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Acidogenic fermentation of organic residual solids: Effect of different alkaline sources on pH, alkalinity, and fermentation performance

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Abstract: Acidogenic fermentation (AF) of wastes provides a biological route to volatile fatty acids (VFA) production, as an alternative to production from fossil hydrocarbons. As VFA are acidic in nature, the pH in AF typically drops, in turn affecting the fermentation performance. The effect of different alkaline substances such as urea, NaOH or $CaCO_3$ on AF of organic residual solids (ORS), a food waste rich fraction of municipal solid waste (MSW), was studied. Enzymes were used as an additive in simultaneous hydrolysis and fermentation (SHF) experiments. Use of urea (66 mg Urea g^{-1} TS) in SHF resulted in a VFA yield of 0.14 g VFA g^{-1} VS after 6 days, 177% higher than the control (no urea addition). pH and ammonia concentrations significantly influenced the bacterial population distribution. In SHF using NaOH/$CaCO_3$, added alkalinity of 11 g $CaCO_3$ L^{-1} did not influence VFA yields or pH control with NaOH addition at pH 7 or higher. This study demonstrates that, while increasing pH up to 8.5 using different alkaline substances improves VFA production, higher pH is detrimental to SHF due to enzyme inhibition. Finally, use of urea as an alkaline source must be considered carefully as the acidic pH naturally reached by AF can inhibit urea hydrolysis.

Keywords: acidogenic fermentation; volatile fatty acids; municipal solid waste; organic waste; urea; anaerobic digestion; alkalinity

1. Introduction

C2-C6 fatty acids, often referred to as volatile fatty acids (VFA), are used by the pharmaceutical, food, chemical and agricultural industries [1] as ingredients in product formulations. Currently, VFA are primarily produced from fossil hydrocarbons [2]. VFA are also an intermediary product of anaerobic digestion (AD), a microbiological process where organic substrates are converted to biogas (CH_4+CO_2) by a mixed culture in the absence of free oxygen [3]. With slight modifications of the operational variables within AD, methane-generating microorganisms can be inhibited and an accumulation of VFA can be achieved [4,5]. This process is commonly known as acidogenic fermentation (AF). AF is complex due to the need to inhibit methanogenesis and parallel pathways such as H_2 generation [6]. The literature suggests multiple strategies to promote VFA production over other products; however, optimum parameters for AF have still not been established [1,6], and full control of the metabolic pathways has not been achieved. A popular approach of using high organic loading rates typically results in substantial pH drops due to the acidic nature of VFA [7] when the substrate used cannot generate sufficient buffering capacity during the fermentation process (e.g., substrates with low nitrogen content). Changes in pH can have a significant effect on VFA yield and speciation [8–10], causing inhibition to acidogenic bacteria at very acidic pH [8], or switching the pathway to methanogenesis if pH drops from alkaline to neutral [9]. One common strategy to control pH and alkalinity in AF processes is the addition of sodium hydroxide (NaOH) to neutralise

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VFA [11–13]. Using NaOH alone to control pH can be challenging, as it requires automation of NaOH dosing, and regular use of NaOH is undesirable for both cost and safety reasons.

A suggested novel strategy to maintain operational pH and alkalinity favourable for AF is the addition of organic nitrogen compounds, including urea ($CO(NH_2)_2$) and nitrogen-rich feedstocks. Typically this is done in AD processes to balance carbon to nitrogen ratios, but it has also been suggested for pH control [14]. The addition of urea or nitrogen-rich feedstocks releases ammonia (NH_3) which in turn increases the pH. Ammonia release from urea is a biological process facilitated by the action of urease enzymes generated by hydrolytic bacteria, and follows equations 1 and 2 [15]:

$$(NH_2)_2CO + H_2O \xrightarrow{\text{urease}} NH_3 + H_2NCOOH \xrightarrow{\text{urease}} 2NH_3(gas) + CO_2(gas) \quad (1)$$

$$NH_3(gas) + H_2O \leftrightarrow NH_4^+ + OH^- \quad (2)$$

Ammonia gas has high solubility in water and when it dissolves it forms an ammonium cation and a hydroxide anion, thus increasing pH [3]. Nitrogen content in the substrate can consequently provide buffering capacity and can affect the AF process: ammonia/ammonium can be inhibitory for methanogens [3] and other VFA consuming microbes [16], particularly if it is present in its free molecular form [17], therefore it can be advantageous in AF when VFA production is desired.

Some AF studies have utilised urea as a nitrogen source for ammonia release and increased alkalinity [18–20]. Urea addition was shown to have a beneficial effect on VFA production from kitchen wastes, resulting in a 20% increase in VFA concentrations compared to the pH control method using NaOH/$Ca(OH)_2$ addition alone [18]. However, the pH was kept at 7 by NaOH/HCl addition and there was no report on VFA composition. The same study looked at the effect of pH on ammonia release from the proteins of the substrate. It was found that ammonia release was highly affected by pH, with pH 7 reaching 5 times higher ammonia concentrations [18]. Another study using sugarcane filter cake found that urea supplementation had a negative effect on AF[19]. VFA yield decreased by 75% due to an increase in pH from acidic to neutral which promoted methanogenesis [19]. In a study looking at the effect of urea supplementation on AF of sewage sludge, it was found that functional bacteria linked to VFA generation were enriched and the availability of organic matter was improved, overall boosting the AF process [20].

Other buffering substances or salts such as $CaCO_3$ can also help stabilise pH and consequently improve AD processes [3,21]. Two previous studies on acidogenic fermentation utilised $CaCO_3$ and $(NH_4)HCO_3$ as buffering agents to maintain stable pH [22,23], however there was no report on how these chemicals affected pH. Another utilised $NaHCO_3$ as a buffer agent [13]; however, NaOH was also used in order to increase pH, making it unclear how the $NaHCO_3$ was affecting the pH control. A study with vegetable and salad waste as substrate used 0.6 M $NaHCO_3$ to prepare the feed, which resulted in neutral pH during the continuous fermentation [24]. It should be noted that there is a difference between alkaline pH and alkalinity, the ability to neutralise acids to maintain a certain pH. Each substance has different influence over pH and alkalinity. For example, $CaCO_3$ is typically used in AD as buffering agent but other carbonate salts (sodium or potassium) are preferred to add alkalinity without causing rapid increases in pH [3].

The aim of the current study was to test the effect of different alkalinity sources on AF using a novel substrate derived from the Fiberight process [25]: The organic residuals solids (ORS) used in this study are akin to the organic fraction of municipal solid waste (MSW), widely considered an important waste stream with a great deal of potential for AF. Firstly, urea addition was studied as a pH control method to overcome natural acidification in the AF of untreated ORS as well as in the simultaneous hydrolysis and fermentation (SHF) of ORS. The effect of total ammonia nitrogen (TAN) available in solution was, for the first time, studied separately from the effect of pH rises caused by TAN release. In

addition, a study of the microbial diversity affected by TAN and pH was undertaken. Secondly, the effect of addition of alkalinity via $CaCO_3$ supplementation at different pH values (7-10) in the SHF of ORS was investigated as an innovative strategy to avoid undesired pH drops in AF.

2. Materials and Methods

2.1. Materials

The organic residual solids (ORS) used as substrate were collected from (a) the Fiberight R&D pilot plant in Southampton, UK and (b) an industrial plant using the Fiberight process to treat MSW in Maine, USA. Both ORS types arise from a patented process involving the removal of recyclables, pulping and washing [25]. The ORS consists of suspended solids in the wash water which are then recovered via filtration. ORS was stored at -18°C until used in experiments.

Inoculum containing AD microbes was obtained from a mesophilic anaerobic digester treating municipal wastewater biosolids at Millbrook wastewater treatment plant, Southampton (UK). Inoculum was sieved and incubated for 24 hours at 35±2°C prior to any experiment.

Enzyme complexes for testing purposes were provided by a private third party. These enzymes were classified based on their functionality: Complex A (a mixture of cellulase/beta-glucosidase/hemicellulose), and Amylase. A mixture (w/w) of 0.81 complex A and 0.19 amylase was used in this study.

2.2. Compositional analysis

Total solids (TS) and volatile solids (VS) from ORS were determined following Standard Method 2540 G [26]. $CaCO_3$ ash percentage was measured by method described in Chavez Guerrero et al. [27]. Total suspended solids (TSS) and volatile suspended solids (VSS) were measured as per method 2540 D [28].

The ORS was freeze-dried using a VirTis benchtop K for 5 days to reach a maximum moisture content of 2-3% (fresh weight basis). Freeze-dried ORS was milled to a particle size of <1mm and stored in air-tight plastic containers until analysis. The freeze-dried milled ORS was used in elemental composition (CHNSO), proteins, carbohydrates, lignin, and lipids analyses.

Elemental composition was determined using a FlashEA 1112 Elemental Analyser (Thermo Finnigan, Italy). The combustion reactor was held at 900°C then the sample was flash combusted in a gas flow temporarily enriched with oxygen at 1700 °C. Birch leaf (B2166) with approximately the following composition (%); C=48.33; H=6.36; N=2.09; S=0.16 was used as a standard for this method. Oxygen content was estimated by difference with total sample dry weight.

Protein content was determined by measuring the total Kjeldahl nitrogen (TKN), which was determined according to Standard Method 1687 [29]. Free ammonia in ORS was assumed to be negligible. TKN was then multiplied by a nitrogen-in-protein factor of 6.25 [30].

Determination of carbohydrates and lignin was carried out through acid hydrolysis following the National Renewable Energy Laboratory (NREL) method [31] with slight modifications: 0.3 g of sample and 3 mL of 72% sulfuric acid were placed in test tube. The tube was placed in 30°C water bath for one hour. The contents were occasionally mixed with a glass stirrer. Sample was transferred with deionized (DI) water to a polypropylene copolymer (PPCO) bottle. DI water was added to dilute sulfuric acid to 4%. The bottle was autoclaved at 121°C for one hour. Sample was filtered using a pre-treated at 105°C for 4 hours filter paper. Filtrate was used for acid soluble lignin (ASL) and sugar quantification. The solids on the filter paper were washed with DI water, dried at 105°C for four hours and treated in the furnace at 550°C for two hours for acid insoluble lignin (AIL) and ash quantification. Sugar analysis was carried out on a Dionex DX-500 HPLC system. Glucose, xylose, galactose, arabinose, mannose and cellobiose were separated at 30°C on a

CarboPac PA1 column (250 x 4 mm) in combination with a CarboPac guard column (25 x 4 mm) (Dionex, Sunnyvale, CA, USA).

Lipid content was determined using Soxhlet extraction with hexane following method 9071B [32] with slight modifications.

2.3. Experiments

The first experiment was designed to provide further insight into the ammonia release mechanisms from urea, and the effect of total ammonia nitrogen (TAN) concentrations achieved by urea addition on the AF of ORS. This experiment consisted of three parts: the first part was designed to check the effect of different pH on urea hydrolysis using AD inoculum. The second and third parts were designed to test different urea quantities on AF of untreated ORS, and simultaneous hydrolysis and fermentation (SHF) of ORS, respectively. The second experiment was designed to test the effect of pH and added alkalinity with NaOH and $CaCO_3$ on SHF of ORS.

PPCO bottles (250 mL) with ORS, water, inoculum and enzymes were prepared accordingly depending on the experimental design. The bottles were placed in an incubator with an orbital shaker set to 200 rpm. In SHF experiments, an enzyme loading of 0.02 g of concentrated solution g^{-1} TS was applied. pH was adjusted when needed and at least every 24 hours using either 4 M HCl or 4 M NaOH, and at the same time any pressure build-up in the headspace due to gas production was released. Samples including liquid fraction and suspended solids were taken before pH adjustment, kept frozen and then defrosted for analysis. Samples were centrifuged and supernatant was used for total organic carbon (TOC), VFA, alkalinity and TAN analysis.

2.3.1. Urea addition experiment - Part I

The experiment took place at adjusted pH values of 4.5, 6 and 8, with two replicates each. Each replicate flask contained 150 mL of AD inoculum, 1 g of urea and 1.5 g of glucose. Control replicates contained inoculum alone and the pH was not adjusted. The experiment was carried out for 4 days at 35°C.

2.3.2. Urea addition experiment - Part II and III

An ORS concentration of 6% (w/w) TS and a substrate/inoculum (S/I) ratio of 40 g^{-1} TS g^{-1} TS was used in a working volume of 200 mL. The experiment took place at urea additions of 13 (A), 66 (B) and 155 (C) mg Urea g^{-1} TS, equivalent to approximately 0.41, 2.07 and 4.83 g TAN L^{-1}, respectively, if 100% urea to TAN conversion is assumed. Conditions were tested in duplicate. In parallel, three pH conditions without urea addition (with 2 replicates for each condition) were run in an effort to replicate the pH values observed in the urea addition experiments. Control replicates had no urea addition and pH was not controlled. Fermentation took place for 6 days at 35°C. The experiment consisted of two parts: Part II, where untreated ORS was used, and Part III, where SHF was performed.

2.3.3. $CaCO_3$ addition experiment

An ORS concentration of 6% (w/w) TS and a S/I of 40 g TS g^{-1} TS was used in a working volume of 200 mL. Two replicates were used to test each pH: 10, 8.5 and 7 by NaOH addition. In parallel, the same pH conditions were tested with the addition of 2.2 g of $CaCO_3$ to each replicate (11 g L^{-1}). No control replicates were used in this experiment. Fermentation took place for 8 days at 35°C.

2.4. Analytical methods

A Jenway 3010 meter (Bibby Scientific Ltd, UK) with a combination glass electrode, calibrated in buffers at pH 4, 7 and 9.2, was used to measure pH values. Alkalinity was measured by titration based on Standard Method 2320B [26]. TAN analysis was based on Standard Method 4500-NH3 [33]. TOC was measured using a TOC-V_{CPH} (high-sensitivity model) system (Shimadzu, UK), using solutions of glucose and acetic acid of known concentrations as check standards. VFA were analysed based on the method developed by the Standing Committee of Analysts [34]. Quantification of VFA was carried out using a

Shimazdu GC-2010 gas chromatograph (Shimadzu, UK), with a flame ionisation detector and a capillary column type SGE BP-21. The quantified VFA were acetic acid (AcH), propionic acid (PrH), iso-butyric acid (i-BuH), butyric acid (BuH), iso-valeric acid (i-VaH), valeric acid (VaH), hexanoic acid (HexH) and heptanoic acid (HepH).

2.5. Microbial community analysis

To carry out microbial community analysis, frozen samples were thawed at 4 °C overnight. The total DNA of the samples was extracted using DNA extraction kits (Tiangen Biotech (Beijing) Co. Ltd., Beijing, China) according to the manufacturer's instructions. The DNA concentration of the samples was measured with the Qubit dsDNA HS Assay Kit and Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the purified genomic DNA was also analysed by agarose gel electrophoresis. The V4 region for 16S rRNA of bacteria and archaea (FWD:GTGYCAGCMGCCGCGGTAA; REV:GGACTACNVGGGTWTCTAAT) was targeted for Ribosomal RNA gene amplification [35] for samples taken at the end of the fermentation in the urea addition experiment – part III. Each amplicon was then sequenced on a paired-end Illumina platform to generate 250 bp paired-end raw reads, and then merged using FLASH (V1.2.7) and pretreated according to the Qiime (V1.7.0) quality controlled process to obtain Clean Tags [36–38]. Chimeric sequences in Clean Tags were detected and removed to obtain the Effective Tags. Sequences analysis was performed by Uparse software v 7.0.1090 using all effective tags [39–41]. Operational Taxonomic Units (OTUs) were obtained by clustering with ≥97% similarity. For each representative sequence, the Qiime in Mothur method was performed against the SSUrRNA database of SILVA138 Database for species annotation at each taxonomic rank [42–44]. To obtain the phylogenetic relationship of all OTUs representative sequences, MUSCLE were applied to compare multiple sequences [45].

2.6. Calculations of yield and degree of acidification

The performance of the bioreactors was evaluated in terms of VFA yield, defined as the mass of VFA produced divided by the mass of volatile solids (VS) fed, Equation 3:

$$\text{VFA yield (g VFA g}^{-1}\text{ VS)} = \frac{C_{VFA} \cdot V}{m_{subs} \cdot VS_{subs} + m_{inoc} \cdot VS_{inoc} + m_{enz} \cdot VS_{enz}} \quad (3)$$

C_{VFA} = VFA concentration (g VFA L^{-1});
V = working volume (L);
m = wet mass (g);
VS = volatile solid content (% wet weight);
'Subs', 'inoc', and 'enz' subscripts refer to substrate, inoculum and enzymes respectively.

The degree of acidification (DoA) was calculated as the concentration of VFA converted to units of TOC and divided by the total TOC measured, following Equation 4:

$$\text{DoA (\%)} = \frac{TOC_{VFA}}{TOC_{TOTAL}} \cdot 100 \quad (4)$$

3. Results and discussion

3.1. Compositional analysis

The composition of the ORS is summarised in Table 1. In this work, all quantified glucose is reported as glucan, as the nature of the analytical method meant that the difference between the various glucose-containing carbohydrates could not be established. The glucan percentage varies between 29-33%, depending on the ORS source. It is believed that most of the glucan is represented by cellulose, due to the fibrous nature of the ORS, and the fact that the washing process probably eliminated soluble starch. Hemicellulose

(the sum of xylose, mannose, arabinose, galactose and cellobiose) content ranged between 6-8%.

Table 1. Characteristics of the ORS from Southampton pilot plant (a) and Maine (b).

Component (% TS basis)	Average value (a)	Standard deviation [1] (a)	Average value [2] (b)
Volatile solids	75.94%	±0.96%	67.22%
CaCO3 ash	5.00%	±0.41%	-
Glucan	33.33%	±2.94%	29.15%
Hemicellulose	5.84%	±0.25%	8.09%
Acid insoluble lignin (AIL)	11.10%	±0.07%	14.46%
Acid soluble lignin (ASL)	1.40%	±0.10%	0.66%
Proteins	8.11%	±0.29%	-
Lipids	4.71%	±0.22%	6.72%
Extractives	-	-	10.65%
Total food content [3]	51.99%	-	-

[1] Based on three sample replicates.
[2] Data obtained from external laboratory Celignis Analytical [46].
[3] Total food content was calculated as the sum of glucan, hemicellulose, proteins and lipids.

Total 'food' (cellulose, hemicellulose, lipids and proteins) for AD microbes represents just over 50% of the dry weight of ORS. Extractives are components that can be extracted by solvents (e.g. ethanol or acetone); for example fats, waxes, proteins, gums, resins, simple sugars, phenolics, pectin, and fatty acids. The main fatty acids detected in the ORS from Maine, USA were palmitic (42%), stearic (27%) and linoleic (18%) [46]. The measured $CaCO_3$ ash in the ORS from Southampton was 5%, which could have been derived from small pieces of eggshell (based on visual inspection). The $CaCO_3$ could also be derived from the paper fraction, since $CaCO_3$ is commonly used as paper filler [47].

Some compositional differences are observed when comparing ORS from the two different sources (A and B). Replicability for this substrate, or any residue derived from MSW, cannot be guaranteed as MSW composition may vary depending on source, location, and season. In this case, variations in composition may also be due to the difference in the scales of the two processes (i.e. pilot plant vs. large scale). It was concluded, however, that the compositional parameters exhibited sufficient similarity to enable result comparison across experiments.

The ORS from Southampton (A) was also subjected to elemental analysis, as shown in Table 2. The results fall within the typical composition range of the organic fraction of MSW (OFMSW): 20.3-45%C, 0.4-1.8% N, 5.9-7.8 %H, ~0% S, 31.0-47.3% O, on a dry weight basis [48,49]. The observed low nitrogen content of the ORS, at 1% (w/w), may suggests a potential requirement for supplementary nitrogen containing buffering agents (e.g. urea).

Table 2. Elemental composition of ORS from Southampton on a dry basis.

Element	**N**	**C**	**H**	**S**	**O**
Mass percentage (%)	1.0%	39.8%	5.4%	0.2%	53.7%
Molar percentage (%)	0.58%	27.49%	44.06%	0.04%	27.83%
Mol per mol of C	0.021	1	1.603	0.002	1.012

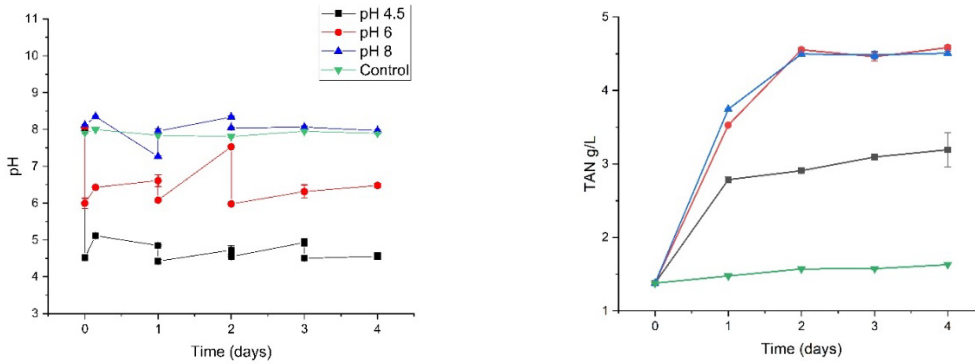
3.2. Part I – pH effect on urea hydrolysis

This part of the experiment was carried out to investigate the effect of different pH values on the bio-hydrolysis of urea into ammonia by AD mixed culture. In addition to two near neutral pH levels (i.e. 6 and 8), a pH of 4.5 was tested for the feasibility of using

urea to raise pH level as a remedy measure when rapid VFA production has caused a severe pH drop. Glucose was added to the digestate to maintain the viability of its microbial population during the test. The estimated S/I ratio was 0.23 g TS g^{-1} TS. As 1 g of urea was added into each test flask with 150 mL of digestate, the nitrogen concentration increase in the digestate was 3.1 g N L^{-1}.

Figure 1, (a) shows the measured pH values for each pH condition tested. On day 0, pH adjustment by acid addition to pH 4.5 and 6 replicates was followed by instant foaming. This could have been caused by the release of CO_2 as a result of acidification. For most replicates small pH adjustments were needed each day. Only one major adjustment was needed on day 2 for the pH 6 replicates, as pH had increased beyond desired levels. No notable pH change was observed in the control replicates to which urea was not added. Despite the moderate S/I ratio, evident acidification occurred within the first day in the pH 8 replicates, causing pH to decrease from 8.35 on day 0 to 7.3 at the end of day 1 despite the increase in TAN concentration. This is probably explained by the production of VFA as well as, to a lesser extent, the CO_2 generated by urea hydrolysis and other metabolic reactions. However, VFA production was not evaluated in this part of the experiment. Natural pH increases can be explained by the combined effect of TAN increase and VFA consumption for biogas production. This combined effect was most obvious on pH 6 duplicates between day 1 and 2.

Figure 1, (b) shows the TAN concentration for each pH condition tested. Ammonia release plateaued within 2 days in all cases, with a slight increase from day 2 to day 4 for pH 4.5 replicates. The highly acidic pH (pH 4.5) reached a maximum of 3.19 g TAN L^{-1}, whereas pH 6 and 8 resulted in 4.58 and 4.51 g TAN L^{-1}, respectively. Based on the difference between TAN values in the urea replicas and the control, it was considered that all of the urea was hydrolysed at pH 8 and pH 6, whereas only 58% was hydrolysed at pH 4.5 after 4 days. It is important to highlight that urea degradation can also occur by non-biological routes and this phenomenon is dependent on temperature and pH [50]. In the absence of urease or microorganisms, it was noted that higher temperatures led to higher degradation and that most stable pH range was 4-8 [50]. The TAN release observed in the current study, therefore, cannot be entirely attributed to biological degradation of urea. Nevertheless, the TAN release at pH 4.5, despite the potential acidic effect on degradation, was considerably lower than at pH 6 and 8. As the addition of chemicals for pH adjustment took place immediately after urea addition, comparative data analysis was undertaken to indicate whether urea hydrolysis in pH 4.5 replicates was primarily biological or chemically-driven. The pH 8 replicates were considered the most natural biological processes due to minimal chemical addition. Consequently, the kinetic rates of pH 8 replicates were compared to those of pH 4.5 replicates. Based on the kinetics observed at pH 8, where it took 0.43 days to reach the same concentration as seen in pH 4.5 replicates on day 1, it was concluded that urea hydrolysis in pH 4.5 replicates was predominantly driven by chemical action, specifically acid hydrolysis. However, the slow release of TAN from day 1 to day 4 in pH 4.5 replicates does not align with the kinetic rate of the first 24 hours, indicating a likely slow (inhibited) biological hydrolysis process. In agreement with these findings, the TAN release in the co-fermentation of WAS and food waste was also negatively affected by acidic pH (4-5) and reached a maximum at pH 7-9 after 12 days of fermentation [51].



(**a**) (**b**)

Figure 1. (a) pH readings from urea hydrolysis experiment, adjusted by HCl or NaOH addition; (b) TAN concentration resulting from urea hydrolysis at different pH values (1 g of urea and 1.5 g of glucose in 150 mL of AD digestate). Error bars represent the values of the two replicates used to calculate the average result

These results confirmed that highly acidic pH inhibits biological ammonia release from nitrogen sources and urea. This inhibition is caused by a protonation of both ionizable groups in the urease enzyme at pH<5.3 [52]. Therefore, careful consideration must be taken when choosing the fermentation conditions, particularly pH, if using urea and other natural nitrogen sources as a methanogenic inhibitor or buffering agent.

3.3. Part II – Urea addition effect on AF of untreated ORS

This part of the experiment looked at the effect of TAN concentration adjusted by urea addition on the AF of untreated ORS.

The measured ammonia concentrations at the end of the fermentation were 0.53, 2.33, 5.83 and 0.13 g TAN L^{-1} for conditions A, B, C and control, respectively. In all cases, the TAN concentration detected represented ≥90% of theoretical values based on the mass of urea added.

Figure 2, (a) shows the pH values for each condition tested. B and C replicates had a pH drop in the first 24 hours and a sharp rise in the next 24 hours. This is explained by a rapid acidification, followed by neutralisation as ammonia is released at a slower rate. Despite this, the addition of urea led to a pH of 5.6 and 6.2 at the end of day 1 for B and C replicates respectively, compared to a pH of 5.1 without urea addition in the control replicates. No further marked drop in pH was observed in B and C replicates after day 2, and pH values above 7 and 8 were achieved by day 3 for B and C replicates, respectively. The pH equivalent replicates for B and C had very unstable pH, with pH dropping by 0.4-1.8 units within 24 hours. This could simply be explained by the fact that NaOH was added on a daily basis to increase the pH of each pH equivalent set to that of A, B or C, as opposed to a slow release of buffering agent in urea replicates. Control replicates also showed an upward trend in pH values from day 1 onwards, despite the absence of external buffering agent. This could be explained by the $CaCO_3$ present in the ORS, which represents approximately 5% of its dry weight. The $CaCO_3$ might be gradually dissolving and neutralising the VFA, acting as a buffering agent.

Figure 2, (b) shows the VFA yields for each condition tested. Based on these results, nitrogen addition seemed to have a positive impact on VFA yields from untreated ORS. Maximum yield was achieved by B-replicates on day 5 and C-replicates on day 6 (0.14 g VFA g^{-1} VS for both), corresponding to concentrations of 8.42 and 9.14 g VFA L^{-1}, respectively. These values correspond to an increase of approximately 70% in VFA yields with respect to the control replicates, where the VFA concentration on day 6 was 4.61 g VFA L^{-1}. In contrast, VFA yield for A-replicates only increased by 20% with respect to the control

replicates. These findings are similar to results from the co-fermentation of waste activated sludge (WAS) with corn gluten meal, with a 13.3-fold increase in VFA concentration corresponding to an increase in TAN released from the substrate from approximately 0.18 to 0.63 g TAN L^{-1} [53]. However, the VFA increase cannot be attributed entirely to an increase in TAN, as the extra TAN resulted from further substrate addition. In another study, urea addition of 0.2 g urea g^{-1} TSS (measured ~0.36 g TAN L^{-1}) resulted in 5.1-fold increase in VFA concentration compared to the control in the fermentation of sewage sludge alone [20]. The same study reported that higher TAN concentrations (~0.51 g TAN L^{-1}) negatively affected VFA production [20]. This is 4.6 times lower than the optimum TAN concentration for VFA production found in this study.

When studying the pH equivalent replicates, a similar pattern was observed, with increased alkalinity/pH leading to higher yields in the last two days of the fermentation and reaching similar yields to the corresponding urea replicates. The VFA concentrations in the pH equivalent replicates A, B and C on day 6 were 4.90, 7.04 and 7.92 g VFA L^{-1}, respectively. However, these replicates, which were adjusted with NaOH, took longer to reach maximum yields, indicating that daily NaOH addition as buffering strategy can lag VFA production when compared to ammonia release from urea. Continuous automated NaOH dosing might reduce or eliminate this lag; however, this was not investigated in the current study.

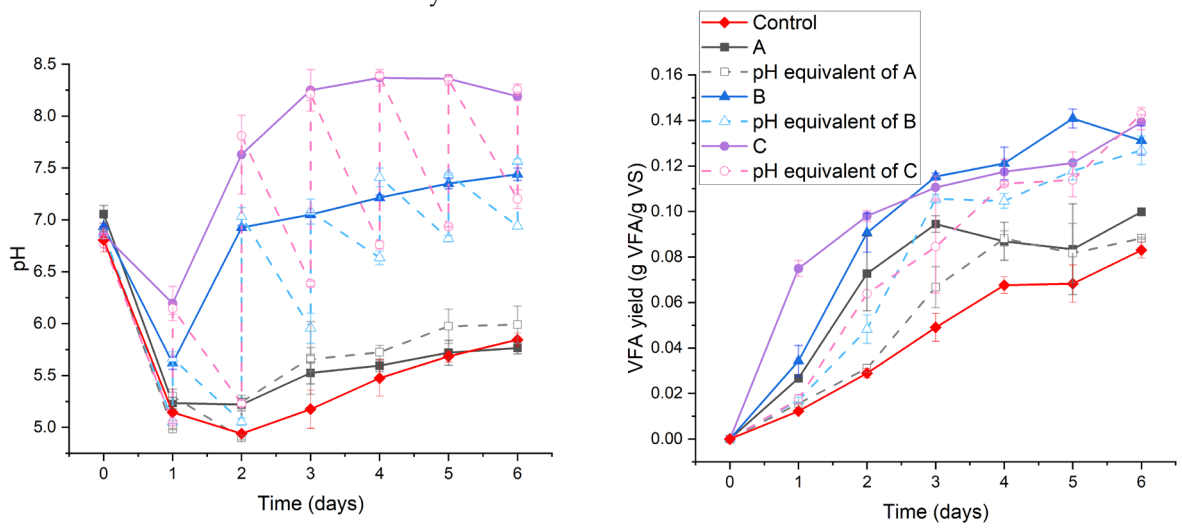


Figure 2. (a) pH readings and; (b) VFA yield of different ammonium concentrations modified by urea addition, using untreated ORS at 7.5% TS, S/I of 40 g TS g^{-1} TS and 35°C. The pH of 'pH equivalent' replicas was modified using NaOH or HCl. Error bars represent the values of the two replicates used to calculate the average result

Nitrogen/urea addition had a notable effect on the VFA profile (Figure 3). Increasing TAN concentration resulted in a switch from mostly BuH production towards a balanced production of AcH and PrH. This is explained by the changes in pH, as BuH production is predominant in slightly acidic pH [6]. However, some differences are observed when comparing the results to those for the pH equivalent replicates. The A-replicates had a balanced composition of AcH, PrH and BuH, whereas the A-pH equivalent replicates were predominantly comprised of AcH and BuH with small proportions of PrH and VaH. This difference was observed despite both sets of replicates following the same pH profile throughout the experiment. A similar observation is made when comparing both A-replicates and their pH equivalent replicates to the control, which followed a very similar pH profile throughout the fermentation but produced considerably higher BuH concentrations. This indicated that VFA profile is also affected by ammonium concentration, independently of pH values. In a study on VFA production from sewage sludge at 35°C and

pH 7, urea addition resulted in an increase in the proportion of AcH, in detriment to PrH and i-VaH [20]. The previously reported effects of urea addition on VFA proportions differ from the results of this study, likely due to variations in substrate and inoculum compositions.

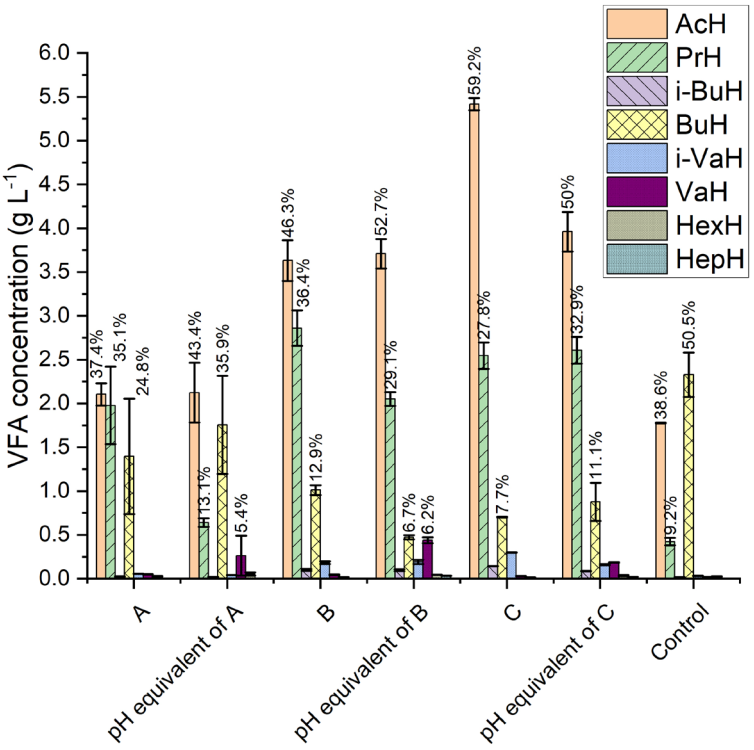


Figure 3. VFA profile on day 6 of the fermentation of different TAN concentrations modified by urea addition, using untreated ORS at 7.5% TS, S/I of 40 g TS g^{-1} TS and 35°C. The pH of 'pH equivalent' replicas was modified using NaOH or HCl. Percentages are the average of two replicates.

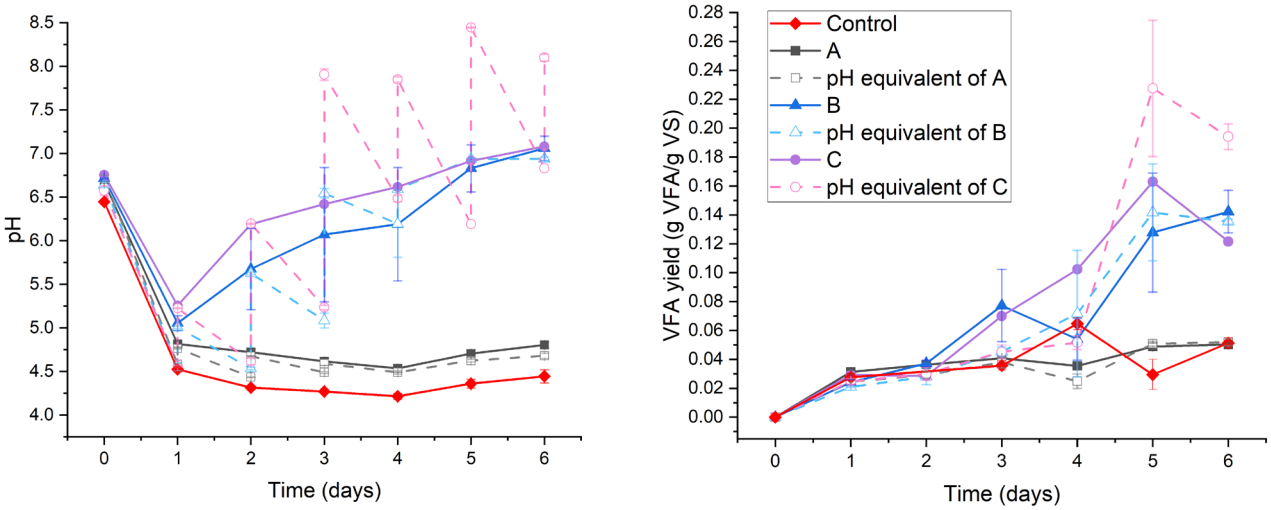
3.4. Part III - Urea addition effect on SHF of ORS

Prior to this experiment, a consecutive hydrolysis and fermentation (CHF) experiment was carried out, where the ORS was first pretreated with 2% enzyme loading for 24 hours at 50°C preceding the fermentation. After enzymatic pretreatment, the pH of all replicates fell to 3.8-4.3. It is believed, based on previous work, that this low pH after pretreatment results from lactic acid production, which is a stronger acid than the targeted VFA. The inoculum and urea were added after pretreatment of the ORS. The low pH values were maintained throughout the fermentation despite the urea addition. After 7 days of fermentation the TAN concentrations were 0.18, 0.44, 1.05 and 0.10 g TAN L^{-1} for A, B, C and control, respectively. These values represent 16-18% of the maximum theoretical urea hydrolysis yield. As seen in Part I, the low pH led to the inhibition of urease enzymes, resulting in low ammonia release from urea. As a consequence of this, pH was not increased during fermentation and, consequently, acidogenesis was inhibited, resulting in undesirably low VFA concentrations at the end of the fermentation (<1 g VFA L^{-1}) and low VFA yields (<0.01 g VFA g^{-1} VS). Therefore, simultaneous hydrolysis and fermentation (SHF) was adopted as an alternative strategy to avoid conversion of sugars by other fermentation pathways to stronger acids, such as lactic acid, with a subsequent sudden pH drop.

It is important to highlight that with SHF there is a higher risk of enzymes being degraded by AD microbes, and temperature conditions are not optimum for hydrolysis (50°C). In this experiment, the same conditions were tested as in the CHF experiment, except that enzymes were added at the same time (or as close as practically possible) as the inoculum. Fermentation took place for 6 days.

Figure 4, (a) shows the pH values from this experiment. All reactors experienced a rapid pH drop within a day as a result of VFA release. After that, the A-replicates did not show a pH rise, with pH remaining below 5 throughout the fermentation. B replicates reached a neutral pH (6.6-7.1) by day 5. One of the C replicates reached pH 8.2 by day 5 of the fermentation, whereas the identical replicate stayed at slightly acidic (pH<6 throughout the fermentation), leading to large error bars for C replicates. At the end of the fermentation TAN concentrations were 0.49, 2.20, 4.03 (average of 5.63 and 2.42) and 0.08 g TAN L^{-1} for A, B, C and control, respectively. The urea hydrolysis yield was 92-106% for all A-B-C replicates except one of C-replicates (only 45% of urea hydrolysed). The reason for this discrepancy is unknown.

Figure 4, (b) shows the VFA yields for this experiment. A lag of at least 2 days in VFA production was observed for all replicates, probably as a result of pH instability in the first 2 days. Towards the end of the fermentation, B and C-replicates had higher VFA yields than A-replicates and the control replicates. In this case, pH equivalent replicates behaved much more like their corresponding urea replicates, with the exception of C-pH equivalent replicates, which showed higher yields than C-replicates. This is explained by the high pH variability in C-pH equivalent replicates, as seen in Figure 4, (a), which resulted in a higher average pH compared to C replicates in the last 3 days of the fermentation (7.3 vs 6.9).



(**a**) (**b**)

Figure 4. (a) pH readings and; (b) VFA yield VFA for SHF of ORS at 7.5% TS, S/I of 40 g TS g^{-1} TS, 35°C and 2% enzyme load with different ammonium concentrations from urea addition. The pH of 'pH equivalent' replicates was modified using NaOH or HCl. Error bars represent the values of the two replicates used to calculate the average result. C-replicate VFA yields are based on one replicate only (replicate with near 100% urea hydrolysis)

Figure 5 shows the VFA profiles for each condition tested. When these are compared to the VFA profile for untreated ORS (Figure 4), there are clear differences for all conditions. This might be due to the observed lag phase in the first 4 days and the potentially different metabolic pathways followed in SHF compared to untreated ORS fermentation. In the case of A-replicates, A-pH equivalent replicates, and control replicates, VFA

concentrations were low (<3.1 g VFA L-1), potentially because of inhibition caused by low pH. Under these conditions, AcH was the predominant acid (>70%). BuH and AcH were the predominant acids for B and C-replicates. Clear differences were observed from the pH equivalent replicates, particularly for C-replicates. The BuH ratio (BuH concentration divided by total VFA concentration) was 0.31 for the C-replicates, whereas it was only 0.08 for the C-pH equivalent replicates. This is likely explained by the higher average pH in the pH equivalent replicates, as BuH production is promoted under acidic pH.

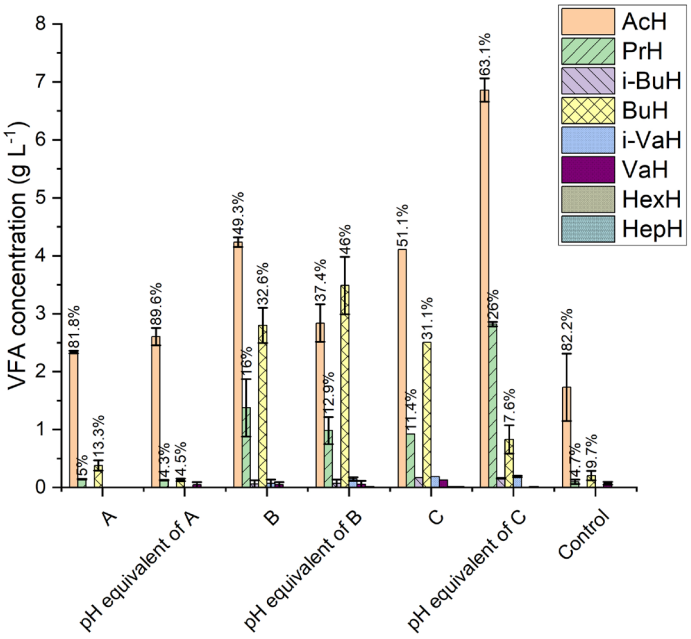


Figure 5. VFA profile on day 6 for SHF of ORS at 7.5% TS, S/I of 40 g TS g^{-1} TS, 35°C and 2% enzyme load with different ammonium concentrations from urea addition. The pH of 'pH equivalent' replicates was modified using NaOH or HCl. Error bars represent the values of the two replicates used to calculate the average result. C-replicate results are based on one replicate only (replicate with near 100% urea hydrolysis)

3.5. Part III - Biodiversity and relative abundance

Samples from Part III taken at the end of the experiment were subjected to microbial community analysis (see Appendix A).

Archaea content was ≤0.1% for all samples, indicating that the conditions tested were favourable for methanogenic inhibition. Bacteria represented >99.9% of the root (i.e. of total microbes); therefore, in this section, percentages refer to percentages of Bacteria unless stated otherwise.

In the control samples (~0.08 g TAN L^{-1}), the majority of bacteria belonged to the phylum Firmicutes (92-99%), primarily comprised of *Lactobacillus* (78%-83%). *Lactobacillus* main pathway leads towards lactate production, which explains the low pH (~4.5) and the low VFA yield (~0.05 g VFA g^{-1} VS) in the control samples. Between 5-19% was comprised of Clostridia, with a high proportion of *Clostridium* (18%) in one of the replicates. Only small proportions of Proteobacteria (2%), Actinobacteriota (2%) and Bacteroidota (3%) were observed in the other replicate.

A-pH equivalent replicates (pH ~4.5) were very similar to the control ones in terms of microbial relative abundance, with *Lactobacillus* representing (83-91%). Some Clostridia (4-7%), Bacteroidota (2%) and Proteobacteria (0-1%) were also observed.

Microbial community composition of the A-replicates (0.46 g TAN L^{-1}) was noticeably different to that of the controls and the pH equivalent replicas, with a high proportion

of *Lactobacillus* (55-67%) and *Clostridium* (18-31%). Some Actinobacteriota (0-1%), Proteobacteria (1-2%) and Bacteroidota (3%) were also detected. A-replicates and A-pH equivalent replicates had an almost identical pH profile throughout the fermentation. Therefore, the differences in relative abundance are attributed to the difference in TAN concentrations.

B-pH equivalent replicates had *Lactobacillus* (27-28%) and other genera within Bacilli (4-11%). The other major component of Firmicutes was Clostridia (21-26%). The main Clostridia genus in one replicate was *Pygmaiobacter* (14%), whereas *Anaerofilum* (10%) was the most dominant in the other, with only 1% of *Pygmaiobacter*. These replicates had a relatively high proportion of Bacteroidota (22-32%) and smaller proportions of Actinobacteriota (6-7%) and Proteobacteria (2-3%). Among the Bacteroidota, there were unclassified *Weeksellaceae* (6-14%), *Bacteroides* (3-11%), and *Prevotella* (7-11%).

B-replicates (2.08 g TAN L^{-1}) contained a high proportion of Bacilli (42-48%), with *Lactobacillus* (29-45%) as the main genus and a proportion of *Enterococcus* (8%) in one of the replicates. The next most abundant class was Clostridia (6-18%), followed by Actinobacteriota (6-11%) and Proteobacteria (5-11%). The genera of Clostridia and Proteobacteria varied between replicates, but Actinobacteriota was mostly comprised of *Propionibacterium* (57-74% of Actinobacteriota). *Propionibacteria* are able to metabolize carbohydrates, pyruvate and lactate to propionate, acetate, and carbon dioxide [54].

C-pH equivalent replicates had a high relative abundance of Bacilli (19%), with *Lactobacillus* (72-77% of Bacilli) as the predominant genus, accompanied by Negativicutes (20-23%) and Clostridia (11-12%). The main Negativicutes was *Veillonella* (99-100% of Negativicutes). Clostridia was diverse, with 24-32% of Clostridia represented by *Pygmaiobacter*. Bacteroidota (21-33%) and Proteobacteria (10-26%) were also relatively abundant. The main Bacteroidota were unclassified *Weeksellaceae* (22%) and *Bacteroides* (6%). A proportion of the Proteobacteria was comprised of *Escherichia-Shigella* (8%), known to be a pathogen closely related to *E.coli* [55]. The metabolic products of *E. Shigella* under anaerobic conditions include ethanol, acetic acid, succinic acid, formic acid, hydrogen and carbon dioxide [56]. Some proportion of Actinobacteriota (3%) was also observed.

C-replicates showed great differences in relative abundance. In one of the replicates (5.63 g TAN L^{-1}), with higher pH and VFA yields, Bacilli (27%) and Clostridia (30%) were observed as the main Firmicutes (58%). In this case, the Bacilli were comprised of *Lactobacillus* (12%) and *Enterococcus* (8%). Considerable proportions of Bacteroidota (28%) and Proteobacteria (13%) were also observed. In this case, *Fermentimonas* comprised 90% of Bacteroidota, and *Paenalcaligenes* 72% of Proteobacteria. In the other replicate (2.42 g TAN^{+} L^{-1}), which showed lower pH and VFA yield, the majority of the abundance was represented by *Lactobacillus* (66%). This is explained by the replicate lower TAN concentrations, resulting in insufficient added alkalinity to increase pH. This replicate had a microbial community closer composition to the A-replicates, probably because of the lower ammonium content. The percentage of Clostridia (15%), however, was lower than the values observed in A-replicates , where it was replaced by Actinobacteriota (8%), Proteobacteria (3%) and Bacteroidota (3%).

The results demonstrated that for the same substrate, fermentation conditions such as pH or TAN concentration can highly influence the microbial community compostion in terms of relative abundance.

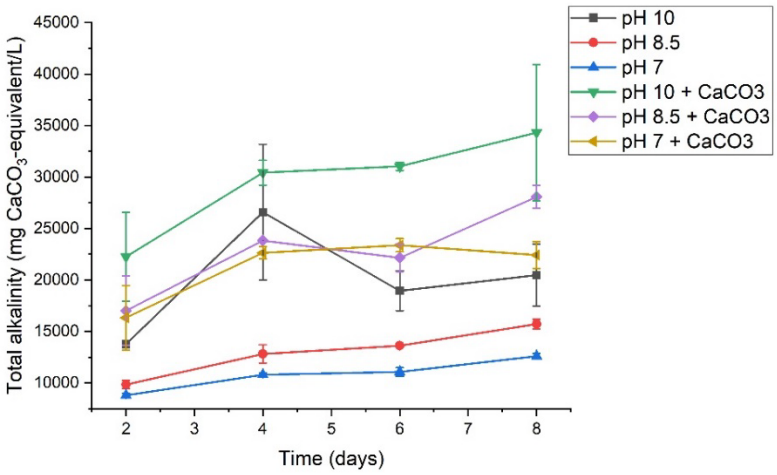
In contrast with previous studies, this experiment observed different phenomena in terms of relative microbial abundance. In the co-fermentation of waste activated sludge with corn gluten meal, an increase in soluble TAN led to a rise in the abundance of Firmicutes and Bacteroidota, while the populations of Proteobacteria and Actinobacteria decreased [53]. In another study using sewage sludge, the addition of urea to increase TAN led to a decrease in Proteobacteria matched with an increase of Firmicutes, Actinobacteria, Planctomycetes and Acidobacteria [20]. These differences from previous studies are most likely due to differences in substrate and inoculum composition.

3.6. CaCO3 addition – pH and alkalinity effect on SHF of ORS

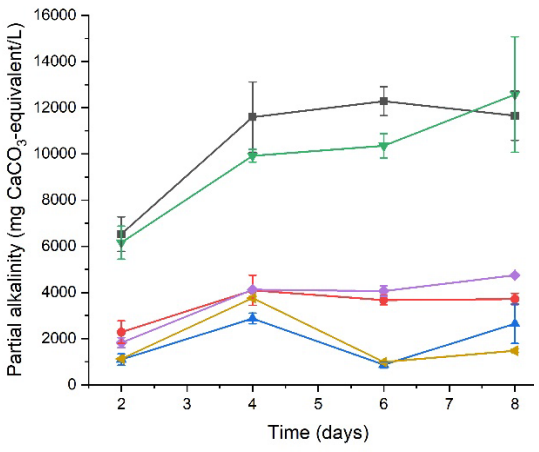
This experiment was designed to test the effect of pH (controlled by daily NaOH addition) and the presence of $CaCO_3$ (i.e. added alkalinity) on SHF of ORS.

After 24 hours of SHF, it was observed that the pH 10 and pH 10 + $CaCO_3$ replicates did not show a noticeable degree of liquefaction (as indicated by poor mixing when shaken), unlike the other replicates at lower pH which showed signs of disturbance when shaken. This indicated that enzyme activity was negatively affected by pH 10, and hydrolysis yield was insufficient for effective acidogenic fermentation. Some level of enzyme inhibition was expected, as typically the optimum pH for hydrolytic enzymes such as amylase and cellulase is slightly acidic (pH~5) [57,58].

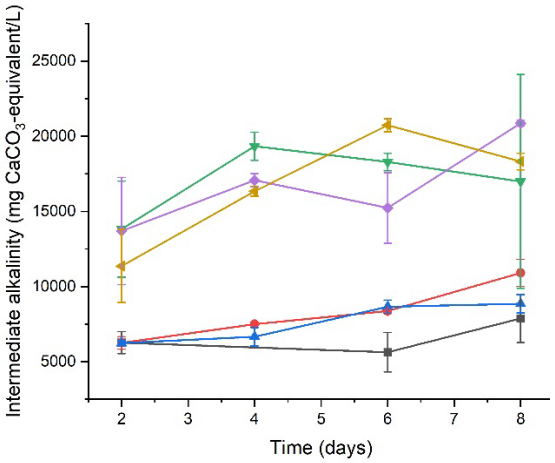
Figure 6 shows the alkalinity results for this experiment. As expected, the replicates with higher (total) alkalinity were pH 10 + $CaCO_3$, followed by the other replicates with $CacO_3$. Higher NaOH addition resulted in higher partial alkalinity and higher pH. The presence of $CaCO_3$ resulted in higher intermediate alkalinity for all pH tested. This is an indication that $CaCO_3$ might be useful as buffering agent for slightly acidic pH (~5.7). In contrast, NaOH addition seemed to be related to partial alkalinity values. Error bars for replicates pH 10 and pH 10 + $CaCO_3$ were relatively large. This might be attributed to poorer sample homogeneity due to the reduced degree of hydrolysis. However, a clear relationship between pH and alkalinity can be seen.



(a)



(b)

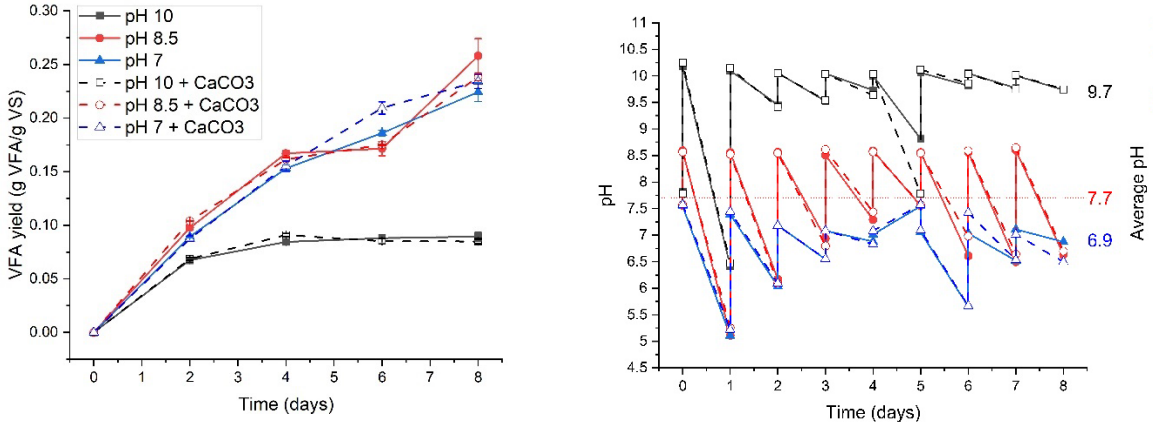


(c)

Figure 6. (a) Total; (b) partial and; (c) intermediate alkalinity for different controlled pH with and without $CaCO_3$ addition of SHF at 7.5% TS, S/I of 40 g TS g^{-1} TS, 35°C and 2% enzyme loading. Error bars represent the values of the two replicated used to calculate the average result

Figure 7, (a) shows the VFA yields from this experiment. The negative effect of pH 10 (with or without $CaCO_3$) on hydrolysis is reflected in the low yields achieved (~0.09 g VFA g^{-1} VS after 8 days). There are no major differences in yields for pH 7 and 8.5, whether $CaCO_3$ is present or not, with the exception of pH 7 + $CaCO_3$ on day 6, whose yield is higher than the rest. In one study, pH 8.5 was more favourable than pH 7 for VFA production from molasses-spent wash [59]. The addition of $CaCO_3$ showed a positive influence on VFA production, but the pH values tested were lower than in the current study (6-8.5) [59]. In the acidogenic fermentation of potato peel waste, the VFA production was considerably higher at neutral pH compared to pH 5 and 11, despite reaching similar levels of substrate solubilisation at pH 11 [11]. In a study looking at the effect of pH (4-11) on the co-fermentation of WAS and food waste, it was found that maximum VFA concentration of 8.24 g COD L^{-1} was achieved at pH 8 on day 4, closely followed by pH 9 [51]. The estimated yield based on initial experimental conditions was 0.5 g VFA g^{-1} VS. In the current study, maximum VFA yield of 0.26 g VFA g^{-1} VS was achieved on day 8 by pH 8.5 replicates, closely followed by the pH 8.5 + $CaCO_3$, pH 7 and pH 7 + $CaCO_3$ replicates (0.22-0.24 g VFA g^{-1} VS). This contrast with other results found in the literature highlights the influence of substrate characteristics over the fermentation performance. In another study looking at the effect of pH, temperature and retention time on the acidogenic fermentation of sewage sludge, it was found that increasing pH from 7 to 8.5 using fly wood ash had negligible or even detrimental effects on VFA yields under mesophilic conditions and retention times of 2-4 days [60]. While the majority of wood ash is reported to be comprised of $CaCO_3$ (23-57% depending on wood source [61]), some other alkaline components (carbonates and hydroxides [62]) contributed to pH modification and possibly, inhibition to acidogenic activity. In the fermentation of potato solid waste, using other alkaline substances such as $NaHCO_3$ to increase alkalinity to 3 g $CaCO_3$ L^{-1} (final pH 5.7-6.5) resulted in a marked increase in VFA yield; however the final pH of the reactors without added alkalinity was very low (3.9-4.2) [63]. Another study using $NaHCO_3$ and $KHCO_3$ to provide alkalinity to the fermentation of olive oil mill wastewater found that increasing alkalinity from 4 to 5 g $CaCO_3$ L^{-1} increased VFA production considerably, particularly at higher substrate concentrations [64]. However, increasing alkalinity further had a detrimental effect [64].

In the current work controlling pH was challenging, particularly during the first 2 days (see Figure 7, (b)). pH 10 replicates had a more stable pH after day 5, probably due to the fall in VFA production. The presence of $CaCO_3$ did not have a strong influence on pH. The average of all pH readings taken for each replicate was estimated, and the results are shown in the graph by the dotted horizontal lines. The average pH of pH 8.5 replicates was far from the design value, with an average of 7.7. This could explain the similarities in VFA yield for pH 7 and pH 8.5 replicates. It is likely that more significant differences would have been observed if pH was continuously monitored and controlled, but it was not possible in this work.



(**a**) (**b**)

Figure 7. (a) VFA yields and; (b) pH reading for different controlled pH with and without $CaCO_3$ addition of SHF at 7.5% TS, figure 2of 40 g TS g^{-1} TS, 35°C and 2% enzyme loading. Error bars represent the values of the two replicates used to calculate the average result.

Figure 8 shows the VFA profile on day 8 of the SHF. The conditions tested had a marked effect on VFA profile, with AcH being the predominant VFA in all cases (≥58%). Despite the similar VFA yields in pH 7 and pH 8.5 replicates, the 0.8 difference in average pH was still sufficient to affect the VFA profile. AcH proportion was benefitted by increased pH, with or without $CaCO_3$ addition. The opposite was observed for BuH proportion. Meanwhile PrH proportion was higher in pH 8.5 conditions (with or without $CaCO_3$). Similar VFA distributions were previously reported in the co-fermentation of WAS and food waste from pH 7 and above; however, AcH proportion was the highest at pH 4, though total VFA concentration at pH 4 was low (<1 g L^{-1}) [51]. It was noted that $CaCO_3$ supplementation showed a notable impact on VFA distribution from molasses-spent wash, leading to higher concentrations of BuH and AcH, and lower concentrations of PrH [59]. An increase in AcH and a decrease in PrH and BuH were also observed in the fermentation of potato solid waste when alkalinity was increased using $NaHCO_3$ [63]. However, the pH in that study was not monitored throughout the fermentation, and the changes in VFA distribution might be attributed to differences in pH. In the present study, CaCO3 supplementation had minimal impact for pH 8.5 and pH 10 conditions; however, the presence of $CaCO_3$ had a noticeable effect on pH 7 replicates. While the main acids were AcH, PrH and BuH for pH 7 with or without $CaCO_3$, the percentage of HexH was 6.6% higher for the replicates with $CaCO_3$. Considerable proportions of VaH were only observed in pH 7 replicates, with or without $CaCO_3$. Considerable proportions of i-VaH and i-BuH were observed in pH 10 and pH 10 + $CaCO_3$ replicates, whereas i-VaH and i-BuH were negligible in the other conditions tested.

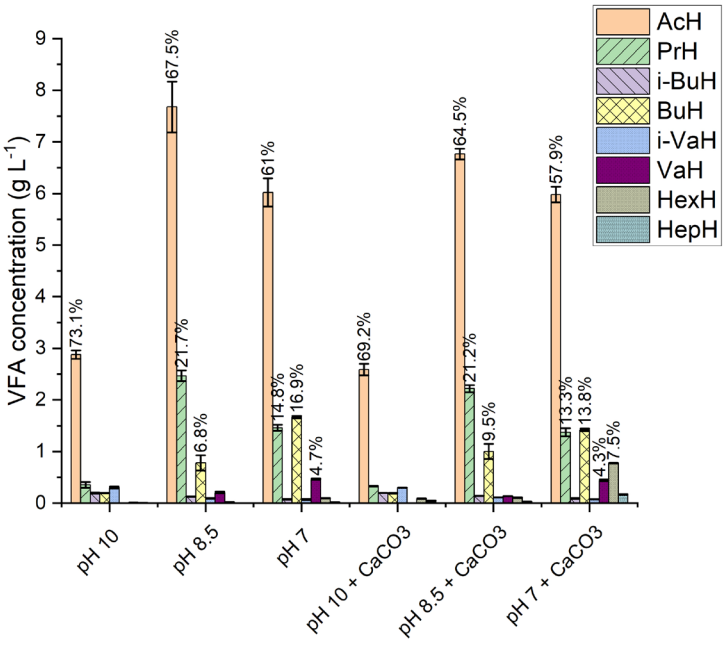


Figure 8. VFA profile on day 8 for different controlled pH with and without $CaCO_3$ addition of SHF at 7.5% TS, S/I of 40 g TS g^{-1} TS, 35°C and 2% enzyme loading. Percentages are the average of two replicates.

Figure 9 shows the degree of acidification (DoA) for each condition tested in this experiment. The DoA achieved by pH 10 replicates is low (<50%), indicating that pH 10 not only affected hydrolysis, but also fermentation. This could be a side effect of high solids content and low fluidity rather than a negative effect from high pH, as it widely reported in the literature that, while perhaps not an optimum, acidogens are not inhibited at pH 10 under mesophilic conditions [9]. In fact, clear signs of inhibition were only observed at pH 11 for short batch fermentations (<10 days) [65]. DoA for the other conditions reached 80-86% with no marked differences among them. This is a further indication that alkalinity modified by NaOH or $CaCO_3$ addition has no notable effect on VFA yields. In contrast, in the fermentation of potato solid waste differences in DoA were reported, with increased alkalinity using $NaHCO_3$ leading to increased DoA [63]. However, due to the low final pH, it is believed that lactic acid production took over from VFA production, though lactic acid concentrations were not reported.

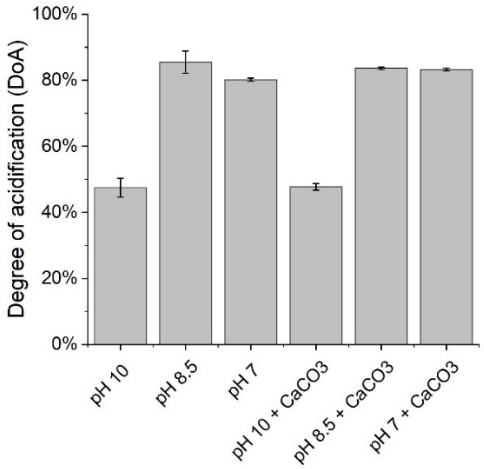


Figure 9. DoA values on day 8 for controlled pH with and without $CaCO_3$ addition of SHF at 7.5% TS, S/I of 40 g TS g^{-1} TS, 35°C and 2% enzyme loading. Error bars represent the values of the two replicates used to calculate the average result.

4. Conclusions

The findings of this study provide further insight into the effect of fermentation conditions such as pH, $CaCO_3$ content, and TAN content, and how they influence performance variables of VFA production such as yield and degree of acidification as well as microbial community composition.

This work demonstrated the importance of aligning fermentation conditions with optimal urea hydrolysis conditions. An acidic pH of 4.5 can inhibit urea hydrolysis, preventing the release of buffering ammonium cations. Additionally, in this work, a minimum of 3-4 days was required to achieve the maximum ammonia release from urea. Therefore, careful consideration must be taken when using urea as buffering agent and the pH and HRT conditions must be designed accordingly, preferably at around neutral pH (6-8) and with a minimum retention time of 3-4 days, depending on other fermentation conditions and substrate.

In contrast, when AF was undertaken within the optimum pH range for urea hydrolysis, the use of urea as buffering agent led to higher VFA yields compared to NaOH at the same pH values.

TAN concentration greatly affected the type of VFA produced and the microbial community composition, partly due to the change in pH and partly due to differences in nitrogen availability. It was found that around 2-3.8 g TAN L^{-1} was the optimum value for VFA production in SHF and 2.2-5.4 g TAN L^{-1} in fermentation alone.

Though previous studies have shown alkaline conditions to be favourable for VFA production, this work demonstrated that adding NaOH to increase pH 7 to 8.5 did not result in improved VFA yields from ORS. Further NaOH addition to reach pH 10 is highly unfavourable for SHF aiming at VFA production, due to hydrolysis inhibition.

In this study, $CaCO_3$ addition to increase alkalinity did not affect VFA yields and only had an impact on intermediate alkalinity; therefore, it was concluded that $CaCO_3$ is not effective as buffering agent when alkaline pH values are desired.

Author Contributions: Conceptualization, M.RS.; methodology, M.RS.; formal analysis, M.RS. and Y.Z.; writing—original draft preparation, M.RS.; writing—review and editing, M.RS. and S.H.; supervision, Y.Z. and S.H.; project administration, S.H.; funding acquisition, S.H. All authors have read and agreed to the published version of the manuscript.

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Appendix A

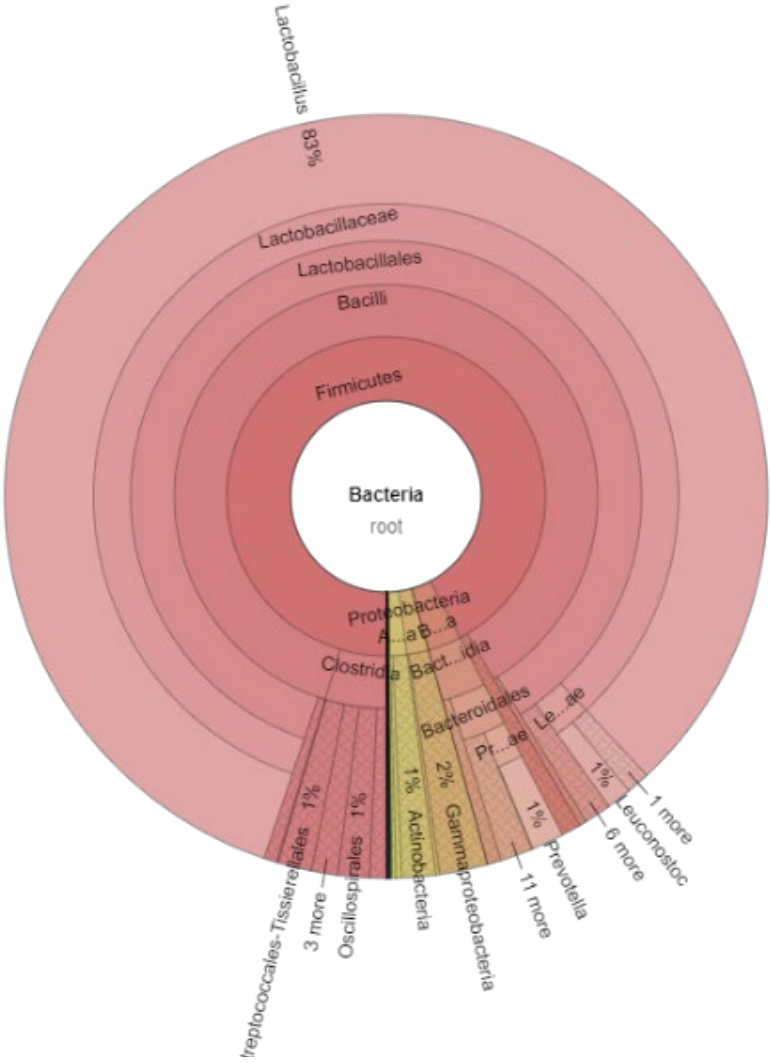

Figure A1. Microbial relative abundance for urea addition experiment part III, Control–Replicate 1

Figure A 2. Microbial relative abundance for urea addition experiment part III, Control–Replicate 2

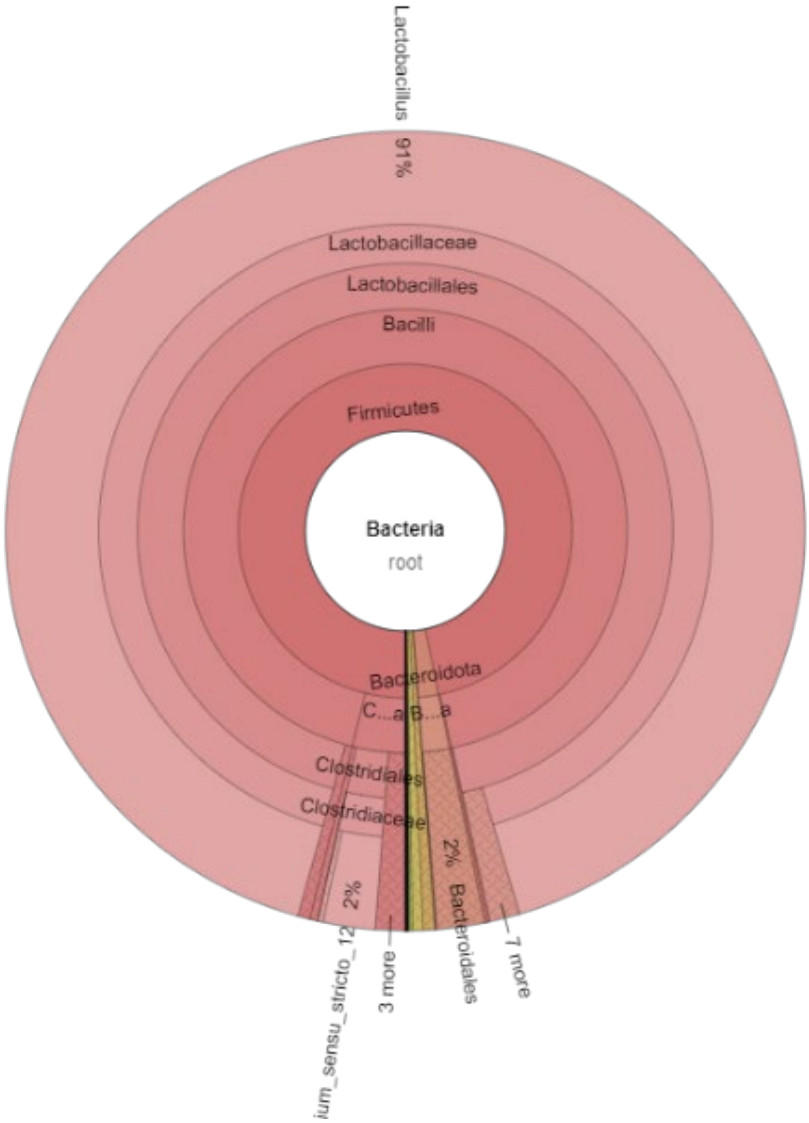

Figure A 3. Microbial relative abundance for urea addition experiment part III, A-pH equivalent–Replicate 1

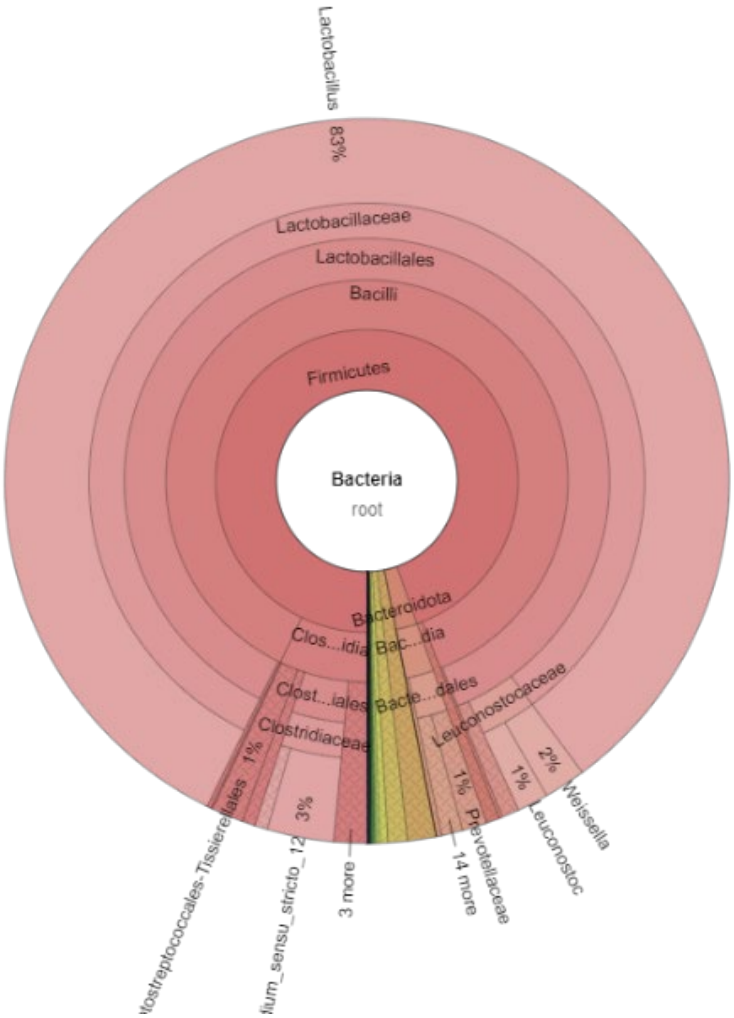

Figure A 4. Microbial relative abundance for urea addition experiment part III, A-pH equivalent–Replicate 2

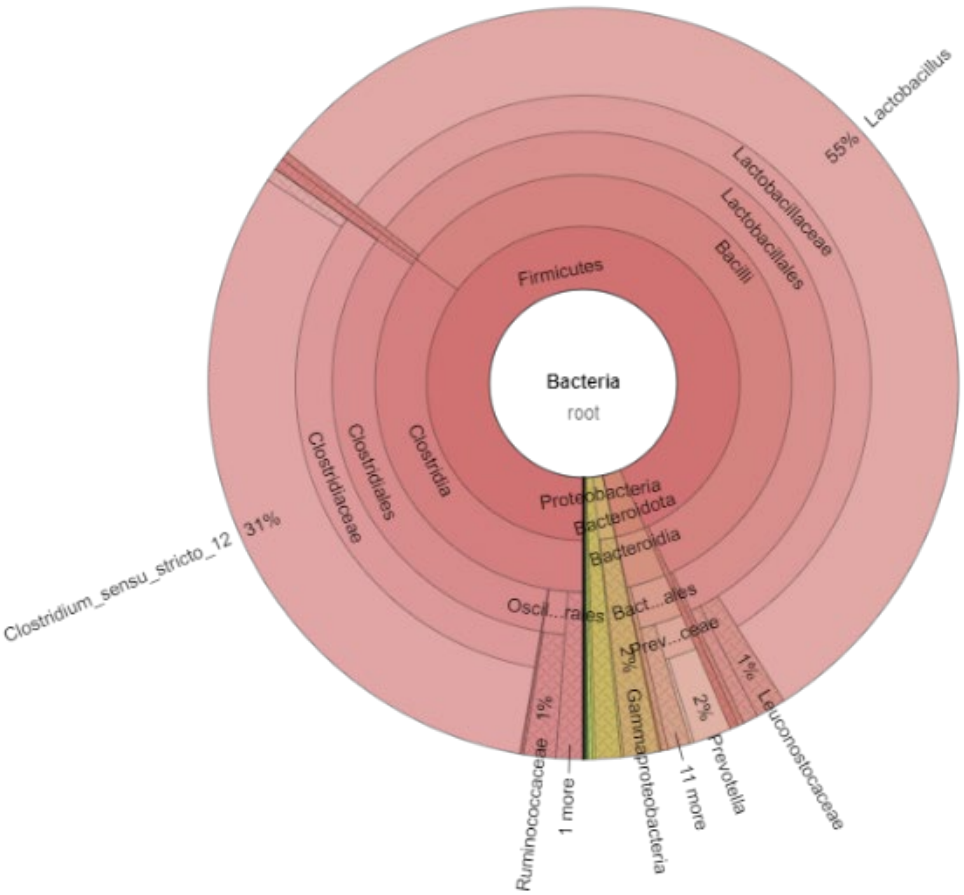

Figure A 5. Microbial relative abundance for urea addition experiment part III, A–Replicate 1

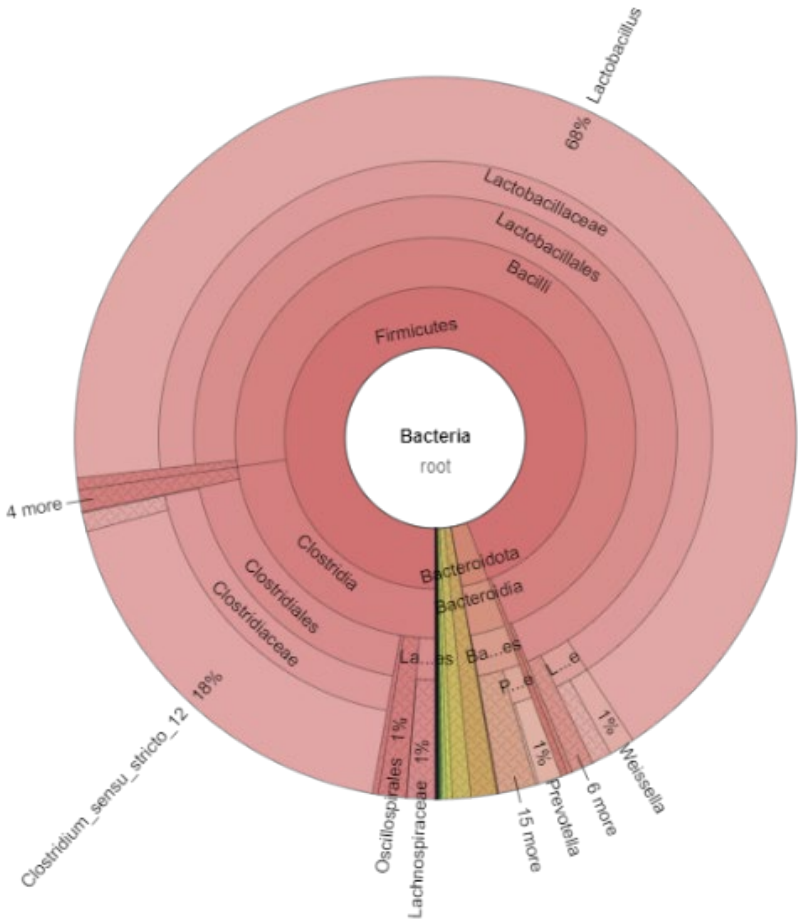

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Figure A 6. Microbial relative abundance for urea addition experiment part III, A-Replicate 2 739

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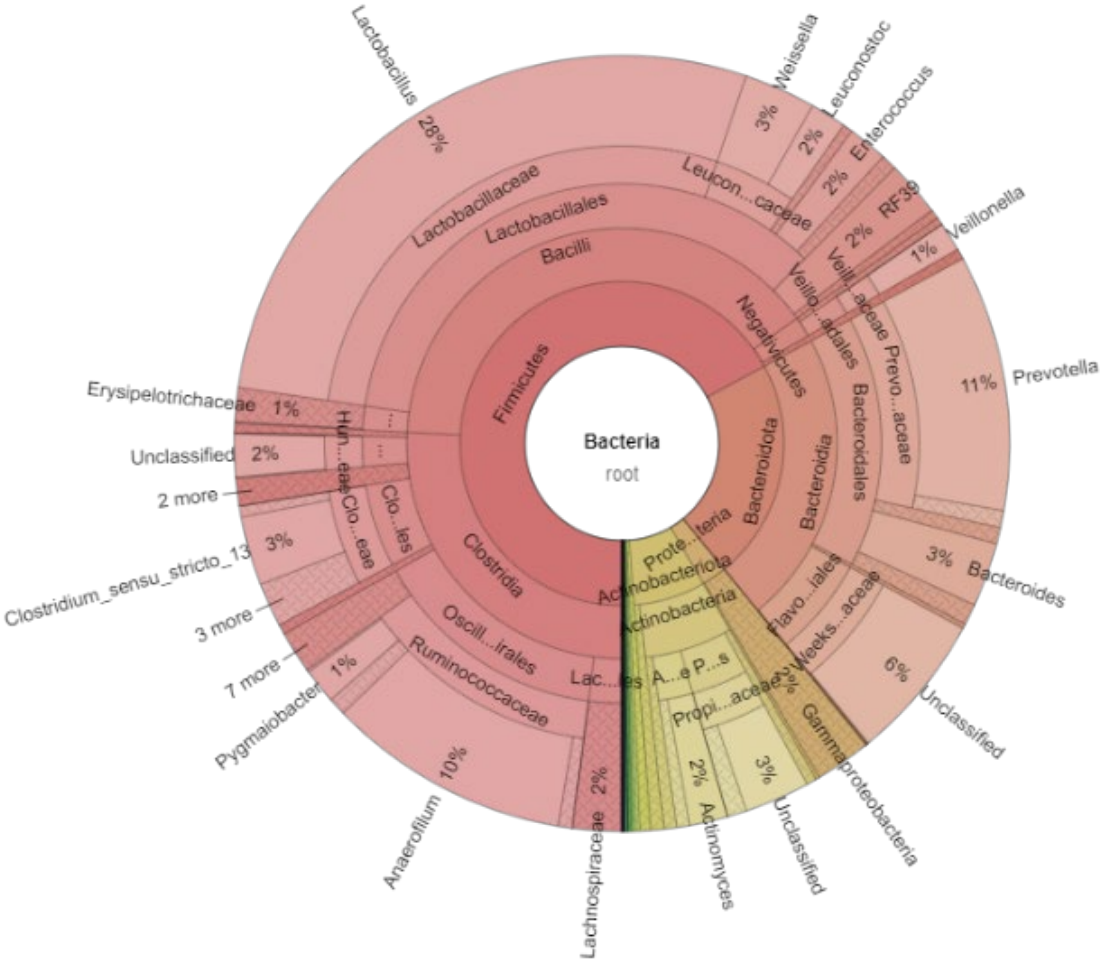

Figure A 7. Microbial relative abundance for urea addition experiment part III, B-pH equivalent–Replicate 1

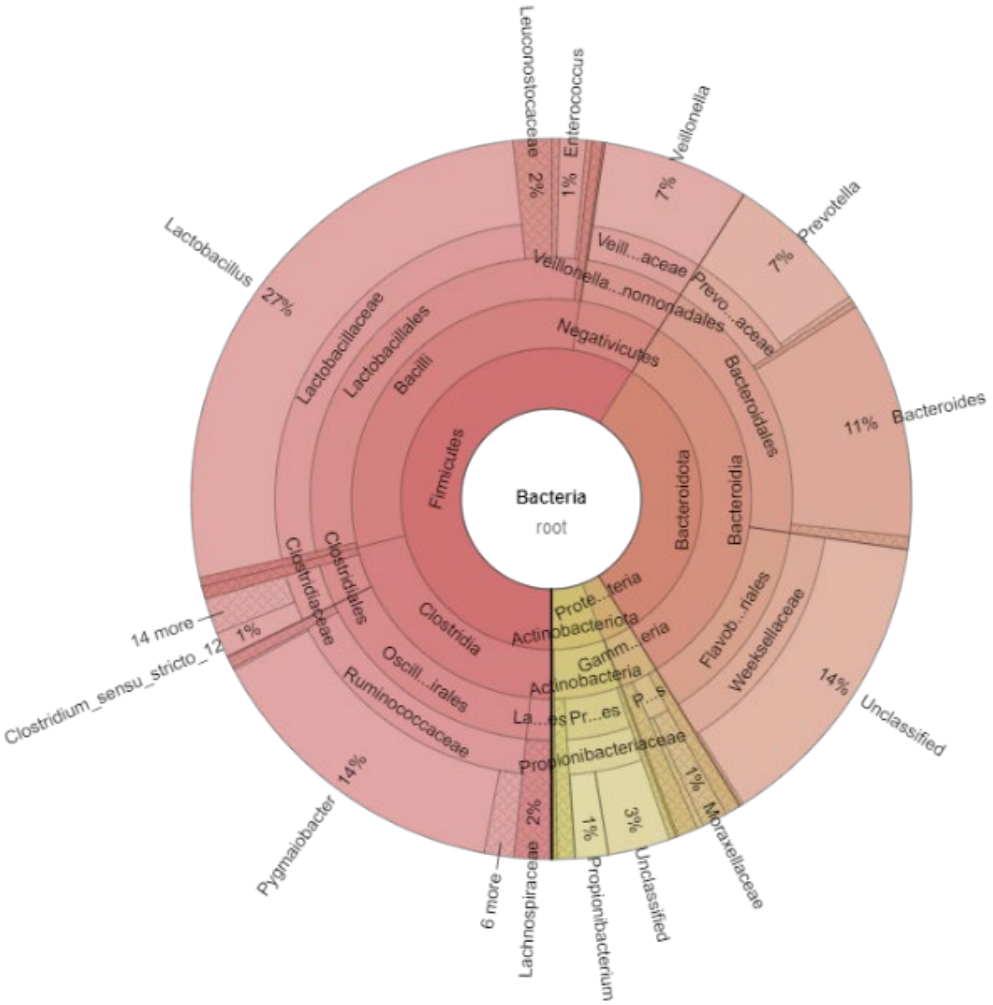

Figure A 8. Microbial relative abundance for urea addition experiment part III, B-pH equivalent–Replicate 2

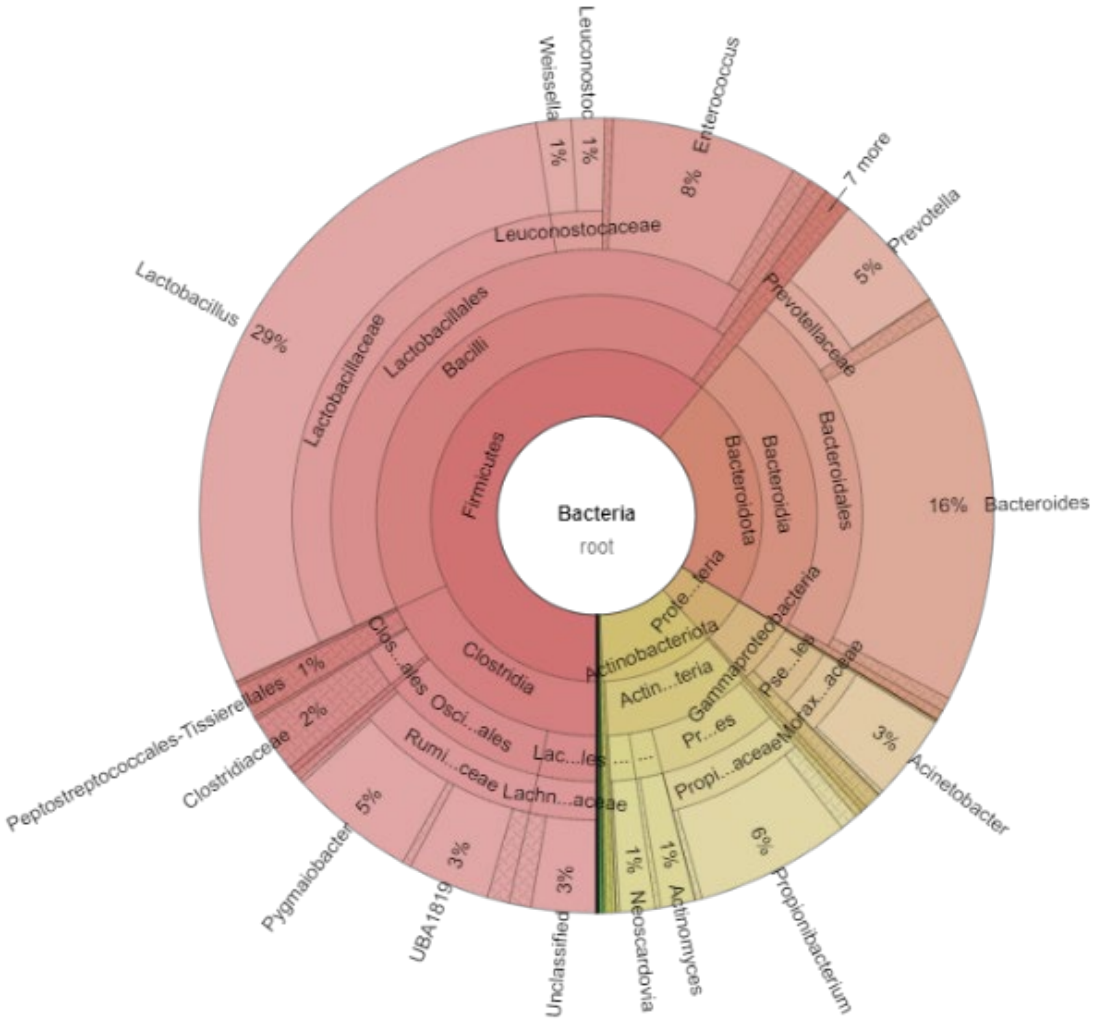

Figure A 9. Microbial relative abundance for urea addition experiment part III, B–Replicate 1

Figure A 10. Microbial relative abundance for urea addition experiment part III, B-Replicate 2

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Figure A 11. Microbial relative abundance for urea addition experiment part III, C-pH equivalent–Replicate 1 795 796

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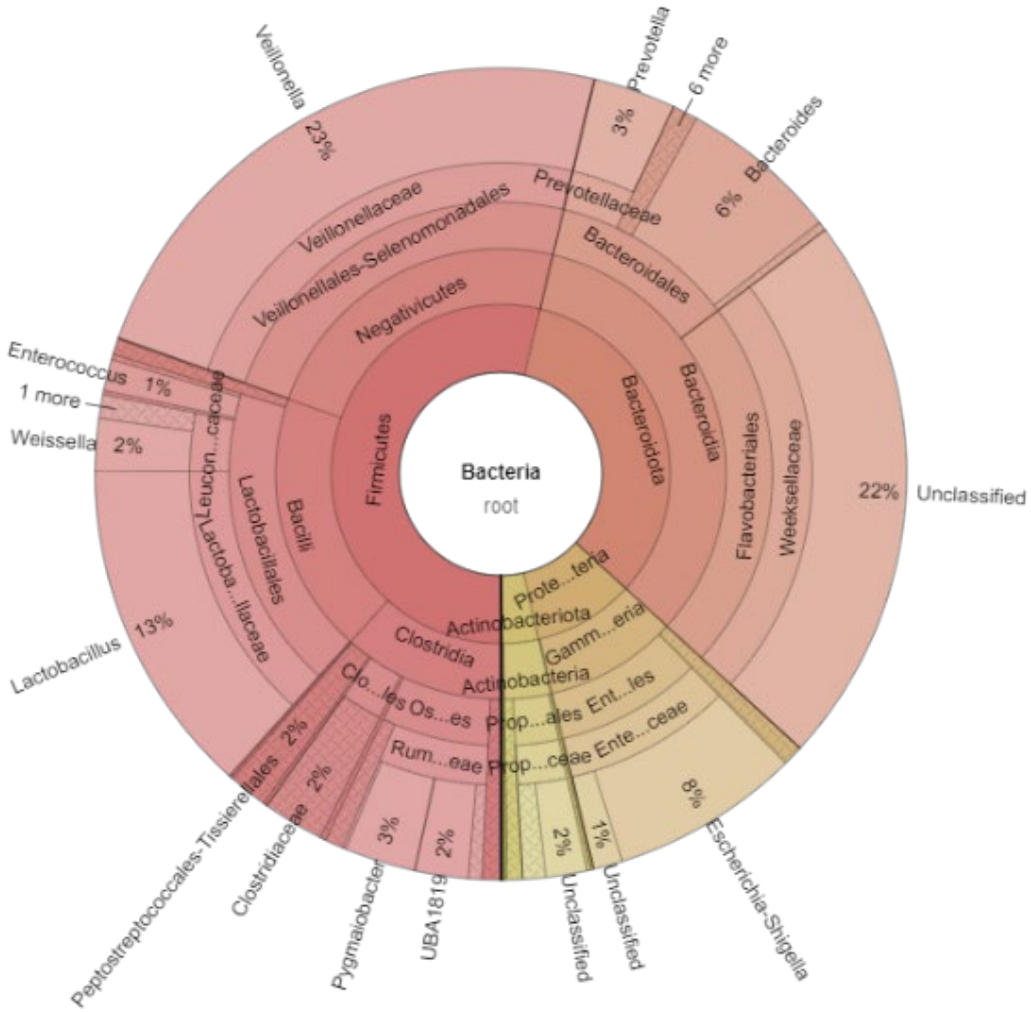

Figure A 12. Microbial relative abundance for urea addition experiment part III, C-pH equivalent–Replicate 2

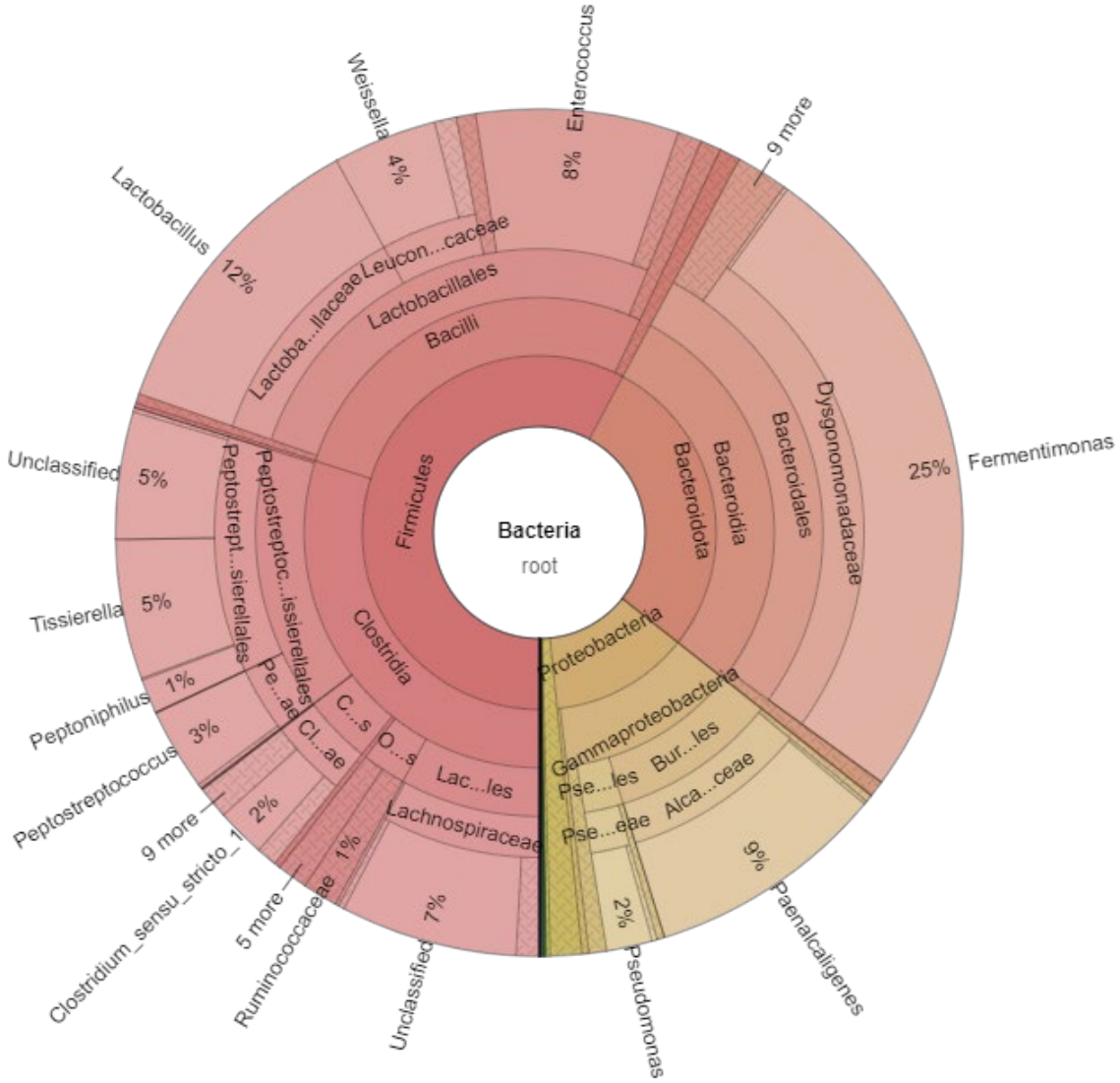

Figure A 13. Microbial relative abundance for urea addition experiment part III, C–Replicate 1

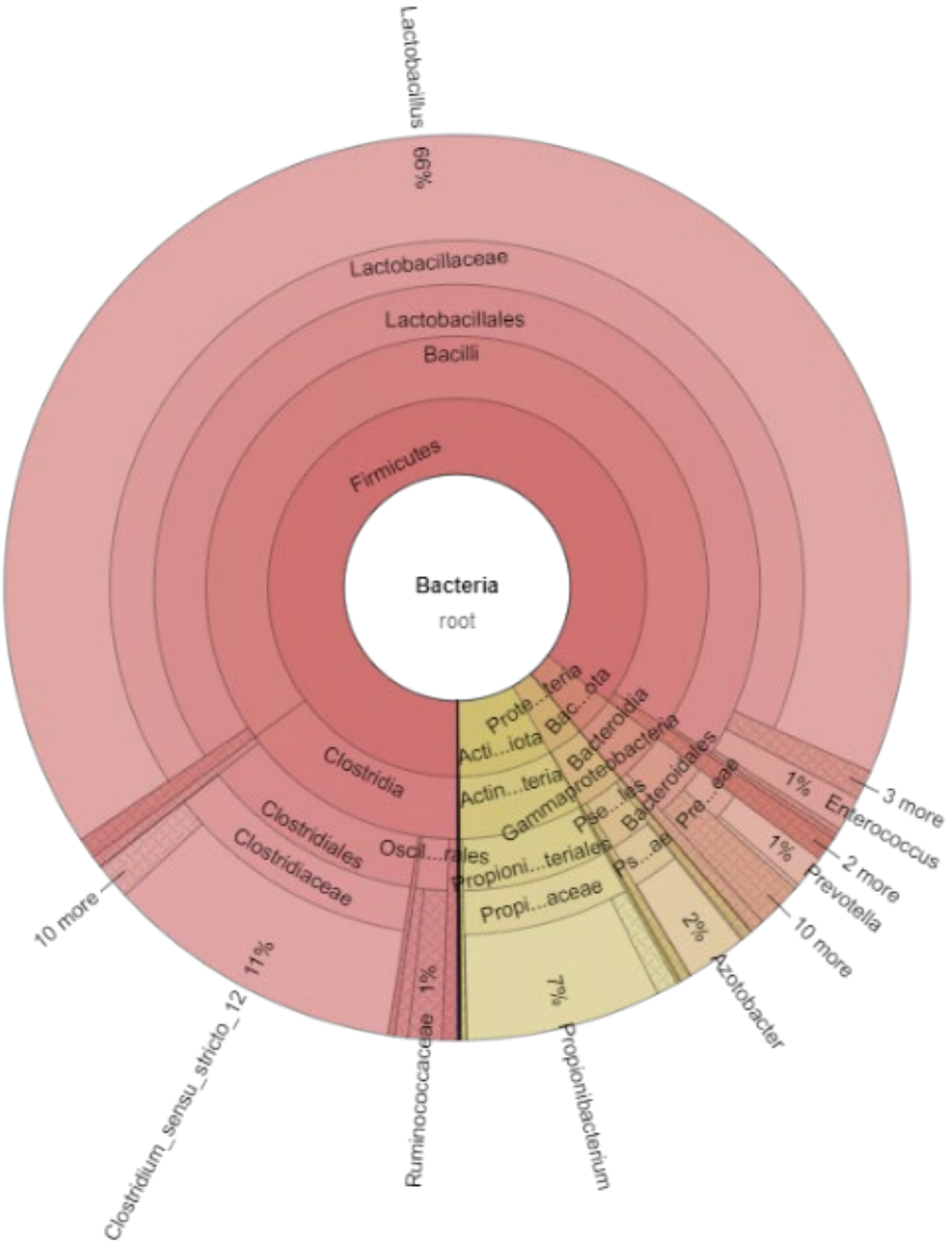

Figure A 14. Microbial relative abundance for urea addition experiment part III, C–replicate 2

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