timepoint using:

$$[M]_t = ((1 - A_k) \cdot [A]_t)$$

In this model, degradation rates were calculated from when VFAs started being produced, up until 96 hours after feeding, as the 92-hour gap after this timepoint is too long relative to the other timepoints to determine the degradation rate with accuracy. As we know there are no unknown VFAs adding to the hexanoate concentration for the hexanoate-treated sludge, we can use the k-value of hexanoate from the hexanoate-treated dataset for the keratin- and casein-treated datasets as it can be assumed they would be similar. The butyrate, acetate, and methane concentrations are then calculated in that order, minimizing discrepancy between model and GC-FID data to get a best-fit for each VFA per dataset.

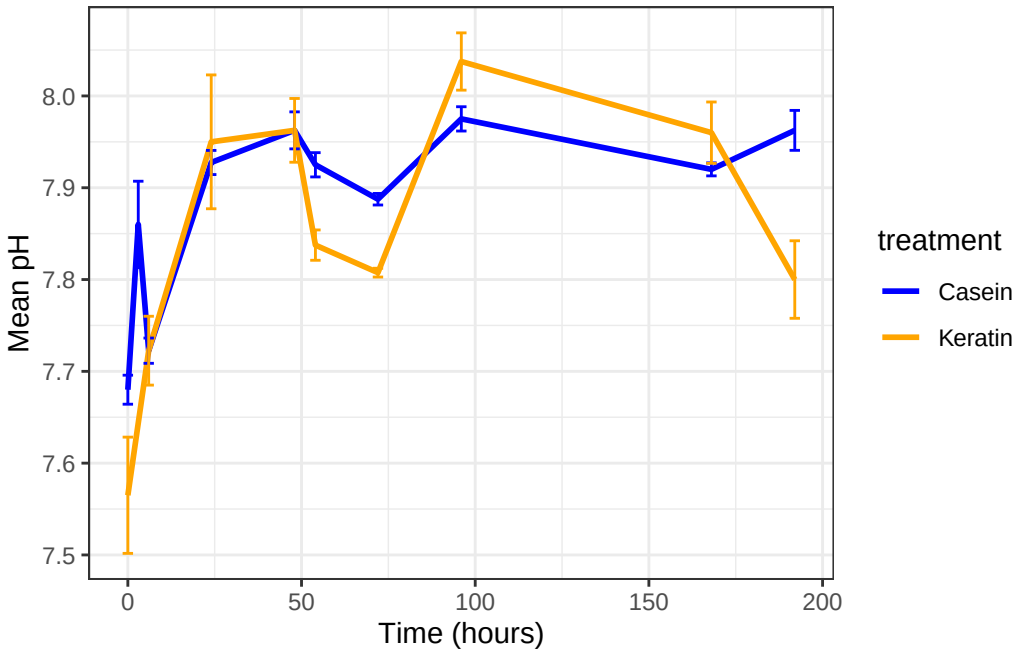
Statistics

All statistical significance tests and data visualisation were performed using R (version 4.3.3) (R Core Team, 2023) with tidyverse packages (Wickham et al., 2019). Statistical significance was assigned at $P < 0.05$. Unless otherwise stated, error bars shown in figures are standard error of mean.

Results

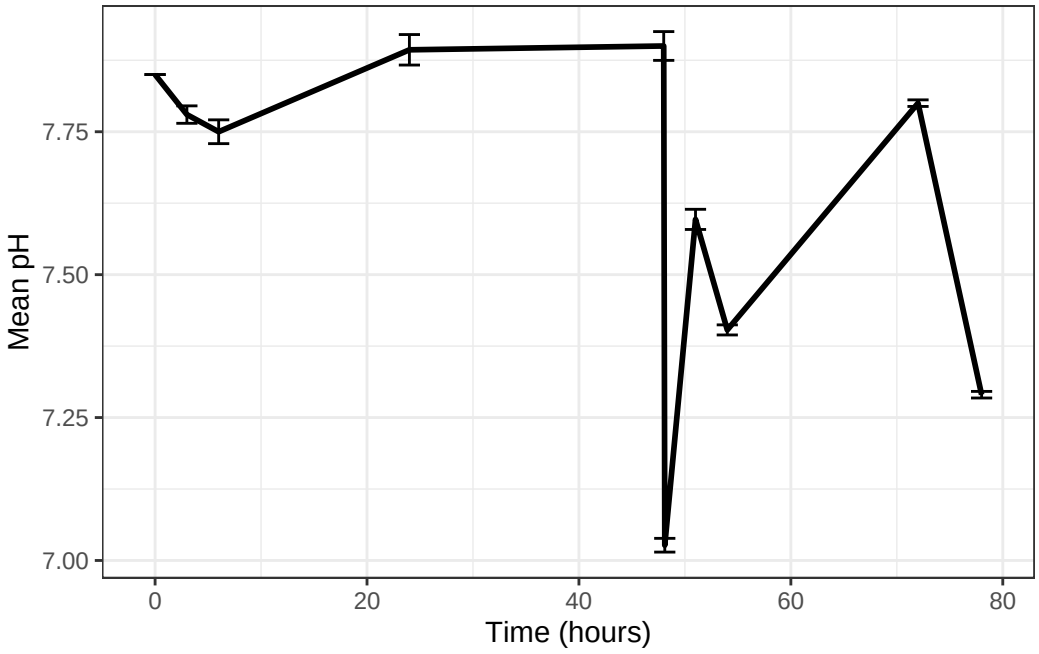
Analysis of pH and VFA accumulation

The two-way repeated-measures ANOVA of the mean pH trend over time for casein and keratin treatments in Figure 1 revealed a lack of significant divergence in pH trends between the two treatments ($F[1,64] = 1.801$, $P = 0.184$). Despite this, two-sample t-tests revealed that the keratin treatment drops significantly lower than the casein treatment at 54 and 72 hours ($P = 0.0068$, $P < 10^{-4}$, respectively). Keratin was also significantly lower at 192 hours ($P = 0.0223$). However, both treatments never went below a pH of 7.5, unlike the hexanoate treatment (Figure 2), which went from a mean pH of 7.9 immediately before the second feeding to 7.03 immediately after.



(a)

Figure 1: **Comparison between casein and keratin pH**. The mean pH over time (hours) for casein and keratin treatments was determined (n = 4). Two-way repeated-measures ANOVA found no significant difference in pH trend between treatments (F[1,64] = 1.801, P = 0.184). Two-sample t-tests only revealed significance at 54 hours (P = 0.0068), 72 hours (P = 10^{-4}), and 192 hours (P = 0.0223).

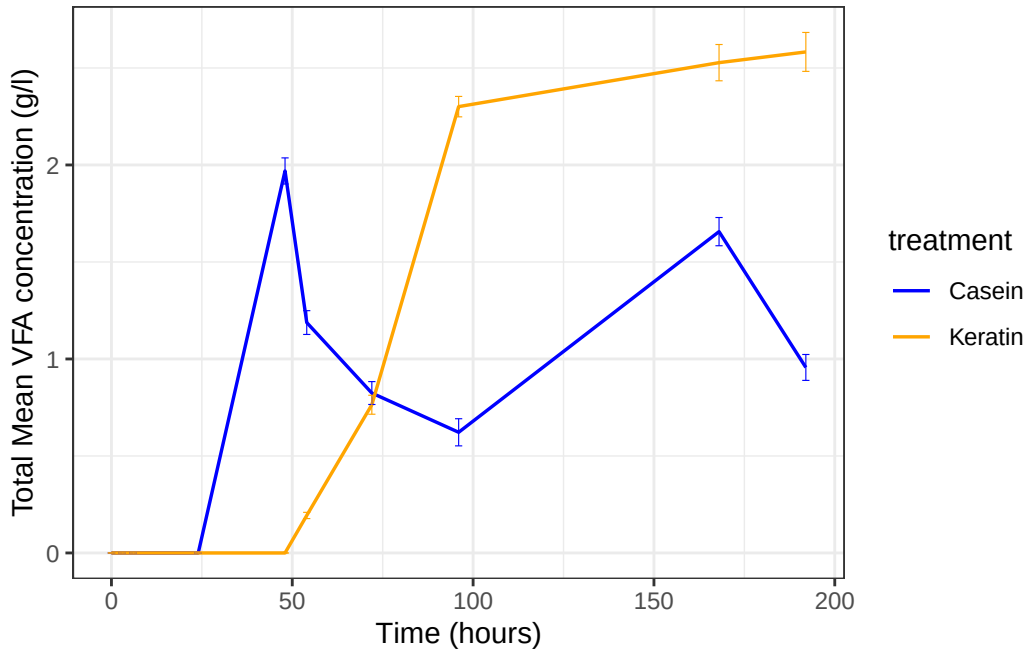


(a)

Figure 2: **Trend in hexanoate pH over time**. Mean pH over time (hours) for hexanoate treatment (n=3), with feeding at 0 and 48 hours.

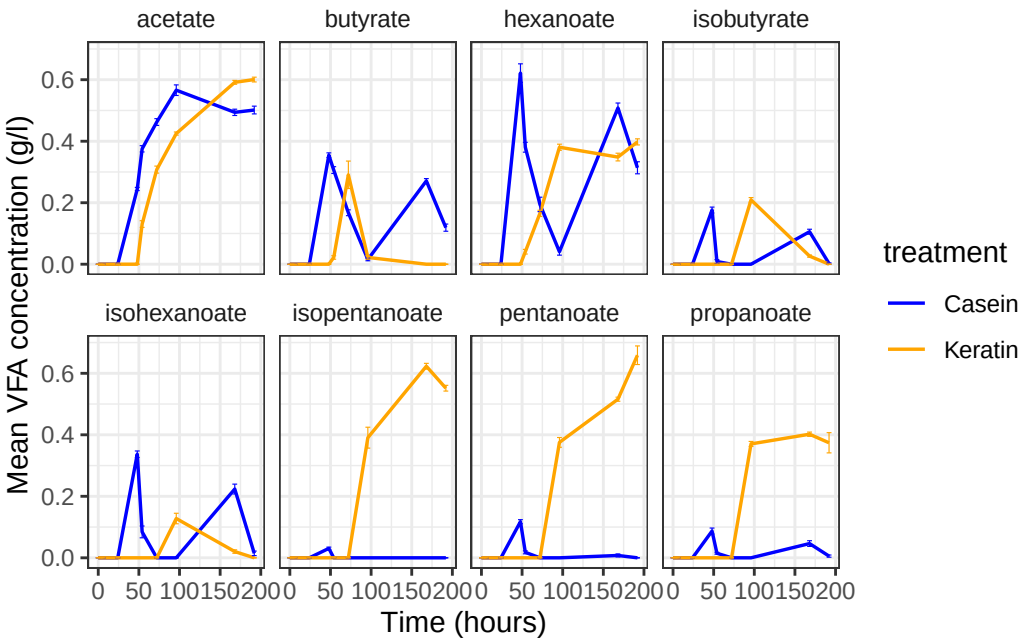
The mean total VFA concentration (g/l) over time for casein and keratin (Figure 3) reveals that the VFA concen-

tration in keratin is always increasing after 48 hours, with its rate in production slowing down after 96 hours. Whereas casein initially increases in concentration after 24 hours but then decreases until 96 hours, where we see it increase again to 168 hours before reducing at 192 hours. To investigate this further, we looked at the mean concentration (g/l) for each VFA over time (Figure 4). The main finding is the large increase in isopentanoate, pentanoate, and propanoate concentrations in the keratin treatment from 72 hours, whereas they are consistently low in the casein treatment.



(a)

Figure 3: **Trend in total VFA concentration for casein and keratin**. Shows the trend in mean total vfa concentration (g/l) over time (hours) for both casein and keratin treatments (n=4).



(a)

Figure 4: **Trend in concentration of each VFA for keratin and casein**. Shows the mean concentration (g/l) of each VFA over time (hours) for casein and keratin (n=4).

Modelling VFA degradation

To estimate the VFA degradation rates of hexanoate, butyrate, and acetate, iterative inverse modelling was performed. The model gave us a mean k-value of 0.83 for hexanoate, a mean k-value of 0 for butyrate, and a k-value of 0.37 for acetate. For casein, mean k-values of 0.8 and 0.61 were obtained for butyrate and acetate, respectively, whereas for keratin, mean k-values of 0 and 0.69 were obtained for butyrate and acetate, respectively (Figure 5). Two-way t-tests revealed that the k-value for acetate was significantly lower for the hexanoate treatment than casein (P = 0.0063) and keratin (P = 0.0076). It also showed that acetate's k-value for casein was significantly lower than for keratin (P = 0.0065). The k-value of butyrate was also significantly higher for casein than it was for hexanoate ($P < 10^{-4}$) and keratin ($P < 10^{-4}$).

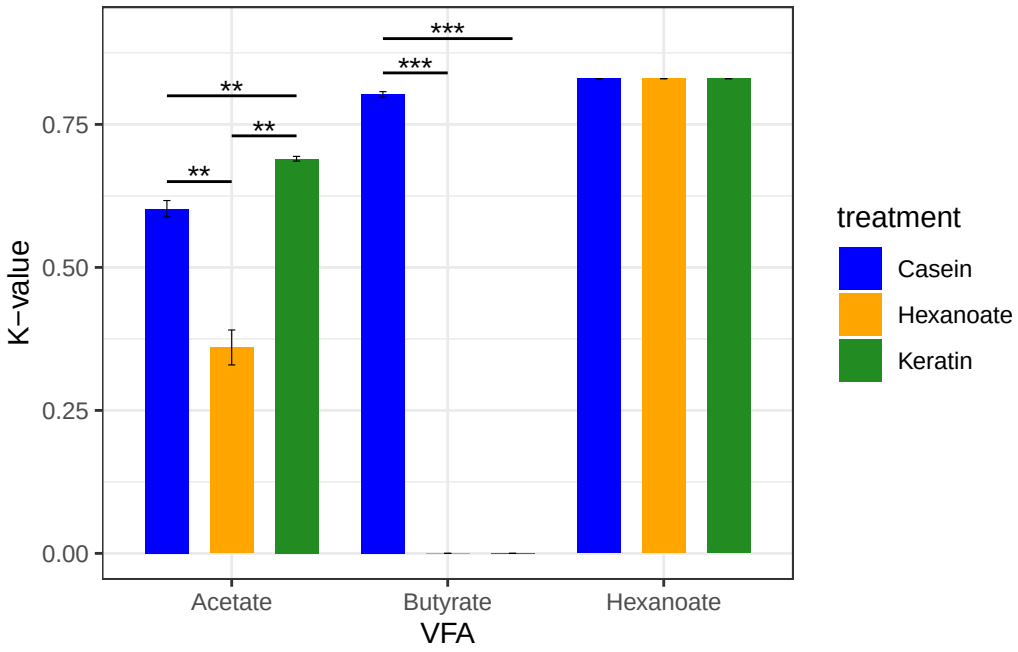
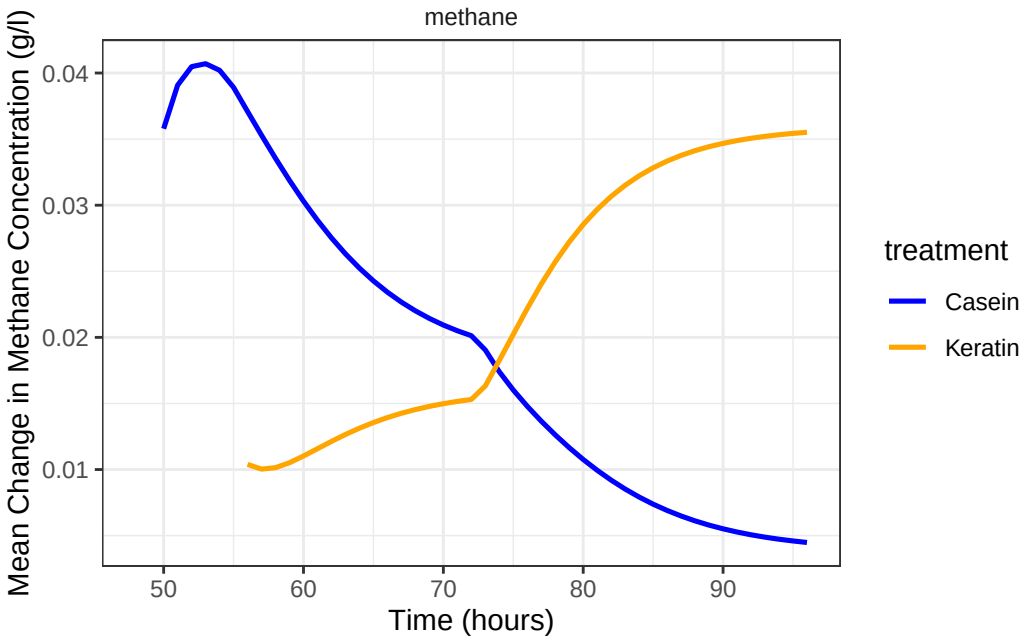


Figure 5

Significant difference in VFA k-values between treatments. Mean k-value of acetate, butyrate, and hexanoate for each treatment (n = 4, 4, 3 for casein, keratin, and hexanoate, respectively). Statistical analysis by paired sample t-test, *P < 0.05, **P < 0.01, ***P < 0.005.

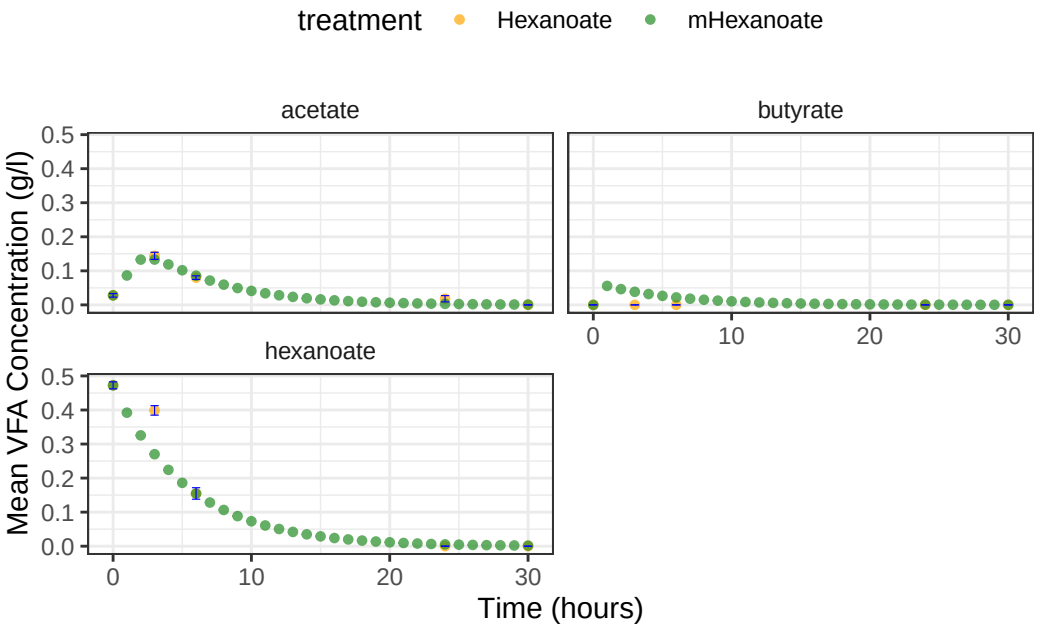
It can be assumed that the rate of methane production is equal to the rate of acetate degradation. Therefore, the first-order production rate of methane for the hexanoate, keratin, and casein treatments are 0.37, 0.61, and 0.69, respectively. Figure 6 compares the rate of methane production for casein and keratin. The graph shows that the rate of methane production for casein is initially high, at ~0.04g/l/hr, but decreases over time, reaching close to 0.005g/l/hr at 96 hours. Whereas keratin starts low at roughly 0.01g/l/hr, but increases to ~0.035g/l/hr. Despite these contrasting rates of methane production, both treatments end up producing roughly the same total methane, at 0.95g/l for keratin, and 0.93g/l for casein.



(a)

Figure 6: **Rate of methane production over time** Shows the mean change in methane concentration (g/l) over time (hours) for casein and keratin treatments.

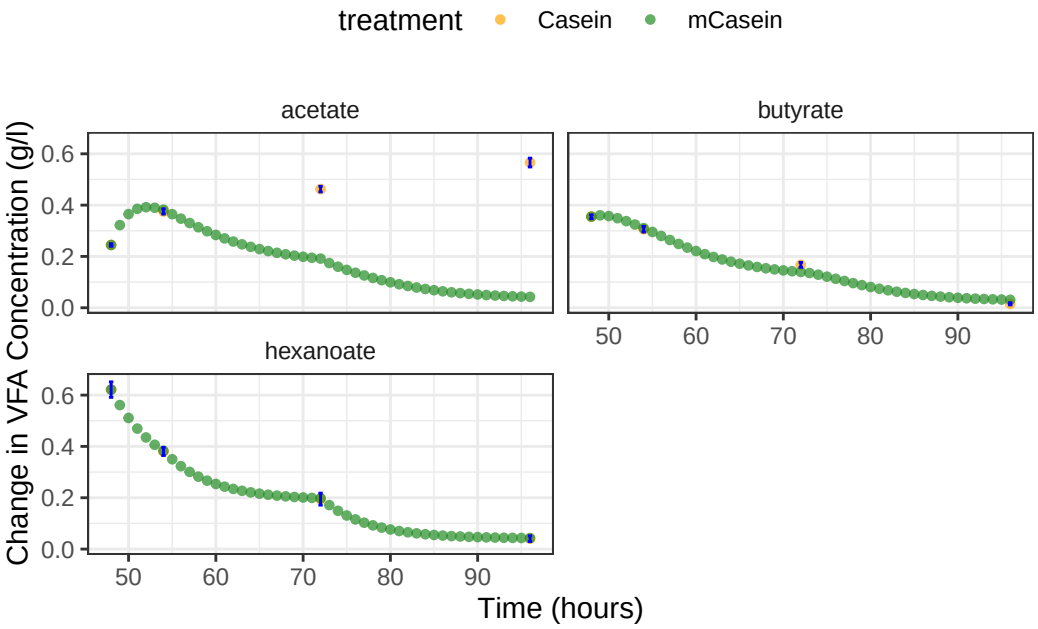
To measure the accuracy of the model, the GC-FID data was compared to model data to test for significant differences using one-way t-tests. The hexanoate model appears to fit well with most of the GC-FID data, with only a few significant discrepancies (Figure 7). The model made the butyrate concentration too high at 3 hours (P = 0.0056) and 6 hours (P = 0.0204) after feeding. It also modelled the hexanoate concentration too low at 3 hours (P = `h3h`) after feeding.



(a)

Figure 7: **Comparing model and GC-FID VFA data for hexanoate treatment**. Average acetate, butyate, and hexanoate concentrations (g/l) over time (hours) between the model and GC-FID data for the hexanoate-treated sludge (n=3). One-sample t-tests show significance at 3 hours for butyrate (P = 0.0056), 6 hours (P = 0.0204), and hexanoate at 3 hours (P = h3h).

For casein and keratin, the model fits well with hexanoate due to the additions from octanoate allowing it to match the GC-FID data. However, casein's model drastically underestimates the concentration of acetate at 72 hours (P = 0.0053) and 96 hours (P < 10^{-4}) after feeding (Figure 8). The model also slightly overestimates butyrate at 96 hours (P = 0.0338). On the other hand, keratin's model severely underestimates acetate's concentration at 72 hours (P = 0.0017) (Figure 9). It also underestimates butyrate at 72 hours (P = 0.0091) but slightly overestimates it at 96 hours (P = 0.0178).



(a)

Figure 8: **Comparing model and GC-FID VFA data for casein treatment**. Average acetate, butyrate, and hexanoate concentrations (g/l) over time (hours) between the model and GC-FID data for casein-treated sludge (n = 4). Statistical analysis by one-sample t-test found significance between GC-FID and model data for each timepoint. Significant differences found for acetate at 72h (p = 0.0053), 96h ($p < 10^{-4}$), and butyrate at 96h (p = 0.0338).

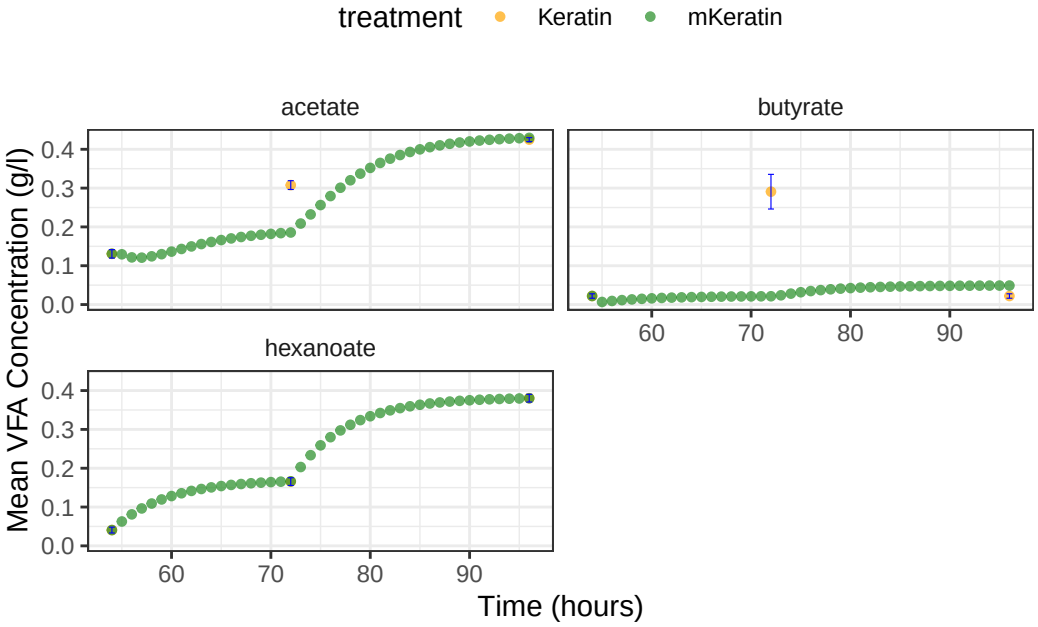


Figure 9

Comparing model and GC-FID VFA data for keratin treatment. Average acetate, butyrate, and hexanoate concentrations (g/l) over time (hours) between the model and GC-FID data for keratin-treated sludge. Statistical analysis

by one-sample t-test to find significance between GC-FID and model data for each timepoint. Significant differences found for acetate at 72h ($p = 0.0017$), and butyrate at 72h ($p = 0.0091$) and 96h ($p = 0.0178$).

Discussion

The lack of significance in the two-way repeated-measures ANOVA indicates that the two treatments cause similar effect on pH over time. However, this model assumes homogeneity of variance, whereas, in Figure 1, the standard deviation for casein is a lot smaller compared to keratin for most pH values. This may have reduced the power to detect true differences between groups. The two-sample t-tests revealed that keratin was significantly more acidic than casein at 54, 72, and 192 hours. This correlates with the mean total VFA concentration of casein and keratin in Fig.3, where keratin is increasing in VFAs during these timepoints, whereas casein is decreasing. This accumulation of VFAs indicates an imbalance between VFA production and consumption rate (Babel et al., 2004; Siegert and Banks, 2005) which could be an indicator of system instability for keratin (Fischer et al., 1983).

The accumulation of iso-pentanoate, as seen for the keratin treatment in Figure 4, has been reported to be one of the best indicators for process instability, along with iso-butyrate accumulation (Hill and Holmberg, 1988). The keratin treatment reaches a peak accumulation of isopentanoate at just over 600mg/l, which is above the reported threshold level of 50mg/l to indicate system instability (Deublein and Steinhauser, 2008). Despite an absolute VFA level to indicate system instability is not entirely feasible due to there being many different types of AD systems, it can't be denied that an accumulation of this magnitude is likely a problematic sign. Another study, by Hill and Bolte (1989), reported levels of isopentanoate above 15mg/l indicated that failure has already occurred, where a long recovery period was necessary. It could be argued that the higher concentration of leucine, in the keratin treatment, at 40mg/l, compared to the casein treatment, at 23mg/L, played a role, due to being a precursor of iso-pentanoate (Firkins et al., 2015). However, the difference in amino acid is relatively small compared to the difference in iso-pentanoate accumulation.

The nitrogen present in both protein treatments likely contributed to pH stability, with the pH never dropping below 7.5 for either treatment. In contrast, hexanoate, as shown in Figure 2, exhibited significant fluctuations. This difference is probably attributed to the buffering capacity of ammonia generated from the degradation of amino acids in both protein treatments, as noted in previous studies (Fricke et al., 2007; McCarty, 1964; Zhang et al., 2016).

Both protein treatments have nitrogen concentrations too low to cause ammonia inhibition directly (Duan et al., 2012; Hejnfelt and Angelidaki, 2009; Kotsyurbenko et al., 2004; McCarty, 1964), at 33.33mg/l and 76.9mg/l for casein and keratin, respectively. However, is important to note that both treatments are slightly outside the optimal pH range of 6.5 – 7.5 for methanogens (Liu et al., 2008; Zhang et al., 2015), which is likely due to the presence of ammonia, a weak base, in both systems. On the other hand, the hexanoate treatment stays relatively closer to the optimal pH range. However, if more sodium hexanoate were to be added, such as for industrial use, the likelihood of the pH going below the optimal range increases. Hence the comparison between Figure 1 and Figure 2 shows the importance of having ammonia as a buffer in the system.

The iterative inverse model told us that the hexanoate treatment had the fastest acetate degradation, at k = 0.37, indicating that 63% of acetate in the system was consumed per hour (Figure 5). Since acetate degradation produces methane, it can be assumed that faster acetate degradation means a faster methane production rate. Casein having a faster acetate degradation (k = 0.61) than keratin (k = 0.69) was a surprise, since we expected the higher concentration of sulfur, at 24mg S/l to benefit the methanogens more than casein's at 2mg S/l due to both being within the reported optimal range (Scherer and Sahm, 1981). However, it may be the case that the upper-boundary of this optimal range allows for a higher likelihood of AD inhibition with no additional benefit. It could also be the case that the type of AD system used in this study can tolerate less sulfur than the system used by Scherer and Sahm (1981). The significantly higher butyrate k-value for casein than hexanoate and keratin (p = 10^{-4}, p = 10^{-4}, respectively) is also surprising, considering butyrate is usually consumed a high rate relative to the other VFAs in the system (Wang et al., 1999).

Figure 6 tells us that methane production is overall decreasing for casein whereas it's increasing for keratin. This may be due to the degradation of casein starting before keratin, during the 24-hour gap between sampling at the 24- and 48-hour timepoints. This may be because the casein feedstock is more easily broken down than keratin, possibly due to the addition of HCl to the feedstock to help with dissolving the protein. It could also be that the hydrolysing bacteria in the casein AD system being more efficient than those in the keratin AD system.

However, there is reason to be skeptical of the model data results, as iterative inverse modelling makes many assumptions when calculating the k-values of different VFAs. The model data fits well with the GC-FID data for the hexanoate treatment (Figure 7). This is because hexanoate is being added directly to the sample, so we know that there is no other material out there that is making hexanoate through metabolism, hence the modeled degradation rate of hexanoate can be reliable. This point can be extended for acetate and butyrate as well, as no other material other than the VFAs included in the model should be adding to them through metabolism. However, there is a slight but significant over-representation of the butyrate data at 3 and 6 hours (p = 0.0056, p = 0.0204, respectively). This likely comes from the assumption that the addition of new material from hexanoate at each hour is not being degraded whilst it's being added. If this were the case, the model butyrate would likely match with the GC-FID data due to the k-value of the model butyrate data being 0. The significant discrepancy for hexanoate at 3 hours (p = 0.0056) may be due to the assumption that the degradation rate is consistent throughout the process, whereas it is more realistic that the rate would fluctuate slightly.

The main discrepancy in the model casein data (Figure 8) is the acetate being significantly underrepresented at 72 and 96 hours (p = 0.0053, p = 10^{-4}, respectively). This could be due to the assumption that no other material is adding to the acetate, other than butyrate, hexanoate, and octanoate. We see in Figure 4 that there is degradation of isobutyrate, isohexanoate, pentanoate, propanoate, and a small amount of isopentanoate between 48 and 54 hours for the keratin-treatment. This degradation should have contributed to the acetate concentration without affecting the hexanoate and butyrate concentrations, due to being odd-number or branched carbon chains. Hence the model butyrate fits well with the GC-FID data, with only a minor yet significant over-representation at 96 hours (p = 0.0178).

Whereas the keratin model significantly over-represents acetate and butyrate at 72 hours (p = 0.0017, p = 0.0091,

respectively). Figure 4 does not help as much in this scenario, as there are no signs of major degradation specifically between 54 and 72 hours that would contribute this much to the concentration of butyrate. It could be argued that a large amount of hexanoate was produced and degraded between the 54- and 72-hour sampling point, meaning it wouldn't appear in the results.

To limit the number of assumptions made from using a first-order kinetic model, the continuous monitoring of every VFA, through using an online analysing machine, would provide us with a better understanding of the cause of certain VFA accumulations, as well as offer a degradation rate based off less assumptions. More research into the impact of high nitrogen and sulfur feedstocks independently and combined is needed to provide a better understanding of how nitrogen and sulfur affect the AD system when separate and when together, giving insight into their synergy.

Conclusion

With signs of AD system instability based on the iso-pentanoate accumulation for the keratin treatment, it appears that the high sulfur and nitrogen synergistically create a more negative effect on the AD system than the high nitrogen and low sulfur casein treatment. The keratin treatment also had a lower k-value for acetate in keratin treatment, indicating a slower rate of methane production, however, due extensive list of assumptions made by the iterative inverse model, it cannot be confirmed that using reportedly beneficial amounts of nitrogen and sulfur results in a faster or slower methane production than lower amounts of sulfur. However, it can be confirmed that the addition of nitrogen to both systems appear to buffer pH at around 7.5 – 8, unlike the nitrogen-depleted AD system. Although, this may have slightly negatively impacted methanogenesis due to being more alkaline than the reported optimal pH range of 6.5 – 7.5 for methanogens.

Word count: 3593

Supplementary

Files

File S1

Model.xlsx

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