Diverse profile of fermentation byproducts from thin stillage

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SUPPLEMENTAL RESULTS

Detailed community analysis

In the R1$_{TS}$ experiment, two highly abundant *Prevotella* 7-related OTUs, (AB818575.1.1466 and EU887831.1.1496) accounted for 27.7±13.4% and 9.6±5.4% of the 16S rRNA gene sequence reads per sample, respectively. *Prevotella* are common rumen inhabitants and have been shown to produce C4 from LA and carbohydrates (Esquivel-

Elizondo et al., 2017; Fraga et al., 2018). *Pediococcus* (MF436194.1.1509) was the most prominent *Lactobacillaceae* in the R1$_{\text{TS}}$ bioreactor at 13.3±7.0% relative abundance, whereas the abundance of other genera of this family was much lower. Like other heterofermentative *Lactobacillaceae,* pediococci produce LA and C2 from carbohydrates (Dicks & Endo, 2014; Zheng et al., 2020). Two *Lachnospiraceae* OTUs, *Acetitomaculum* (GU303559.1.1495) and *Lachnospiraceae* NK3A20 group (AB746782.1.1535), were present at approximately 3-4% relative abundance. *Acetitomaculum* are another typical rumen inhabitant noted for C2 production from carbohydrates (Greening & Leedle, 1989; Li et al., 2012; Rainey, 2011). Unique to the abundant OTUs in R1$_{\text{TS}}$ was an OTU related to *Dialister* (New.ReferenceOTU88), a genus of Clostridia which is noted for the ability to metabolize succinate and produce C3 (Morotomi et al., 2008; Wade, 2011), present at 2.7±2.4% relative abundance. Conversely, an *Acetobacter*-related OTU (FJ157231.1.1401), present at 3.6±2.1% in the R1$_{\text{TS}}$ community, was also present as an abundant microbial community member in all five bioreactors (Fig. 3). *Acetobacter* are common in fermented foods, but have also been observed in other mixed culture fermentations (Andersen et al., 2017). They are typically noted for the ability to produce C2 from carbohydrates and other carbon sources including glycerol, and subsequent mineralization of C2 (Cleenwerck et al., 2008; Cleenwerck et al., 2002; Komagata et al., 2014).

Shifts in the microbial community occurred when the solids were removed from TS in the R2$_{\text{SR-TS}}$ experiment, including the selection of an OTU related to *Furfurilactobacillus* (AJ564009.1.1368), a heterolactic fermenter, at 18.9±4.7% relative abundance (Fig. 3). A second lactobacillus that was enriched to a lower abundance (6.4±2.5% relative abundance) in the R2$_{\text{SR-TS}}$ reactor was related to *Lacticaseibacillus* (JN561696.1.1371), a homolactic fermenter. Both lactobacilli have been isolated from diverse substrates such as cereal grain fermentations and corn liquor (Zheng et al., 2020). The *Lachnospiraceae* NK3A20 group (AB746782.1.1535) OTU that was present in R1$_{\mathrm{TS}}$ was also abundant in R2$_{\mathrm{SR\text{-}TS}}$, at 10.6±4.2% abundance. Additional *Clostridia* including OTUs related to *Pseudoramibacter* (AB036759.1.1480), which are known or predicted to produce MCFA from carbohydrates (Holdeman et al., 1967), glycerol and LA (Scarborough et al., 2020), and related to the [*Eubacterium*] *nodatum* group (GQ458236.1.1465) were abundant under the R2$_{\mathrm{SR\text{-}TS}}$ reactor conditions at 8.4±8.1% and 3.1±3.1%, respectively. Two *Prevotella* 7 OTUs (EU887831.1.1496 and AB818575.1.1466), initially observed in R1$_{\mathrm{TS}}$, decreased in relative abundance, to 3.7±1.5% and 11.8±5.2%, respectively.

Decreasing the SRT to 1 day in R3$_{\mathrm{LowSRT}}$ resulted in the washout of the *Lachnospiraceae*-related OTUs, but other OTUs from the *Clostridia* remained abundant in the microbial community, such as *Pseudoramibacter* (AB036759.1.1480) and [*Eubacterium*] *nodatum* group (GQ458236.1.1465) at 8.7±4.5% and 1.8±1.3% relative abundance, respectively (Fig. 3). Additionally, *Pediococcus* (MF436194.1.1509) remained a prominent OTU in the microbial community at 12.4±5.1% relative abundance. *Companilactobacillus* species are a frequent presence in food fermenting communities (Zheng et al., 2020), and the two related OTUs (AJ496791.1.1570 and AJ417738.1.1567) from R3$_{\mathrm{LowSRT}}$ maintained a consistent low-level abundance at 1.2±0.7% and 2.0±1.2%, respectively. Conversely, *Furfurilactobacillus* (AJ564009.1.1368) was the most abundant lactobacillus in the community, at 22.5±4.7%. The *Prevotella* 7 OTUs further decreased in abundance to ca. 10% (AB818575.1.1466) and 3% (EU887831.1.1496).

Operating the bioreactor at thermophilic temperatures (55 °C) had a pronounced effect on the composition of the microbial community in $R4_{T\text{-}pH}$. *Prevotella* OTUs were absent from the abundant members of the microbial community and *Clostridiales* OTUs were reduced in relative abundance as compared to the reactors operating at mesophilic temperatures (Fig. 3). Instead, *Acetobacter* OTUs accounted for approximately 35% of the reads in $R4_{T\text{-}pH}$, and a single OTU (AJ419838.1.1440) dominated at 29.7±6.0% relative abundance. The second most abundant OTU corresponded to *Furfurilactobacillus*, while other lactobacilli were present at lower abundances. In addition, a *Bacillus* OTU (KP297896.1.1456) was enriched in $R4_{T\text{-}pH}$, with 6.8±1.5% relative abundance.

The $R5_{T\text{-}pH\text{-}LowSRT}$ microbial community had only 11, the fewest number of, OTUs greater than 1% relative abundance among all the bioreactors. In general, the prominent OTUs in the $R5_{T\text{-}pH\text{-}LowSRT}$ bioreactor were similar to those observed in $R4_{T\text{-}pH}$, corresponding to a microbial community dominated by *Acetobacter* (AJ419838.1.1440, 28.7±6.1%) with a few lesser abundant *Lactobacillaceae*-related OTUs (Fig. 3). Two additional *Lactobacillaceae* OTUs, *Lactiplantibacillus* (JN043518.1.1483) at 11.5±5.1% relative abundance, and *Levilactobacillus* (JF763842.1.1470), a lactobacillus associated with beer-spoilage (Zheng et al., 2020), was present at 4.8±3.9%.

SUPPLEMENTAL FIGURES

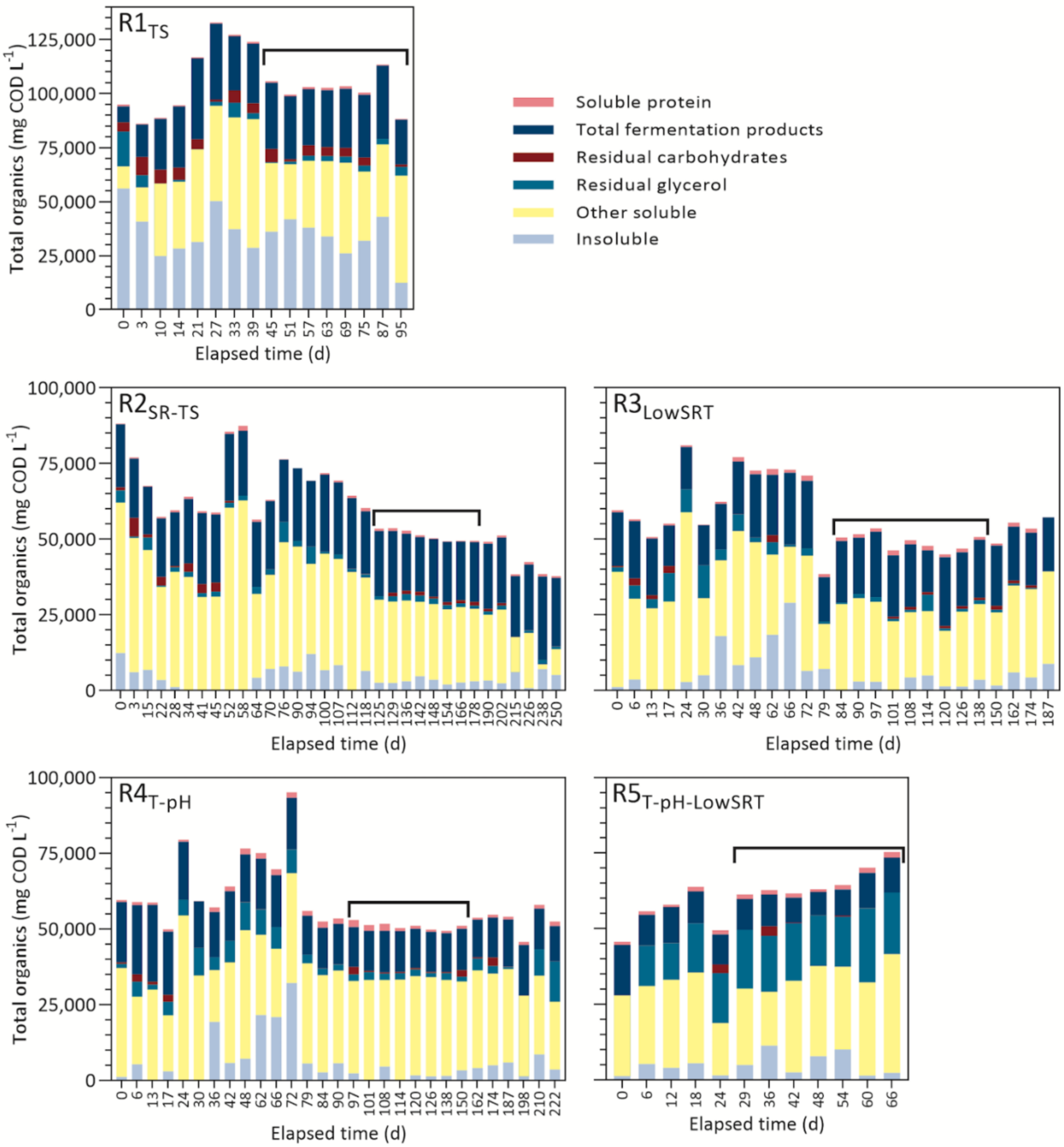

Figure S1. Dynamics of bioreactors ran under different operating conditions. $\text{R3}_{\text{LowSRT}}$ operated at a 1 d SRT starting at 48 d. $\text{R4}_{\text{T-pH}}$ was operated at 55°C starting at 24 d, pH was reduced to 4.5 by 54 d, and increased to 5.0 by 120 d. $\text{R5}_{\text{T-pH-LowSRT}}$ was initially at thermophilic temperatures and operated at a 1 d SRT at 19 d. Insoluble portion of the total organics represents the difference between total and soluble COD of the effluent and can be inferred to be accumulated microbial biomass, with the exception of $R1_{TS}$ which also contains influent-derived solids. Other soluble COD represents the remaining measured COD that was uncharacterized by other analytical methods plus metabolites detected at trace concentrations. Total carbohydrates are the sum of unconsumed glucose, xylose, and cellobiose remaining in the effluent. Total fermentation products represent the sum of all carboxylic acids and ethanol. Brackets mark a window of approximately 50 d during which bioreactor operation was more stable following the initial dynamic phase.

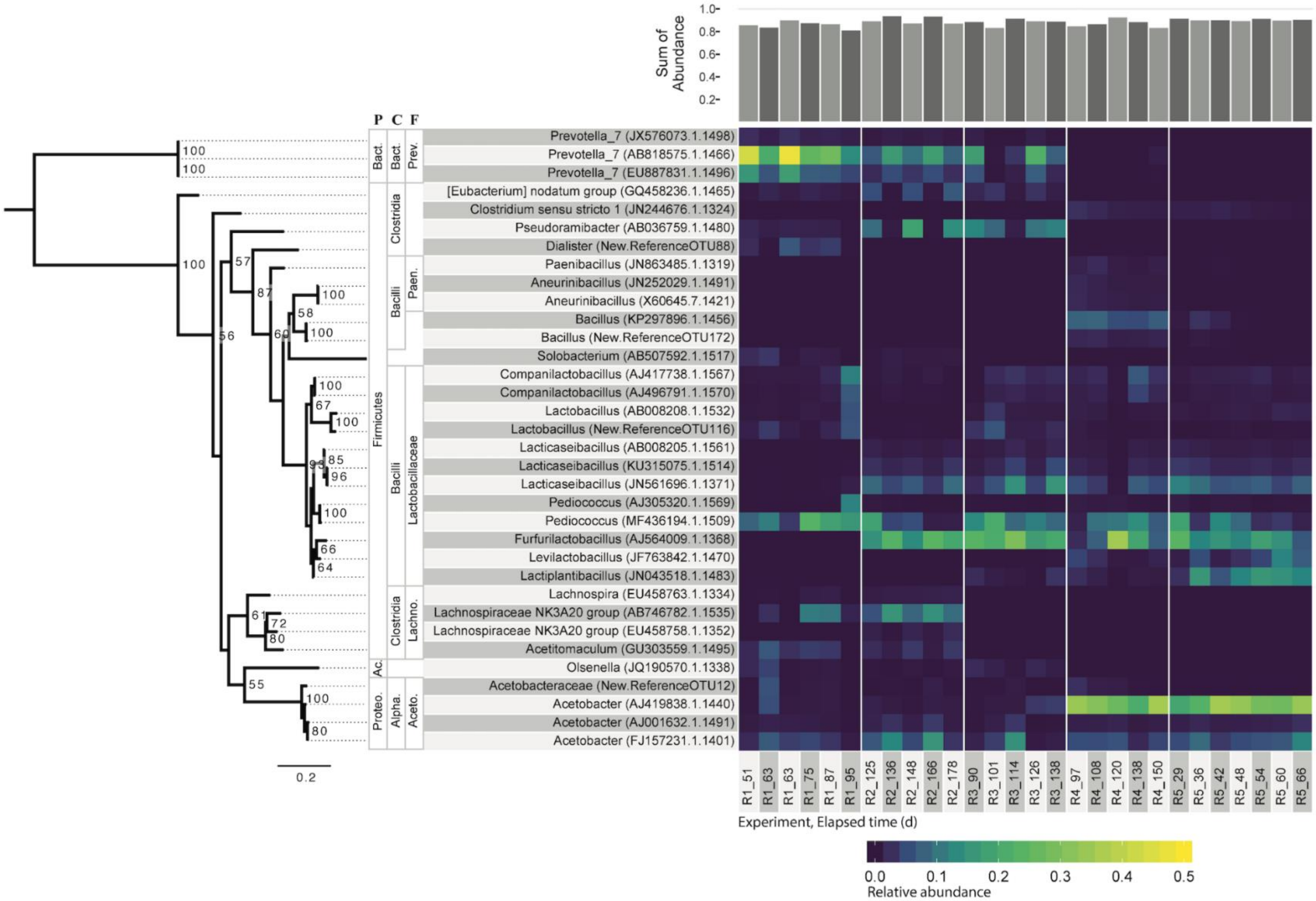
Sum of Abundance
1.0
0.8
0.6
0.4
0.2
P
C
F
Bact.
Bact.
Prev.
Firmicutes
Clostridia
Bacilli
Paen.
Bacilli
Lactobacillaceae
Clostridia
Lachno.
Ac.
Proteo.
Alpha.
Aceto.
Prevotella_7 (JX576073.1.1498)
Prevotella_7 (AB818575.1.1466)
Prevotella_7 (EU887831.1.1496)
[Eubacterium] nodatum group (GQ458236.1.1465)
Clostridium sensu stricto 1 (JN244676.1.1324)
Pseudoramibacter (AB036759.1.1480)
Dialister (New.ReferenceOTU88)
Paenibacillus (JN863485.1.1319)
Aneurinibacillus (JN252029.1.1491)
Aneurinibacillus (X60645.7.1421)
Bacillus (KP297896.1.1456)
Bacillus (New.ReferenceOTU172)
Solobacterium (AB507592.1.1517)
Companilactobacillus (AJ417738.1.1567)
Companilactobacillus (AJ496791.1.1570)
Lactobacillus (AB008208.1.1532)
Lactobacillus (New.ReferenceOTU116)
Lacticaseibacillus (AB008205.1.1561)
Lacticaseibacillus (KU315075.1.1514)
Lacticaseibacillus (JN561696.1.1371)
Pediococcus (AJ305320.1.1569)
Pediococcus (MF436194.1.1509)
Furfurilactobacillus (AJ564009.1.1368)
Levilactobacillus (JF763842.1.1470)
Lactiplantibacillus (JN043518.1.1483)
Lachnospira (EU458763.1.1334)
Lachnospiraceae NK3A20 group (AB746782.1.1535)
Lachnospiraceae NK3A20 group (EU458758.1.1352)
Acetitomaculum (GU303559.1.1495)
Olsenella (JQ190570.1.1338)
Acetobacteraceae (New.ReferenceOTU12)
Acetobacter (AJ419838.1.1440)
Acetobacter (AJ001632.1.1491)
Acetobacter (FJ157231.1.1401)
100
100
100
57
87
56
60
58
100
100
100
67
100
93
85
96
100
66
64
61
72
80
55
100
80
0.2
R1_51
R1_63
R1_63
R1_75
R1_87
R1_95
R2_125
R2_136
R2_148
R2_166
R2_178
R3_90
R3_101
R3_114
R3_126
R3_138
R4_97
R4_108
R4_120
R4_138
R4_150
R5_29
R5_36
R5_42
R5_48
R5_54
R5_60
R5_66
Experiment, Elapsed time (d)
0.0
0.1
0.2
0.3
0.4
0.5
Relative abundance

Figure S2. OTUs with greater than 1% average relative abundance in the microbial community during periods of stable reactor conditions (ca. 50 d) for five different bioreactor operational conditions: R1, $R1_{TS}$; R2, $R2_{SR\text{-}TS}$; R3, $R3_{LowSRT}$; R4, $R4_{T\text{-}pH}$; R5, $R5_{T\text{-}pH\text{-}LowSRT}$. Taxonomy was assigned by aligning representative sequences to the SILVA Database (version 138.1). OTU IDs, in parenthesis, are represented by accession numbers where alignment to the database was greater than 99% or were otherwise assigned *de novo*. Phylogenetic tree is rooted in the OTUs representing the genus *Prevotella*. Bootstrap values greater than 50 are shown. Scalebar indicates the number of nucleotide substitutions per sequence site. Phyla abbreviations: Bact., *Bacteroidetes*; Ac., *Actinobacteria*; Proteo., *Proteobacteria*. Class (C) abbreviations: Bact., *Bacteroidia*; Alpha., *Alphaproteobacteria*. Family (F) abbreviations: Prev., *Prevotellaceae*; Paen., *Paenibacillaceae*; Lachno., *Lachnospiraceae*; Aceto., *Acetobacteriaceae.*

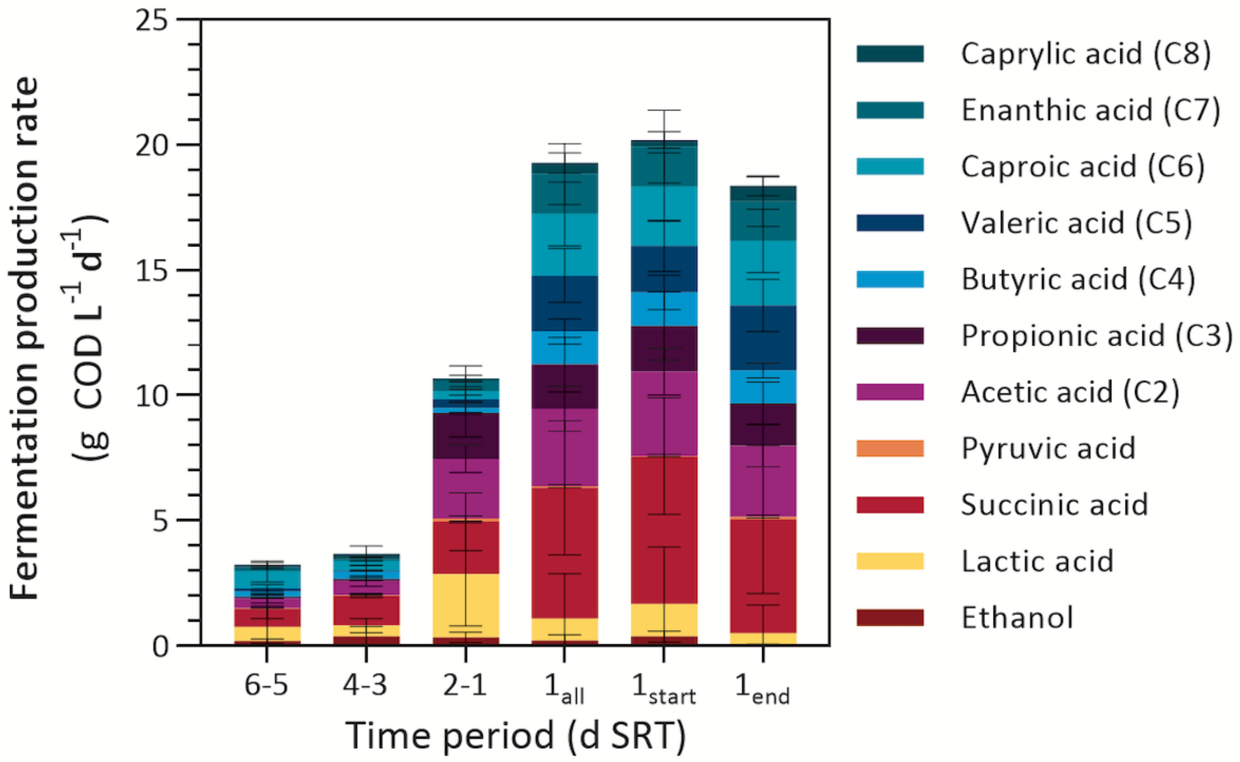

Figure S3. Fermentation product formation rate in $R3_{LowSRT}$ from a 6 d SRT to a 1 d SRT, averaged across three samples per time period. The production rate during the 1 d SRT phase was averaged across all samples at this retention time (*n*=18), the first half of the 1 d SRT phase (*n*=9), and last half of 1 d SRT operation (*n*=9).

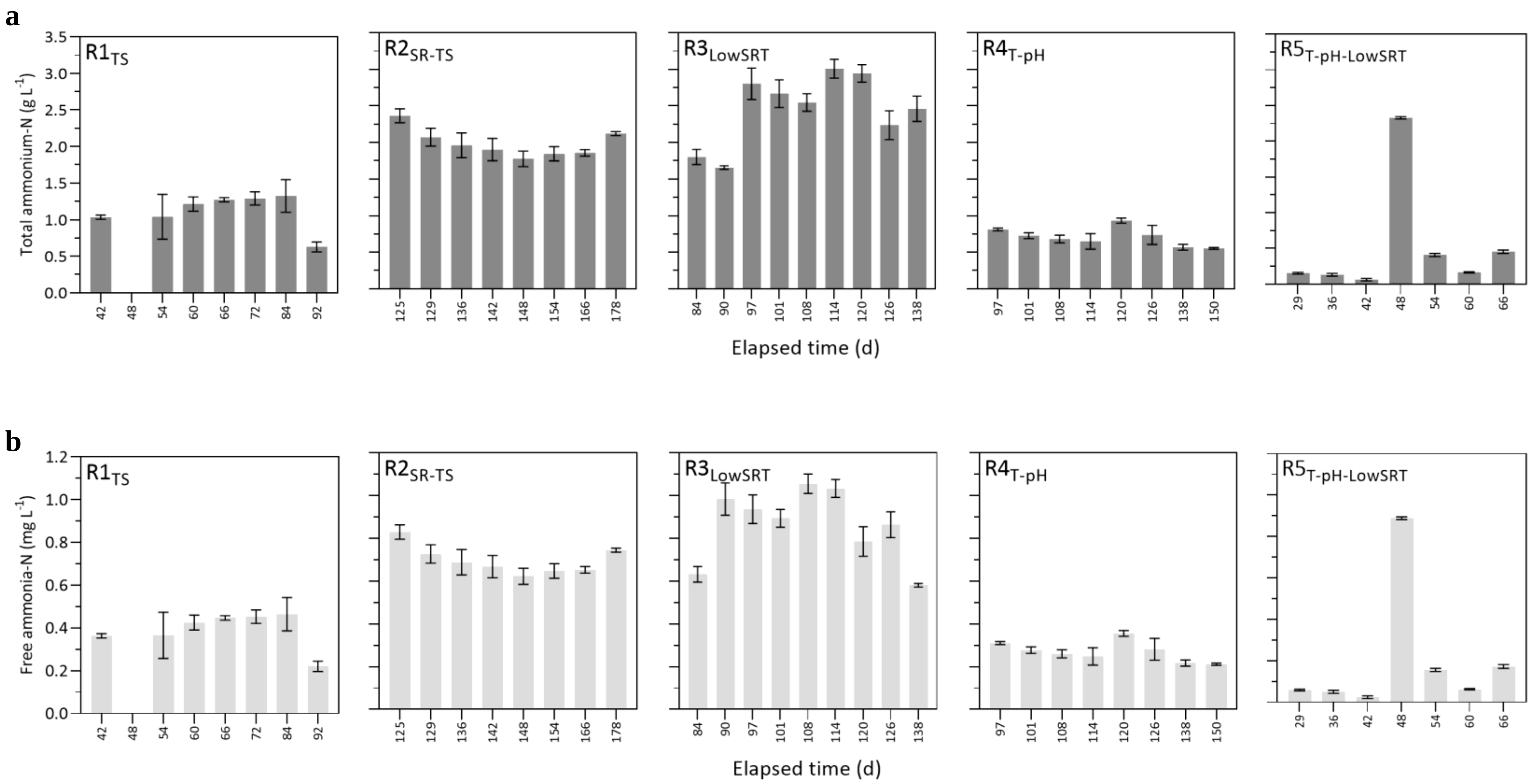

Figure S4. Total ammonium nitrogen (TAN) concentration (a; sum of NH_4^+-N and NH_3-N), and free ammonia nitrogen (FAN) concentration (b; ideal equilibrium calculation) during indicated periods of reactor stability. Note the difference in units between the two figures.

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