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ACIDOGENIC FERMENTATION OF WASTEWATER: INFLUENCE OF OPERATIONAL CONDITIONS AND ORGANIC COMPOSITION OF THE SUBSTRATE

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PhD Thesis

Doctoral program in Environmental Science and Technology

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Bellaterra, Cerdanyola del Vallès

September 2023

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MARÍA EUGENIA SUÁREZ OJEDA, Professora Agregada del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

CERTIFIQUEM:

Que l'enginyera química Ana Vázquez Fernández ha realitzat sota la nostra direcció, el treball que amb títol "ACIDOGENIC FERMENTATION OF WASTEWATER: INFLUENCE OF OPERATIONAL CONDITIONS AND ORGANIC COMPOSITION OF THE SUBSTRATE" es presenta en aquesta memòria, i que constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

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Bellaterra, 1 de setembre de 2023

Dr. Julián Carrera Muyo

Dra. María Eugenia Suárez Ojeda

"The proper use of science is not to conquer nature

but to live in it."

Barry Commoner

Agradecimientos

Todavía no me acabo de creer que esté escribiendo los agradecimientos de la tesis. Ha sido un camino más difícil de lo que me imaginaba. Sin embargo, me ha permitido compartir tiempo con muchas (pero muchísimas) personas, con las que me he sentido arropada siempre y de las cuales también me llevo recuerdos geniales que espero no se borren de mi memoria de pez.

En primer lugar, tengo que dar las gracias a mis directores de tesis. Maria Eugenia, gracias por haber confiado en mí y darme esta oportunidad. Julián, gracias por estar siempre disponible para cualquier duda y darle las vueltas que hiciese falta hasta que estuviese todo claro. A los dos, gracias especialmente por el apoyo estos últimos meses.

Por supuesto, esto no sería posible sin mis compañeros de planta piloto. Cuidar de un reactor con aguas residuales durante semanas/meses y todo lo que eso conlleva, creo que genera un vínculo incondicional con tus compañeros de lab. Congcong, you are probably one of the kindest people I ever met. I cannot count all the beautiful and crazy things we lived together, and I miss so much to have you as neighbour! You are a truly friend, thanks always for your support during your time in Barcelona and now from Beijing. MengQi, my crazy grandaugher (with shared parents), I still remember the first time I saw you in the corridor and you gave me a cat figure (How did you know?). Less than a year later, we started being roomates. Thanks for the infinite time we shared and for everything you've done for me. Marina, gracias por tu generosidad siempre, por acogerme en tu casa alguna que otra vez y por los miles de stickers. Me has ayudado a desconectar en los momentos de más estrés con tus historias y ocurrencias. Tenemos un viaje pendiente. Lluc, gracias por ser una persona con la que da gusto trabajar: siempre con una sonrisa y dispuesto a ayudar. Además de un fenómeno que monta reactores de la nada y una enciclopedia de las WWTP. Era genial coincidir contigo en el lab y escuchar tus onomatopeyas de fondo. Oscar, compañero de lab y de despacho y nuestro ¿community manager?, gracias por hacernos creer siempre que llegaremos lejos. Me alegro mucho de todas las cosas que están por llegar en tu vida. Javi, la persona que empezó conmigo desde el día 1 y con la que probablemente haya pasado más tiempo en el lab. Gracias por ser nuestro 'jefe' de planta piloto e implicarte tanto con los demás, te he echado mucho de menos. Xènia, mi maestra en planta piloto y personal trainer, gracias por todo lo que me has enseñado, dentro y fuera del lab y por haber estado (y seguir estando) siempre para escucharme. Aina, la persona más resolutiva y 'chill' del mundo, gracias por tus consejos siempre y por todo lo compartido dentro y fuera de la uni. Oriol Carbó, gracias por los mil debates sobre los BMPs y el buen rollo siempre. Laura y Carolina, que pena no haber compartido tiempo de lab con vosotras. Laura, muchas gracias por tu energía radiante siempre, no tengo duda que te irá genial. Carolina, gracias por todas esas conversaciones sobre los MCCA, me has ayudado mucho. ¡Te deseo muchos éxitos!

Gracias también por supuesto a todo el resto de miembros de GENOCOV, incluyendo profesores y estudiantes (tanto los que continúan como los que se han ido), por todo lo compartido en los laboratorios y en los seminarios, pero también en calçotadas, comidas

internacionales, xantar galego o el ecoSTP. En especial, gracias a Juan Baeza, por tu ayuda en el laboratorio, sobre todo con las conexiones eléctricas del PLC, aún sin ser tu doctoranda. Gracias a mis compañeras cuidadoras del GC: Pili, Zainab y Mariella. Pili, gracias por ser mi diseñadora gráfica, mi agenda de seminarios y por tantos momentos divertidos, tanto en la uni como fuera, incluyendo todas tus celebraciones multitudinarias o los audios "cantando" clásicos durante el confinamiento. Zainab, gracias por ser mi fiel compañera cuando la uni estaba desierta. Mariella, contigo me he reído muchísimo, espero que algún día te animes a venir conmigo a teatro. Hablando del GC, quiero agradecer a Mireia Baeza, por estar siempre pendiente de ayudarnos con el equipo y sus múltiples problemas. Pía, una parte de esta tesis sin duda es gracias a ti, gracias por todas tus lecciones de bioestadística desde el otro lado del charco, por tus audios saludándome (hola Pía!) y por tu risa contagiosa. Lorena, gracias por ofrecerte a ayudarnos y escucharnos siempre. Chiara, gracias por cantarme desde la otra punta del pasillo, era genial tenerte por aquí. Manu, compatriota e o mellor anfitrión do planeta, graciñas por ser como es, estar contigo é sinónimo de pásalo ben. Gracias a Dani González por tantas invitaciones a cafés y ser tan majo siempre. Thanks to all the team Lunch (the ones already mentioned and Serena, Pietro, Alex Ricoveri, Àlex Gaona, Eric and Francesco) for the time shared under the sun during what was for me the best moment of the day.

También quiero agradecer por supuesto a los técnicos del DEQBA: Rosi, Manuel y Pili por su ayuda siempre que lo necesité. Rosi, gracias por la ayuda incalculable durante las prácticas de EEQII y por tantas y tantas cosas en las que me has salvado la vida con mis experimentos. Me pasaría la mañana charlando contigo. Manuel, muchas gracias por tu ayuda y tanta paciencia con el cromatógrafo de gases, con el que tanto te di la tabarra. Pili, gracias por hacer que el laboratorio general sea un sitio más ordenado. Gracias a Toni Casablancas, de Planta Pilot de Fermentació por supervisarme tantas veces con la caldera y el autoclave grande. Gracias también al personal de mantenimiento, que tantas veces me ayudo con las conexiones eléctricas y al personal de limpieza, especialmente a Luisa, por hacernos más amenas las mañanas.

Gracias también a mis compañeros del despacho QC-1117, aka 'Juntas', sobre todo por aguantar mis desvaríos en la temporada de escritura. David, una de las primeras personas que conocí en Barcelona, gracias por todo lo vivido y por tu humor negro que nos ha salvado tantos días. Edu, gracias por escucharme y aconsejarme tantas veces todos estos años y por supuesto, por regalarnos tus bailes. Kike, mi 'funsionario' favorito, ¡quién me iba a decir que aquel chico que me dio miedo por afirmar que Lugo-Santiago se hacía en 45 mins, sería años después mi compi de piso! Gracias por tu sabiduría en todos los temas del mundo, por tus habilidades de bricolaje, por tu paciencia y tu buena 'onda' a todas las horas del día. Elena, gracias por tus notitas en las manzanas pochas, las voy a echar de menos.

Pero en el DEQBA, he tenido la suerte de conocer a otras muchas personas super majas. Jesica, amora, gracias por ser mi confidente y mi apoyo tantas veces (que suerte tuve contigo). Eres super buena amiga y una persona brillante. Diana, la comadre, gracias por ser siempre tan encantadora, por haberme enseñado tantas palabras y por todo lo

compartido. Esther, gracias por ser un ejemplo de como afrontar esta aventura que es la tesis (alguna que otra vez te hecho caso). Maria Pinel, gracias por todas las sobremesas de café en el despacho en las que discutimos de temas tan trascendentales como cuál es el centro de Sabadell. Nathaly, gracias por looks compartidos cada día en verano y por tu cariño. Carlos Ruiz, gracias por compartir tu buena energía siempre. Gracias a Shamim, Romina, Kaidi, Zewen, Xudong, David Cueto, Mira, Franc, Laia, Dani Salom, Arnau Sala, Goli, Nico, Yerko, Darly, Anna, Roberto, Jose, Caro, Garazi, Nuria y demás compañeros con los que me cruzaba cada día por los pasillos.

A mi familia cerdanyolenca por aguantarme conviviendo bajo el mismo techo: Marc Keys, Juan, Marc Dalmau y otra vez, Mengqi y Kike. Marc Keys, la bondad personificada, gracias por escucharme tantas veces, por tus delirios mientras dormías que dan para unas cuantas novelas y por acompañarme en las cosas buenas y no tan buenas durante tantos años de convivencia. Juan, mi fiel compañero de confinamiento, gracias por ayudarme a desconectar con tu espíritu festivo, nuestros temazos eurovisivos del año de la polca y algún que otro platito de comida. Marc Dalmau, gracias por informarnos siempre de la actualidad y por no perder la esperanza en cambiar el mundo a mejor.

Ás miñas amigas picheleiras e lucenses, polo seu apoio a distancia e porque con vós, parecera que os anos non pasan. Blanca, gracias por entenderme casi a la perfección, será por eso de nuestro tipo de personalidad. María, gracias por todas las aventuras vividas en Barcelona (qué tiempos tan felices) y por las visitas para rememorarlos después. Amy, gracias por las llamadas de los fines de semana, por las visitas y por tu cariño infinito. Silvia, gracias por tu honestidad, que valoro mucho y por tus muestras de interés aun viéndonos contadas veces al año. Sonia, gracias por ser la mejor organizadora de eventos y una compañera de payasadas y por darme privilegios como administradora. Ruth, gracias por tu memoria tergiversadora que hace que nuestras historias pasadas parezcan aún más divertidas. Helena, gracias por tus chistes científicos inigualables. Marina, gracias por mostrarnos siempre la mejor actitud ante cualquier situación.

Por último, gracias a mi familia por recibirme siempre como si no pasara el tiempo. A los Vázquez, por los skypes de todos los viernes. A los Fernández, por su punto de locura que llevamos en los genes. A mis padres, por preocuparse siempre por mí, por acogerme en casa siempre que podía volver y por las visitas a un campus desértico en agosto. A mi hermano, por ayudarme a entender los métodos estadísticos usados en esta tesis (ya lo decía la abuela Fina, ‘tachuncrá’) y por visitarme para comer en el campus.

Financial support

This work was supported by the grant ORIGEN PID2021-126102OB-I00 funded by MCIN/AEI/10.13039/501100011033 and by ERDF A way of making Europe from the European Union and the grant CTQ2017–82404-R funded by the Spanish Ministerio de Economía, Industria y Competitividad (MINECO), Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER, EU). The author also acknowledges a predoctoral grant from Universitat Autònoma de Barcelona (PIF 2018-2019). The author is a member of the GENOCOV research group (Grup de Recerca Consolidat de la Generalitat de Catalunya, 2021 SGR 00515, www.genocov.com).

Summary

Making the switch to a circular economy model is one of the main challenges to be faced by the society in the twenty-first century. In this framework, resource recovery from waste or wastewater contributes to the environmental sustainability since it decreases the use of raw materials and the generation of waste. Therefore, the development of sustainable processes to transform the organic matter contained in waste or wastewater into valuable products is gaining attention in research. Short chain carboxylic acids (SCCAs) are among the possible products that can be recovered from organic matter. SCCAs are a type of fatty acids which are considered building block chemicals since they can be used as feedstock in many industries. Applications of SCCAs include polyhydroxyalkanoates (PHA) synthesis, medium chain carboxylic acids (MCCAs) synthesis, hydrogen synthesis, etc. Biological synthesis of SCCAs from waste or wastewater by using microbial mixed cultures would be an environmentally friendly alternative to the chemical synthesis process which is currently implemented at industrial scale. SCCAs can be produced from organic matter in an anaerobic process known as acidogenic fermentation. Nevertheless, this process still needs to be investigated before being scaled up. One of the main limitations of this process that still needs to be solved is the control of the SCCA production yield and the resultant SCCA composition.

This thesis starts with a literature review (Chapter 1) which aimed to explore the state-of-the-art of the acidogenic fermentation process and find the research gaps. This review pays special attention to the impact of the operational parameters on the SCCA production yield and on the SCCA composition distribution obtained depending on the organic composition of the substrate. The operational conditions (pH, temperature and volumetric organic loading rate or OLRv) that lead to high SCCA production yields depending on the majoritarian organic components of the substrate (carbohydrates, proteins or lipids) were determined according to the results found in literature. However, the influence of the operational parameters and the organic composition of the substrate on the SCCA composition distribution obtained was still not clear. It was hypothesized that the different parameters analyzed might have a joint effect on the SCCA composition obtained. Moreover, it was detected that authors were not usually considering the impact of the ratio between substrate and biomass concentrations on the SCCA production yield and SCCA composition distribution obtained. Therefore, the present study proposes the use of the specific organic loading rate (OLRs) as a parameter to quantify the ratio

between the substrate amount fed per biomass amount in the reactor (expressed in g COD g^{-1} VSS L^{-1}, where COD stands for chemical oxygen demand and VSS denoted volatile suspended solids). Also, standard effectiveness parameters to evaluate the process performance are suggested to facilitate the comparation of the results between different acidogenic fermentation studies. These effectiveness parameters include bioconversion, degree of acidification, acidified COD, carboxylic acid (CA) production yield and odd-to-even ratio (Chapter 3). In view of the observations obtained from the literature review, the experimental part of this study aimed to further explore the effect of the OLRv, OLRs and substrate organic composition on the process effectiveness parameters, effluent composition and biomass composition.

First, in Chapter 4, acidogenic fermentation experiments were performed in a sequencing batch reactor (SBR) fed by simulated wastewater only composed by food-grade sugar as carbon source. The objective was to study the effect of the OLRv, OLRs and influent carbon-to-nitrogen ratio on the process effectiveness parameters, effluent composition and biomass composition when using a simulated wastewater only composed by a model carbohydrate compound. Each parameter was tuned individually while keeping constant the other two. By increasing the OLRv from 6.0 to 11.0-15.8 g COD L^{-1} d^{-1}, the effluent changed from being composed mainly by a mixture of butyric and acetic acid to being comprised by a mixture of acetic acid and ethanol. Consequently, the degree of acidification, acidified COD and the CA production yield decreased due to the change in the synthesis of butyric acid to ethanol. The increase of the OLRs from 2.3 up to 3.8 g COD g^{-1} VSS d^{-1} favoured chain elongation reactions leading to caproic acid increase. Finally, the influent carbon-to-nitrogen ratio had low impact on the process performance. Under all the conditions tested, the biomass was mainly composed by the following genera: *Bifidobacterium*, *Ethanoligenens*, *Clostridium IV* and *Clostridium sensu stricto.*

In Chapter 5, acidogenic fermentation experiments were performed in a SBR fed by simulated wastewater only composed by whey protein isolate as carbon source. The objective was to study the effect of the OLRv and OLRs on the process effectiveness parameters, effluent composition and biomass composition when using a simulated wastewater composed only composed by a model protein compound. Both parameters were modified separately. Tuning the OLRv between from 6.5-18.5 g COD L^{-1} d^{-1} and the OLRs between 1.9-3.3 g COD g^{-1} VSS d^{-1} did not significantly affect the effectiveness

parameters. The degree of acidification, acidified COD and the CA yield obtained were higher than the obtained using a substrate only formed by food-grade sugar under the same conditions of OLRv and OLRs. The effluent was composed by acetic acid (15.4-18.1%), isovaleric acid (15.0-17.5%), propionic acid (11.5-15.5%), butyric acid (11.9-15.8%), isocaproic acid (7.2-11.2%) and isobutyric acid (7.2-9.1%), no matter the OLRv and OLRs applied. When increasing the OLRv from 6.5-12.5 to 18.5 g COD L^{-1} d^{-1}, chain elongation reactions were favoured provoking a significant increase of isocaproic acid. By increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1}, the odd-to-even was slightly increased due to a significant increase of propionic and valeric acids. Under all the conditions tested, the main genera detected in the reactor were *Petrimonas*, *Pyramidobacter*, *Aminobacterium*, *Alistipes* and *Peptostreptococcus.*

Finally, in Chapter 6, acidogenic fermentation experiments were performed in a SBR fed by simulated wastewater composed by mixtures of food-grade sugar and whey protein isolate in different ratios under constant OLRv and OLRs. It was aimed to evaluate the impact of the substrate organic composition on the process effectiveness parameters, effluent composition and biomass composition. Influents containing both carbohydrates and proteins led to significantly higher CA production yields in comparison to influents only consisting of carbohydrates or proteins. Besides, the higher the protein proportion in the influent, the higher the number of compounds and the branched CA proportion in the effluent. Moreover, it was found a linear correlation for the composition of three CAs with the substrate composition: isobutyric and isovaleric acids were directly proportional and butyric acid was inversely proportional to the protein proportion (% COD) in the substrate. The influent organic composition also had a great impact on the resultant biomass composition, but it was possible to link the main effluent components to one or more genera present in the reactor.

Resumen

Hacer la transición hacia un modelo de economía circular es uno de los principales retos a enfrentar por la sociedad en el siglo XXI. En este contexto, la recuperación de recursos a partir de residuos o aguas residuales contribuye a la sostenibilidad ambiental ya que disminuye el uso de materias primas y la generación de residuos. Por ello, el desarrollo de procesos sostenibles para transformar la materia orgánica contenida en los residuos o las aguas residuales en productos con alto valor añadido es un tema de interés creciente en investigación. Los ácidos carboxílicos de cadena corta (SCCAs, del inglés *'short chain carboxylic acids'*) son uno de los posibles productos que se pueden recuperar a partir de la materia orgánica. Los SCCAs son un tipo de ácidos grasos que se consideran productos químicos elementales dado que se pueden usar como materia prima en muchas industrias. Las aplicaciones de los SCCAs incluyen la síntesis de polihidroxialcanoatos (PHA), de ácidos carboxílicos de cadena media (MCCAs, del inglés *'medium chain carboxylic acids'*), de hidrógeno, etc. La síntesis biológica de SCCAs a partir de residuos o aguas residuales usando cultivos microbianos mixtos sería una alternativa más respetuosa con el medio ambiente que el proceso de síntesis química que está actualmente implementado a nivel industrial. Los SCCAs se pueden producir a partir de materia orgánica en un proceso anaerobio conocido como fermentación acidogénica. Sin embargo, este proceso aún requiere ser investigado en mayor profundidad antes de ser escalado. Una de las principales limitaciones de este proceso, que todavía necesita ser resuelta, es el control del rendimiento de producción de SCCAs y de la composición de SCCAs resultante.

Esta tesis comienza con una revisión bibliográfica (Capítulo 1) cuyo objetivo es explorar el estado del arte de la fermentación acidogénica e identificar las lagunas en la investigación de este proceso. Esta revisión se enfoca en el impacto de los parámetros operacionales en el rendimiento de producción de SCCAs y en la composición de SCCAs obtenida, dependiendo de la composición orgánica del sustrato. Con los resultados encontrados en la bibliografía, se determinaron las condiciones operacionales (pH, temperatura y velocidad de carga orgánica volumétrica) que conducen a rendimientos altos de producción de SCCAs en función de los componentes orgánicos mayoritarios en el sustrato (carbohidratos, proteínas o lípidos). Sin embargo, la influencia de los parámetros operacionales y la composición orgánica del sustrato en la composición de SCCAs obtenida no quedaba claramente establecida. Se hipotetizó que los diferentes

parámetros analizados podrían tener un efecto conjunto en la composición de SCCAs obtenida. Además, se detectó que los autores no solían considerar el impacto de la ratio entre la concentración de sustrato y la concentración de biomasa en el rendimiento de producción de SCCAs y en la composición de SCCAs obtenida. Por lo tanto, el presente estudio propone el uso de la velocidad de carga orgánica específica (OLRs, del inglés *'specific organic loading rate'*) como un parámetro para cuantificar la ratio entre la cantidad de sustrato alimentado por cantidad de biomasa en el reactor (expresada en g DQO g^{-1} SSV L^{-1}, donde DQO denota demanda química de oxígeno y SSV denota sólidos en suspensión volátiles). Además, en el Capítulo 3 se sugirieren parámetros de efectividad estándar para evaluar el rendimiento del proceso y facilitar la comparación de resultados entre diferentes estudios de fermentación acidogénica. Estos parámetros de efectividad incluyen la bioconversión, el grado de acidificación, la DQO acidificada, el rendimiento de producción de ácidos carboxílicos (CAs, del inglés *'carboxylic acids'*) y la ratio impar-par (Capítulo 3). En vista de las observaciones obtenidas de la revisión bibliográfica, la parte experimental de este estudio tenía como objetivo explorar en mayor profundidad el efecto de la velocidad de carga orgánica volumétrica (OLRv, del inglés *'volumetric organic loading rate'*), OLRs y la composición orgánica del sustrato en los parámetros de efectividad del proceso, en la composición del efluente y en la composición de la biomasa.

Inicialmente, en el Capítulo 4, se llevaron a cabo experimentos de fermentación acidogénica en un reactor biológico secuencial (SBR, del inglés *'sequencing batch reactor'*) alimentado con agua residual simulada compuesta únicamente por azúcar de grado alimentario como fuente de carbono. El objetivo era estudiar el efecto de la OLRv, la OLRs y la ratio carbono/nitrógeno del afluente en los parámetros de efectividad del proceso, en la composición del efluente y en la composición de la biomasa cuando el afluente está compuesto sólo por un carbohidrato modelo. Cada parámetro se modificó individualmente mientras se mantenían constantes los otros dos. Al incrementar la OLRv de 6.0 a 11.0-15.8 g DQO L^{-1} d^{-1}, el efluente pasó de estar compuesto fundamentalmente por una mezcla de ácidos butírico y acético a estar compuesto por una mezcla de ácido acético y etanol. Consecuentemente, el grado de acidificación, la DQO acidificada y el rendimiento de producción de CAs disminuyeron dado el cambio de síntesis de ácido butírico a etanol. El incremento de la OLRs de 2.3 a 3.8 g DQO g^{-1} SSV d^{-1} favoreció las reacciones de alargamiento de cadena, implicando un aumento del ácido caproico.

Finalmente, la ratio carbono/nitrógeno del afluente tuvo poco impacto en el desarrollo del proceso. Bajo todas las condiciones probadas, la biomasa estaba principalmente compuesta por los siguientes géneros: *Bifidobacterium*, *Ethanoligenens*, *Clostridium IV* y *Clostridium sensu stricto.*

En el Capítulo 5, se llevaron a cabo experimentos de fermentación acidogénica en un SBR alimentado con agua residual simulada compuesta únicamente por proteína aislada de suero lácteo como fuente de carbono. El objetivo era estudiar el efecto de la OLRv y la OLRs en los parámetros de efectividad del proceso, en la composición del efluente y en la composición de la biomasa cuando el afluente está compuesto sólo por un compuesto proteico modelo. Ambos parámetros fueron modificados por separado. Modificar la OLRv entre 6.5-18.5 g DQO L^{-1} d^{-1} y la OLRs entre 1.9-3.3 g DQO g^{-1} SSV d^{-1} no afectó significativamente a los parámetros de efectividad del proceso. El grado de acidificación, la DQO acidificada y el rendimiento de producción de CAs eran superiores a los obtenidos cuando el sustrato estaba formado únicamente por azúcar de grado alimentario bajo las mismas condiciones de OLRv y OLRs. El efluente estaba compuesto por ácido acético (15.4-18.1%), ácido isovalérico (15.0-17.5%), ácido propiónico (11.5-15.5%), ácido butírico (11.9-15.8%), ácido isocaproico (7.2-11.2%) y ácido isobutírico (7.2-9.1%), independientemente de la OLRv y la OLRs aplicadas. Al aumentar la OLRv de 6.5-12.5 a 18.5 g DQO L^{-1} d^{-1}, se vieron favorecidas las reacciones de alargamiento de cadena, provocando un aumento significativo del ácido isocaproico. Al aumentar la OLRs de 1.9 a 3.3 g DQO g^{-1} SSV d^{-1}, la ratio impar-par se incrementó ligeramente debido a un aumento significativo de los ácidos propiónico y valérico. Bajo todas las condiciones probadas, los principales géneros detectados en el reactor fueron *Petrimonas*, *Pyramidobacter*, *Aminobacterium*, *Alistipes* y *Peptostreptococcus.*

Finalmente, en el Capítulo 6, se llevaron a cabo experimentos de fermentación acidogénica en un SBR alimentado con agua residual simulada compuesta por mezclas de azúcar de grado alimentario y proteína aislada de suero lácteo en diferentes ratios bajo condiciones constantes de OLRv y OLRs. Se pretendía evaluar el impacto de la composición orgánica del sustrato en los parámetros de efectividad del proceso, en la composición del efluente y en la composición de la biomasa. Los diferentes afluentes compuestos por mezclas de carbohidratos y proteínas llevaron a rendimientos de producción de CAs superiores a los obtenidos con afluentes conteniendo sólo carbohidratos o sólo proteínas. Asimismo, a mayor concentración de proteína en el

afluente, mayor número de compuestos y mayor proporción de CAs ramificados en el efluente. Además, se encontró una correlación lineal para la composición de tres CAs con la composición del sustrato: los ácidos isobutírico e isovalérico eran directamente proporcionales y el ácido butírico era inversamente proporcional a la proporción de proteínas (% DQO) en el sustrato. La composición orgánica del afluente también tuvo un gran impacto en la composición de la biomasa resultante, pero fue posible relacionar los componentes principales del efluente con uno o más géneros presentes en el reactor.

Resum

Fer la transició cap a un model d'economia circular és un dels reptes principals per la societat del segle XXI. En aquest context, la recuperació de recursos a partir de residus o aigües residuals contribueix a la sostenibilitat ambiental, ja que disminueix l'ús de matèries primeres i la generació de residus. Per això, el desenvolupament de processos sostenibles per transformar la matèria orgànica continguda als residus o aigües residuals en productes amb alt valor afegit és un tema d'interès creixent en recerca. Els àcids carboxílics de cadena curta (SCCAs, de l'anglès *'short chain carboxylic acids'*) són un dels possibles productes que es poden recuperar a partir de la matèria orgànica. Els SCCA són un tipus d'àcids grassos que es consideren productes químics elementals degut al fet que es poden fer servir com a matèria primera en moltes indústries. Les aplicacions dels SCCAs inclouen la síntesi de polihidroxialcanoats (PHA), d'àcids carboxílics de cadena mitjana (MCCAs, de l'anglès *'medium chain carboxylic acids'*), d'hidrogen, etc. La síntesi biològica de SCCAs a partir de residus o aigües residuals usant cultius microbians mixts seria una alternativa més respectuosa amb el medi ambient que el procés de síntesi química que està actualment implementat a nivell industrial. Els SCCA es poden produir a partir de matèria orgànica en un procés anaerobi conegut com a fermentació acidogènica. Tot i això, aquest procés encara ha d'esser investigat més a fons abans de poder ser escalat. Una de les principals limitacions d'aquest procés, que encara necessita ser resolta, és el control del rendiment de producció de SCCAs i de la composició de SCCAs resultant.

Aquesta tesi comença amb una revisió bibliogràfica (Capítol 1) que té per objectiu explorar l'estat de l'art de la fermentació acidogènica i trobar els buits en la recerca d'aquest procés. Aquesta revisió s'enfoca en l'impacte dels paràmetres operacionals en el rendiment de producció de SCCA i en la composició de SCCA obtinguda, depenent de la composició orgànica del substrat. En funció dels resultats trobats a la bibliografia, es van determinar les condicions operacionals (pH, temperatura i velocitat de càrrega orgànica volumètrica) que condueixen a rendiments alts de producció de SCCAs en funció dels components orgànics majoritaris al substrat (carbohidrats, proteïnes o lípids) . Tot i això, la influència dels paràmetres operacionals i la composició orgànica del substrat en la composició de SCCAs obtinguda no quedava clarament establerta. Es va fer la hipòtesis de que els diferents paràmetres analitzats podrien tenir un efecte conjunt en la composició de SCCA obtinguda. A més, es va detectar que els autors no solien considerar l'impacte

de la ràtio entre la concentració de substrat i la concentració de biomassa en el rendiment de producció de SCCA i en la composició de SCCA obtinguda. Per tant, el present estudi proposa l'ús de la velocitat de càrrega orgànica específica (OLRs, de l'anglès *'specific organic loading rate'*) com un paràmetre per quantificar la ràtio entre la quantitat de substrat alimentat per quantitat de biomassa al reactor (expressada en g DQO g^{-1} SSV L^{-1}, on DQO denota demanda química d'oxigen i SSV denota sòlids en suspensió volàtils). A més, al Capítol 3 es suggereixen uns paràmetres d'efectivitat estàndard per avaluar el rendiment del procés i facilitar la comparació de resultats entre diferents estudis de fermentació acidogènica. Aquests paràmetres d'efectivitat inclouen la bioconversió, el grau d'acidificació, la DQO acidificada, el rendiment de producció d'àcids carboxílics (CAs, de l'anglès *'carboxylic acids'*) i la ràtio senar-parell (Capítol 3). En vista de les observacions obtingudes de la revisió bibliogràfica, la part experimental d'aquest estudi tenia com a objectiu explorar amb més profunditat l'efecte de la velocitat de càrrega orgànica volumètrica (OLRv, de l'anglès *'volumetric organic loading rate'*), OLRs i composició orgànica del substrat sobre els paràmetres d'efectivitat del procés, a la composició de l'efluent i a la composició de la biomassa.

Inicialment, al Capítol 4, es van dur a terme experiments de fermentació acidogènica en un reactor biològic seqüencial (SBR, de l'anglès 'sequencing batch reactor) alimentat amb aigua residual simulada composta únicament per sucre de grau alimentari com a font de carboni. L'objectiu era estudiar l'efecte de l'OLRv, l'OLRs i la ràtio carboni/nitrogen de l'afluent sobre els paràmetres d'efectivitat del procés, la composició de l'efluent i la composició de la biomassa quan l'afluent està compost només per un carbohidrat model. Cada paràmetre es va modificar individualment mentre es mantenien constants els altres dos. En incrementar l'OLRv de 6.0 a 11.0-15.8 g DQO L^{-1} d^{-1}, l'efluent va canviar d'estar compost fonamentalment per una barreja d'àcids butíric i acètic a estar compost per una barreja d'àcid acètic i etanol. Conseqüentment, el grau d'acidificació, la DQO acidificada i el rendiment de producció de CAs van disminuir pel canvi de síntesi d'àcid butíric a etanol. L'increment de l'OLRs de 2.3 a 3.8 g DQO g^{-1} SSV d^{-1} va afavorir les reaccions d'allargament de cadena, implicant un augment de l'àcid caproic. Finalment, la ràtio carboni/nitrogen de l'afluent va tenir poc impacte en el desenvolupament del procés. Sota totes les condicions estudiades, la biomassa estava principalment composta pels següents gèneres: *Bifidobacterium*, *Ethanoligenens*, *Clostridium IV* i *Clostridium sensu stricto*.

Al Capítol 5, es van dur a terme experiments de fermentació acidogènica en un SBR alimentat amb aigua residual simulada composta únicament per proteïna aïllada de sèrum làctic com a font de carboni. L'objectiu era estudiar l'efecte de l'OLRv i l'OLRs sobre els paràmetres d'efectivitat del procés, la composició de l'efluent i la composició de la biomassa quan l'afluent està compost només per un compost proteic model. Tots dos paràmetres van ser modificats per separat. Modificar l'OLRv entre 6.5-18.5 g DQO L^{-1} d^{-1} i l'OLRs entre 1.9-3.3 g DQO g^{-1} SSV d^{-1} no afectà significativament els paràmetres d'efectivitat del procés. El grau d'acidificació, la DQO acidificada i el rendiment de producció de CA eren superiors als obtinguts quan el substrat estava format únicament per sucre de grau alimentari sota les mateixes condicions d'OLRv i OLRs. L'efluent estava compost per àcid acètic (15.4-18.1%), àcid isovalèric (15.0-17.5%), àcid propiònic (11.5-15.5%), àcid butíric (11.9-15.8%), àcid isocaproic (7.2-11.2%) i àcid isobutíric (7.2-9.1%), independentment de l'OLRv i les OLRs aplicades. En augmentar l'OLRv de 6.5-12.5 a 18.5 g DQO L^{-1} d^{-1}, es van afavorir les reaccions d'allargament de cadena, provocant un augment significatiu de l'àcid isocaproic. En augmentar l'OLRs d'1.9 a 3.3 g DQO g^{-1} SSV d^{-1}, es va incrementar lleugerament la ràtio senar-parell a causa d'un augment significatiu dels àcids propiònic i valèric. Sota totes les condicions estudiades, els principals gèneres detectats al reactor eren *Petrimonas*, *Pyramidobacter*, *Aminobacterium*, *Alistipes* i *Peptostreptococcus*.

Finalment, al Capítol 6, es van dur a terme experiments de fermentació acidogènica en un SBR alimentat amb aigua residual simulada composta per barreges de sucre de grau alimentari i proteïna aïllada de sèrum làctic en diferents ràtios sota condicions constants de OLRv i OLRs. Es pretenia avaluar l'impacte de la composició orgànica del substrat sobre els paràmetres d'efectivitat del procés, la composició de l'efluent i la composició de la biomassa. Afluents contenint mescles de carbohidrats i proteïnes van portar a rendiments de producció de CA superiors als obtinguts amb afluents contenint només carbohidrats o només proteïnes. Així mateix, a més concentració de proteïna a l'afluent, major nombre de compostos i major proporció de CAs ramificats a l'efluent. A més, es va trobar una correlació lineal per a la composició de tres CAs amb la composició del substrat: els àcids isobutíric i isovalèric eren directament proporcionals i l'àcid butíric era inversament proporcional a la proporció de proteïnes (% DQO) al substrat. La composició orgànica de l'afluent també va tenir un gran impacte en la composició de la biomassa

resultant, però va ser possible lligar els components principals de l'efluent a un o més gèneres presents al reactor.

Resumo

Facer a transición cara un modelo de economía circular é un dos principais retos a enfrontar pola sociedade do século XXI. Neste contexto, a recuperación de recursos a partir de residuos ou augas residuais contribúe a sostibilidade ambiental, xa que diminúe o uso de materias primas e a xeración de residuos. Polo tanto, o desenvolvemento de procesos sostibles para transformar a materia orgánica contida nos residuos ou nas augas residuais en produtos con alto valor engadido é un tema de interese crecente en investigación. Os ácidos carboxílicos de cadea curta (SCCAs, do inglés '*short chain carboxylic acids'*) son un dos posibles produtos que se poden recuperar a partir de materia orgánica. Os SCCAs son un tipo de ácidos graxos que se consideran produtos químicos elementais dado que se poden usar como materia prima en moitas industrias. As aplicacións dos SCCAs inclúen a síntese de polihidroxialcanoatos (PHA), de ácidos carboxílicos de cadea media (MCCAs, do inglés *'medium chain carboxylic acids'*), de hidróxeno, etc. A síntese biolóxica de SCCAs a partir de residuos ou augas residuais usando cultivos microbianos mixtos sería unha alternativa máis respectuosa co medio ambiente que o proceso de síntese química que está actualmente implementado a nivel industrial. Os SCCAs pódense producir a partir de materia orgánica nun proceso anaerobio coñecido como fermentación acidoxénica. Sen embargo, este proceso aínda require ser investigado en maior profundidade antes de ser escalado. Unha das principais limitacións de este proceso que aínda necesita ser resolta é o control do rendemento de produción de SCCAs e da composición de SCCAs resultante.

Esta tese comeza cunha revisión bibliográfica (Capítulo 1) que ten por obxectivo explorar o estado do arte da fermentación acidoxénica e identificar as lagoas da investigación deste proceso. Esta revisión enfócase no impacto dos parámetros operacionais no rendemento de produción de SCCAs e na composición de SCCAs obtida, dependendo da composición orgánica do substrato. A partir dos resultados atopados na bibliografía, determináronse as condicións operacionais (pH, temperatura e velocidade de carga orgánica volumétrica) que conducen a rendementos altos de produción de SCCAs en función dos compoñentes orgánicos maioritarios no substrato (carbohidratos, proteínas ou lípidos). Sen embargo, a influencia dos parámetros operacionais e a composición orgánica do substrato na composición de SCCAs obtida non quedaba claramente establecida. Fíxose a hipótese de que os diferentes parámetros analizados poderían ter un efecto conxunto na composición de SCCAs obtida. Ademais, detectouse que os autores

non adoitaban considerar o impacto da razón entre a concentración do substrato e a concentración da biomasa no rendemento de produción de SCCAs e na composición de SCCAs obtida. Polo tanto, o presente estudo propón o uso da velocidade de carga orgánica específica (OLRs, do inglés *'specific organic loading rate'*) como un parámetro para cuantificar a razón da cantidade de substrato alimentado por cantidade de biomasa no reactor (expresada en g DQO g^{-1} SSV L^{-1}, onde DQO denota demanda química de oxíxeno e SSV denota sólidos en suspensión volátiles). Ademais, no Capítulo 3, suxírense uns parámetros de efectividade estándar para avaliar o rendemento do proceso e facilitar a comparación de resultados entre diferentes estudos de fermentación acidoxénica. Estes parámetros de efectividade inclúen a bioconversión, o grado de acidificación, a DQO acidificada, o rendemento de produción de ácidos carboxílicos (CAs, do inglés *'carboxylic acids'*) e a razón impar-par (Capítulo 3). En vista das observacións obtidas da revisión bibliográfica, a parte experimental deste estudo tiña por obxectivo explorar en maior profundidade o efecto da velocidade de carga orgánica volumétrica (OLRv, do inglés *'volumetric organic loading rate'*), OLRs e a composición orgánica do substrato nos parámetros de efectividade do proceso, na composición do efluente e na composición da biomasa.

Inicialmente, no Capítulo 4, leváronse a cabo experimentos de fermentación acidoxénica nun reactor biolóxico secuencial (SBR, do inglés *'sequencing batch reactor'*) alimentado con auga residual simulada composta unicamente por azucre de grao alimentario como fonte de carbono. O obxectivo era estudar o efecto da OLRv, da OLRs e da razón carbono/nitróxeno no afluente nos parámetros de efectividade do proceso, na composición do efluente e na composición da biomasa cando o afluente está composto só por un carbohidrato modelo. Cada parámetro foi modificado individualmente mentres os outros dous mantíñanse constantes. Ó incrementar a OLRv de 6.0 a 11.0-15.8 g DQO L^{-1} d^{-1}, o efluente pasou de estar composto fundamentalmente por unha mestura de ácidos butírico e acético a estar comprendido por unha mestura de ácido acético e etanol. Consecuentemente, o grao de acidificación, a DQO acidificada e o rendemento de produción de CAs diminuíron dado o cambio de síntese de ácido butírico a etanol. O incremento da OLRs de 2.3 a 3.8 g DQO g^{-1} SSV d^{-1} favoreceu as reaccións de alongamento de cadea, implicando un aumento do ácido caproico. Finalmente, a razón carbono/nitróxeno no afluente tivo pouco impacto no desenvolvemento do proceso. Baixo

todas as condicións probadas, a biomasa estaba principalmente composta polos seguintes xéneros: *Bifidobacterium*, *Ethanoligenens*, *Clostridium IV* e *Clostridium sensu stricto.*

No Capítulo 5, leváronse a cabo experimentos de fermentación acidoxénica nun SBR alimentado con auga residual simulada composta unicamente por proteína illada de soro lácteo como fonte de carbono. O obxectivo era estudar o efecto da OLRv e da OLRs nos parámetros de efectividade do proceso, na composición do efluente e na composición da biomasa cando o afluente está composto soamente por un composto proteico modelo. Ambos parámetros foron modificados por separado. Modificar a OLRv entre 6.5-18.5 g DQO L^{-1} d^{-1} e a OLRs entre 1.9-3.3 g DQO g^{-1} SSV d^{-1} non afectou significativamente ós parámetros de efectividade do proceso. O grao de acidificación, a DQO acidificada e o rendemento de produción de CAs eran superiores ós obtidos cando o substrato estaba formado unicamente por azucre de grao alimentario baixo as mesmas condicións de OLRv e OLRs. O efluente estaba composto por ácido acético (15.4-18.1%), ácido isovalérico (15.0-17.5%), ácido propiónico (11.5-15.5%), ácido butírico (11.9-15.8%), ácido isocaproico (7.2-11.2%) e ácido isobutírico (7.2-9.1%), independentemente da OLRv e OLRs aplicadas. Ó aumentar a OLRv de 6.5-12.5 a 18.5 g DQO L^{-1} d^{-1}, favorecéronse as reaccións de alongamento de cadea, provocando un aumento significativo do ácido isocaproico. Ó aumentar a OLRs de 1.9 a 3.3 g DQO g^{-1} SSV d^{-1}, a razón impar-par incrementouse lixeiramente debido a un aumento significativo dos ácidos propiónico e valérico. Baixo todas as condicións probadas, os principais xéneros detectados no reactor eran *Petrimonas*, *Pyramidobacter*, *Aminobacterium*, *Alistipes* e *Peptostreptococcus.*

Finalmente, no Capítulo 6, leváronse a cabo experimentos de fermentación acidoxénica nun SBR alimentado con auga residual simulada composta por mesturas de azucre de grao alimentario e proteína illada de soro lácteo en diferentes proporcións baixo condicións constantes de OLRv e OLRs. Pretendíase avaliar o impacto da composición orgánica do substrato nos parámetros de efectividade do proceso, na composición do efluente e na composición da biomasa. Afluentes contendo carbohidratos e proteínas conduciron a rendementos de produción de CAs superiores ós obtidos con afluentes contendo só carbohidratos ou só proteínas. Amais, a maior concentración de proteína no afluente, maior número de compostos e maior proporción de CAs ramificados no efluente. Ademais, encontrouse unha correlación lineal para a composición de tres CAs coa composición do substrato: os ácidos isobutírico e isovalérico eran directamente

proporcionais e o ácido butírico era inversamente proporcional á proporción de proteínas (% DQO) no substrato. A composición orgánica do afluente tamén tivo un gran impacto na composición da biomasa resultante, pero foi posible relacionar os compoñentes principais do efluente cun ou máis xéneros presentes no reactor.

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Chapter 1:

General Introduction

A modified version of this chapter has been published as:

A. Vázquez-Fernández, M.E. Suárez-Ojeda, J. Carrera, Review about bioproduction of Volatile Fatty Acids from wastes and wastewaters: Influence of operating conditions and organic composition of the substrate, J Environ Chem Eng. 10 (2022). https://doi.org/10.1016/j.jece.2022.107917.

In this chapter, literature related to short chain carboxylic acids (SCCAs) production by acidogenic fermentation processes is reviewed. The objective is to give the reader a general overview of the acidogenic fermentation process, including the state-of-the-art of the SCCAs bioproduction processes, metabolic routes for synthetizing SCCAs and the operational parameters affecting the SCCA production yield and the SCCA composition obtained by acidogenic fermentation of substrates with different organic composition. Then, the bioproduction of polyhydroxyalkanoates (PHAs) and medium chain carboxylic acids (MCCAs) are described in detailed as examples of processes were the SCCAs can be used as feedstock. Finally, conclusions of the state-of-the-art of the SCCA bioproduction process and the detected research gaps are outlined and a thesis overview is also given to explain how this study is covering the aforementioned research gaps. A modified version of this chapter was published as a review article in Journal of Environmental Chemical Engineering [1].

1.1 Short chain carboxylic acids (SCCAs): definition, applications and state-of-the-art of their bioproduction processes

Short chain carboxylic acids are a subgroup of fatty acids, ranging from two to five carbon atoms, including acetic, propionic, isobutyric, butyric, isovaleric and valeric acids. In literature, SCCAs are often referred to as volatile fatty acids (VFAs) or short chain fatty acids (SCFAs). SCCAs have a wide range of applications in numerous areas, thus they are considered building block chemicals. Among the SCCAs, acetic, propionic, and butyric acids are those most industrially produced. The annual global market demand of acetic, propionic and butyric acids was estimated in 18.5 Mt for 2020 [2]. In food industry, acetic acid is used as vinegar as well as food additive and preservative; propionic acid is utilized in the preservation of food grains and in animal feed while butyric acid is employed as flavouring [3–6]. Other applications of acetic acid include terephthalic acid production, which is then used in the manufacture of polyethylene terephthalate (PET), and the production of acetate esters [7]. Propionic acid is also a building block in the pharmaceutical industry, and it is employed in the production of herbicides and propionate esters for perfumes [5]. Butyric acid is widely used in the chemical industry in the production of cellulose acetate butyrate for the synthesis of thermoplastics and in the pharmaceutical industry for the manufacture of drugs with several therapeutic effects [6]. SCCAs also have potential applications as renewable feedstock such as for

polyhydroxyalkanoate (PHA) synthesis [8], production of electricity in microbial fuel cells (MFCs) [9], production of hydrogen [10], production of medium chain carboxylic acids (precursors of biofuels) [11] or even as a source of organic matter for biological nutrient removal [12]. Finally, there are further products that can be obtained from SCCAs, such as fatty alcohols, hydrocarbons, rhamnolipids, sophorolipids, N-acylethanolamines or lycopene [13,14].

Commercial production of SCCAs is normally based on chemical synthesis starting from petrochemical raw sources [15]. Acetic acid is mainly obtained by methanol carbonylation, followed by other processes such as the catalytic oxidation of acetaldehyde, ethylene, or butane [16,17]. Propionic acid is traditionally produced as a by-product of acetic acid production or by the hydroxycarboxylation of ethylene in the presence of a catalyst of rhodium or nickel carbonyl [18]. Butyric acid is usually produced by oxidation of butyraldehyde, that is, synthesized by oxosynthesis of propylene obtained from crude oil. Another conventional process is the extraction of butyric acid from butter, but this method is very expensive [19]. However, due to climate change concerns and fossil fuel depletion, SCCA bioproduction via microbial fermentation is gaining interest. Moreover, the final cost of the SCCAs produced from petrochemical resources depends on the price of fossil fuels.

In the last years, several studies related to fermentative bioproduction of SCCAs from different carbon sources have been published. Most of the SCCA bioproduction by fermentation is carried out by using pure substrates, such as glucose, xylose, or glycerol [20–22]. However, even though the use of pure carbon sources should lead to high yields and productivities, the cost of this kind of substrates is high and raises the overall cost of the process. To reduce the costs for making competitive the fermentative bioproduction process compared to the fossil-based one, different types of waste, such as lignocellulosic biomass, waste activated sludge (WAS), food waste, dairy wastewater, paper mill wastewater or even olive mill wastewater have been proposed as substrates [23–29]. In addition, the use of wastes to bioproduce SCCAs contributes to the implementation of a circular economy model and avoids the use of edible raw materials to produce chemicals [30]. The main disadvantage of using waste as a raw material of the fermentative bioproduction is the cost of the downstream purification process of the final products. Yet, when using pure carbon substrates, side products are not normally produced, so the purification step is inexpensive [16]. Moreover, some types of waste need pretreatment

before fermentation. For example, anaerobic microorganisms are not able to directly digest solid waste, such as lignocellulosic biomass or WAS so physical, chemical or enzymatic pretreatments might be needed [31].

Hence, in the design of a SCCA bioproduction process, the following items must be considered: SCCA production yield, productivity, raw material costs, need of a pretreatment step and downstream processing costs [16,32]. One alternative to increase the productivity, avoid the formation of side products, and use different carbon sources is the utilization of engineered strains [13]. Nevertheless, in this case, the cost of the process will also increase due to the requirement of sterile conditions. For that reason, many researchers are focusing their work on SCCA biosynthesis through anaerobic fermentation by mixed microbial cultures (MMC), which do not require sterile conditions and are considered more robust systems [33].

Thus, SCCA bioproduction by fermentation of wastes using MMC is a potential and true alternative to the conventional processes based on petrochemical resources or to the biological processes based on the use of pure organic feedstocks and pure bacterial strains. Nevertheless, research in this field must overcome two challenges: 1) to understand how to drive the fermentation process to a targeted SCCA or to a specific mixture of SCCAs by tuning the operational conditions or design parameters; 2) to improve the efficiency of the downstream purification methods.

Regarding the downstream purification methods for recovering SCCAs like gas stripping with absorption, adsorption, solvent extraction, electrodialysis, microfiltration, reverse osmosis and nanofiltration, membrane contactor and in-line recovery have been extensively discussed in the literature [34–44] and are out the scope of this introduction section.

In this sense, this chapter only covers the SCCA production by anaerobic fermentation of waste streams by MMC, focusing on the first mentioned challenge. To the best of our knowledge, previous review articles analyzed the influence of the operational parameters on SCCA production yield and SCCA composition for different ‘types of waste’ without considering the particular organic composition the different types of wastes can have. Since these works found contradictory results, especially when analyzing the SCCA composition, this introduction section wants to consider the waste attending to its organic

composition (carbohydrate, protein and lipid content) instead of its classification as a certain ‘type of waste’.

1.2 Metabolic routes for anaerobic production of SCCAs

Short chain carboxylic acids are produced during anaerobic digestion of organic matter. Anaerobic digestion is a well-known technology that is implemented at industrial scale for biogas production [45]. Nonetheless, lately, anaerobic digestion is gaining interest in the production of other value-added products, such as SCCAs and hydrogen. The anaerobic digestion process consists in the biological reduction of organic matter in absence of oxygen or nitrate/nitrite and takes place in four interdependent steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1.1) [46]. When biological process is stopped on the acidogenesis and acetogenesis and the methanogenesis does not take place, the process is usually named anaerobic or acidogenic fermentation instead of anaerobic digestion. Hydrolysis consists in the conversion of hardly soluble compounds such as complex carbohydrates, proteins and lipids into monosaccharides, amino acids, and long-chain fatty acids. In the acidogenic step, the products of the hydrolysis are transformed by several fermentative reactions performed by facultative and obligatory anaerobes into carbon dioxide, hydrogen, SCCAs and other products, such as ethanol and lactic acid. During acetogenesis, SCCAs which cannot be transformed into methane by methanogenic microorganisms, are converted into acetate, hydrogen, and carbon dioxide. Finally, methanogenesis is carried out by two different groups of Archaea: the acetoclastic methanogens produce methane and carbon dioxide from acetate while the hydrogenotrophic methanogens produce methane from hydrogen and carbon dioxide [47,48].

Since anaerobic digestion is performed by MMC, different pathways would take place and several by-products and intermediates can be formed. Hydrolytic bacteria are strict anaerobes that include *Bacteroides*, *Clostridium*, *Micrococcus*, *Butiryvibrio*, *Selenomonas* and *Streptococcus* [47]. The acid-forming bacteria, also known as acidogens, are responsible for SCCA synthesis [49]. After hydrolysis, acidogenic bacteria consume the obtained monosaccharides, amino acids and long-chain fatty acids and store them intracellularly as pyruvates, which later are converted into acetate or other SCCAs [50,51]. Polysaccharides are broken into monosaccharides and then, converted to

pyruvate by following different pathways such as: Embden-Meyerhof pathway (EMP), Entner-Doudoroff pathway, Pentose Phosphate pathway (PPP), Bifidus pathway and Phosphoketolase pathway (PK) [52,53]. Finally, pyruvate is transformed into SCCAs, ethanol, lactic, hydrogen and carbon dioxide [52]. Proteins are broken into polypeptides and amino acids. Subsequently, the latter can be converted into SCCAs by three possible pathways: oxidation-reduction of pairs of amino acids (Stickland reaction), oxidative deamination from an individual amino acid or reductive deamination of an individual amino acid [51,54]. Lastly, hydrolysis of lipids produces glycerol and long-chain fatty acids. The resulting glycerol can be transformed into pyruvate and then, form acetyl-CoA, acetic acid and other SCCAs, while long-chain fatty acids are converted into acetic acid by β-oxidation [50]. Moreover, homoacetogens can produce acetic acid from carbon dioxide and hydrogen through the Wood-Ljungdahl pathway [46,49].

Previous studies have particularly focused on the pathways of methanogens more than that of acetogens and acidogens due to the larger interest in biogas production. Until now, the number of species identified as acidogenic microorganisms is very limited. The most studied phyla able to degrade complex substrates and transform them into SCCAs are *Firmicutes*, *Proteobacteria* and *Bacteroidetes* [55]. The variety of species that can perform the acidogenesis step demonstrates that different fermentation products can be obtained depending on the strains present. However, the leading microorganisms will change depending mainly on the substrate, so the substrate would a key element in a bioproduction process of SCCAs [45].

During methanogenesis, approximately two thirds of the methane generated comes from acetate. Thus, methanogens are considered competitors of acetogens since they use hydrogen, formate and acetate for growing [56]. The use of acetate as a substrate for growth and for methane formation competes with the accumulation of acids and is carried out by strains that belong to the genera *Methanosarcina* and *Methanosaeta* [55]. Accumulation of acids implies a drop in pH that could lead to a reduction of the methane produced, as methanogens require pH over 6.5 [57]. This reduction, in turn, comes with a greater accumulation of acids, as acetate is not consumed [46]. When using easily biodegradable feedstocks, methanogenesis is the rate-limiting step of the process, as methanogens grow very slowly and need very specific substrates [58]. The competition between methanogens and acidogens needs further study and it is of great interest for the industrial use of anaerobic fermentation for SCCA bioproduction [59]. Strategies

promoting the acidogenic step and inhibiting the methanogenesis step must be followed to maximize SCCA bioproduction. Most of these strategies can be satisfactorily implemented by tuning the operational conditions of the process, which will be discussed in the next section.

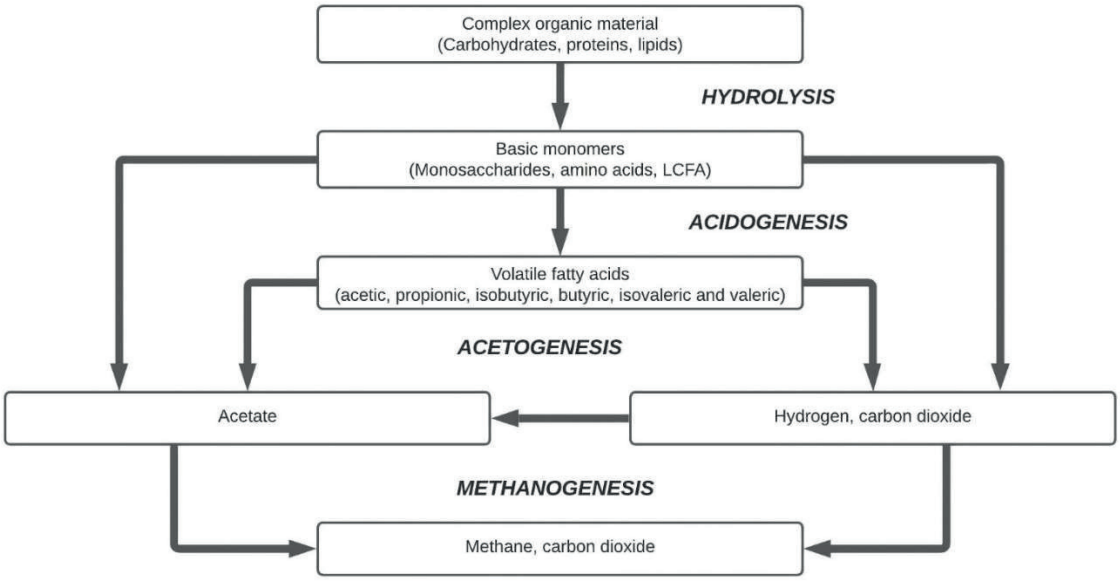


Figure 1.1. Steps in Anaerobic Digestion Process (adapted from Ersahin et al. [60]).

1.3 Operational parameters affecting SCCA production yield

There are several operational parameters that affect the SCCA bioproduction by acidogenic fermentation of waste. But, before starting the analysis, it is important to mention how to quantify the performance of a SCCA bioproduction process. In this sense, to quantify the amount of SCCAs produced, the literature describes two different parameters: SCCA production yield and the degree of acidification. The SCCA production yield (expressed in terms of g COD g^{-1} COD, where COD stands for chemical oxygen demand) can be defined as the amount of SCCAs produced divided by the substrate consumed [61]. Other authors, especially when the substrate is a solid waste, define the SCCA production yield (expressed in terms of g COD g^{-1} VSS or g COD g^{-1} VS) as the amount of SCCAs produced divided by the organic matter contained in the solid waste [62,63] expressed in terms of volatile suspended solids (VSS) or volatile solids (VS). Regarding the degree of acidification, this parameter is calculated (expressed

in percentage) by dividing the total SCCA concentration in the effluent and the total initial COD of the substrate [61].

Recently, many researchers have focused their efforts on studying the effect of several operational parameters on SCCA production, such as: substrate composition [54,64], pH [65,66], temperature [67,68], volumetric organic loading rate (OLRv) and hydraulic retention time (HRT) [69,70]. The aforementioned parameters affect all the steps of the anaerobic digestion, so they can impact on SCCA, hydrogen and methane production and hence it is necessary to understand their influence to maximize SCCA accumulation. Most of the mentioned studies have separately analyzed each parameter. Nevertheless, the influence of each one of these parameters on SCCA production yield might change depending on the type of carbon source used as substrate regarded in terms of its organic composition defined as the proportion between carbohydrates, proteins, or lipids [71]. In this section, we consider the impact of the organic composition of the waste on the SCCA production yield, and then, we sum up the combined influence of some operational parameters: pH, temperature, organic loading rate and HRT according to the organic composition of substrate.

1.3.1 Organic composition of the waste

There are several types of wastes that can be used as substrates in acidogenic fermentation, including: (i) solid wastes, such as tuna waste [72], mushroom compost [73], apple pomace and winterization oil cake [29], (ii) slurry-like wastes, such as primary sludge from a wastewater treatment plant (WWTP) [74], waste activated sludge (WAS) from a WWTP [29,75], food waste [76], organic fraction of municipal solid waste (OFMSW) [77], maize silage [69], and; (iii) liquid wastes or wastewaters, such as dairy wastewater [25], palm oil mill wastewater [78], sugar industry wastewater [79], olive mill wastewater [27,29,61], glycerol [29,80] and paper mill effluent [65]. The difference between solid and slurry-like waste lies in the solids content. In this study, we consider slurry-like waste to have less than 50% in solids content.

Besides, it makes no sense to compare SCCA production yields obtained from different substrates unless the experiments are carried out in exactly the same conditions since several operational parameters might influence SCCA yield. Montiel-Jarillo et al. [29] compared different waste streams to determine their acidogenic potential under

mesophilic conditions after pretreating the biomass to inhibit methanogenic activity. The largest SCCA production yields were obtained when using WAS, olive mill wastewater, winterization oil cake, apple pomace and glycerol (in descending order) [29]. Similarly, Silva et al. [80] previously performed experiments to study the SCCA production of several substrates under mesophilic conditions using an inhibitor of methanogenic bacteria. In this case, the substrates that yielded a highest SCCA production (in descending order) were cheese whey, sugarcane molasses, OFMSW, waste glycerol, winery effluent, olive mill wastewater, soapy slurry waste and landfill leachate [80].

As stated before, the organic composition of the waste would affect SCCA production yield. Therefore, in this introduction, the organic matter would be studied in terms of its composition into three different kinds of macromolecules: lipids, carbohydrates and proteins. Very few studies have analyzed SCCA production yield from the main organic matter components [54,64]. Yin et al. [64], for example, found that glucose led to higher SCCA production yields in comparison to peptone (protein) and glycerol (lipid hydrolysate) and that a mixture of glucose, peptone and glycerol led to an even larger SCCAs production yield than glucose alone [64].

In general, lipidic substrates are less preferred than carbohydrates and proteins in fermentation processes. First, hydrolysis of lipids is slower than hydrolysis of carbohydrates and proteins [64]. Secondly, hydrolysis of lipids produces long chain fatty acids (LCFAs) and glycerol. Glycerol can be converted to SCCAs, but LCFAs could inhibit the metabolism of anaerobic bacteria, as they adhere to the cell walls, and they tend to decrease nutrient transportation [54]. Finally, the acidogenic microorganisms have more difficulties to produce SCCAs from glycerol than from carbohydrates or proteins such as glucose or peptone. Due to their lower degradation rate, lipids tend to accumulate in the degraded waste [64]. The highly reduced nature of carbon atoms in glycerol makes its utilization by microorganisms difficult under fermentative conditions [81]. Nevertheless, the use of acclimatized cultures could be a solution to increase the efficacy of acidogenic fermentation from lipidic wastes [82].

Regarding the fermentation of protein-rich substrates, the hydrolysis of proteins could lead to the release of essential nutrients, but the degradation of some amino acids involves hydrogen consumption reactions [54]. Moreover, proteins are less easily biodegraded than carbohydrates, as their structure is more complex than that of

carbohydrates. Thus, when using wastes with high protein content, hydrolysis could be the rate-limiting step [83]. Additionally, proteins can contain different amino acids, and their composition would also influence SCCA production yield. Shen et al. [83] compared the hydrolysis and acidification of tofu (vegetable protein) and white egg (animal protein). They found that white egg conducted to larger SCCA yield than that of tofu [83].

A parameter that can be used to study the impact on the substrate composition on SCCA production yield is the carbon-to-nitrogen ratio. For example, carbohydrates and lipids contribute to increase the carbon-to-nitrogen ratio of a substrate since they have a higher content in carbon than nitrogen, while proteins tend to decrease the carbon-to-nitrogen ratio because of their high nitrogen content. An optimal carbon-to-nitrogen ratio is needed in any anaerobic digestion process to ensure that nutrients are balanced for the maintenance and growth of the bacteria. Moreover, a low carbon-to-nitrogen ratio could be related to the release of free ammonia or ammonium, which causes acidogenesis inhibition [84]. The optimal range of the carbon-to-nitrogen ratio for anaerobic digestion is 20-30 [85].

Finally, the combination of different kinds of substrates (co-fermentation) generally leads to higher SCCA production yields than the fermentation of different substrates separately. Table 1.1 presents studies showing that SCCA production yield is increased by mixing different substrates. For example, a mixture of carbohydrates and proteins boosts SCCA production yield, rather than using these two substrates individually: Feng et al. [86] found that adding rice (carbohydrate-rich substrate) to WAS (protein-rich substrate) enhances SCCA production in comparison with the fermentation of only WAS [86]. Co-fermentation of more than one kind of substrate can be used to enhance the SCCA production yield by balancing the carbon-to-nitrogen ratio. Besides, co-fermentation processes involve other advantages [87,88]: (i) balancing of micronutrients and moisture, (ii) dilution of inhibitory compounds, (iii) enhancing of the pH-buffer capacity and (iv) providing an active inoculum adapted to the substrate when one or more of the substrates used are waste.

Table 1.1. SCCA production yield obtained by co-fermentation of substrates with different organic composition.

Primary substrate	Secondary substrate	Main component of the primary substrate	Main component of the secondary substrate	SCCA production yield of the primary substrate	SCCA production yield of the mixture (primary and secondary substrates)	Increment of the SCCA production yield obtained by co-fermentation in comparison to primary substrate fermentation*	Reference
Primary sludge from a WWTP	Oleic acid	Proteins	Lipids	119 mg COD g^{-1} VS**	176 mg COD g^{-1} VS**	48%	[87]
Secondary sludge from a WWTP	Oleic acid	Proteins	Lipids	41 mg COD g^{-1} VS**	160 mg COD g^{-1} VS**	288%	[87]
Secondary sludge from a WWTP	Aged refuse	Proteins	Carbohydrates	83 mg COD g^{-1} VSS	184 mg COD g^{-1} VSS	122%	[62]
Secondary sludge from a WWTP	Rice	Proteins	Carbohydrates	101 mg COD g^{-1} VSS	520 mg COD g^{-1} VSS	413%	[86]
Pretreated secondary sludge from a WWTP	Potato peel waste	Proteins	Carbohydrates	132 mg COD g^{-1} VS	344 mg COD g^{-1} VS	160%	[89]
Primary sewage sludge from a WWTP	Organic waste	Proteins	Carbohydrates	250 mg COD g^{-1} VS	301 mg COD g^{-1} VS	20%	[63]
Pretreated secondary sludge from a WWTP	Food waste	Proteins	Carbohydrates and proteins	132 mg COD g^{-1} VS	282 mg COD g^{-1} VS	113%	[89]

* The increment was calculated by dividing the difference between the SCCA yield of the mixture and the SCCA yield of the primary substrate, by the SCCA yield of the primary substrate, expressed as percentage.
** SCCA yields calculated from the reported data

1.3.2 pH and organic composition of the waste

pH is one of the most important key factors affecting the SCCA production yield because it affects the prevalence of acidogenic or methanogenic microorganisms [90,91]. The accumulation of SCCA would lead to a decrease in pH, which can inhibit the methanogenic microorganisms [92]. Acidogenic microorganisms can also be inhibited in acidic environments (pH<5.0) or extremely alkaline conditions (pH>12.0) [93]. Apart from the acidogenesis step, the working pH also has a notable effect on the hydrolysis step [94]. Since the hydrolysis step efficiency is subjected to the complexity of the substrate, optimal pH strongly depends on the nature of the substrate used.

Table 1.2 shows the pHs reported as optimal to lead to high SCCA production yields depending on the substrate used and its organic composition. In general, it can be concluded that acidogenic fermentation of a solid or slurry-like waste, independently of its organic composition, needs an alkaline pH to reach high SCCA production yields. Solid or complex wastes usually require an alkaline pH to boost both, hydrolysis and acidogenesis steps [69]. As an example, when primary or WAS from a WWTP is used, the optimal pH range seems to be 8.0-12.0 [90,95]. In this case, alkaline conditions enhance the SCCA production since they stimulate the hydrolysis of the sludge by the ionization of the charged groups of the extracellular polymeric substances and the consequent release of fermentable carbohydrates and proteins [93,96]. Moreover, an alkaline environment inhibits the growth of methanogens, such as *Methanobacterium sp*. and *Methanobrevibacter sp*., avoiding the consumption of the produced SCCAs [93,96]. Conflicting results have been reported in studies using food waste as substrate. Dahiya et al. [97] performed several batch tests at pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 and found that pH 11.0 led to the highest degree of acidification. In this case, SCCA production might be favoured by a higher availability of hydrolyzed compounds, similarly to the fermentation of sludge. On the contrary, Zhang et al. [98] carried out batch experiments at pH 5.0, 7.0, 9.0 and 11.0 and reported that the SCCA concentration and production yield were maximized at pH 7.0. Moreover, Zhang et al. [98] quantified the degree of solubilization of the substrate and found that pH 7.0 conducted to the maximum degree of solubilization for carbohydrates, proteins and lipids in comparison to the rest of pH conditions tested. These differences might arise from the different organic composition of the food waste, which was not reported by Dahiya et al. [97].

Table 1.2. Optimal pH reported to maximize SCCA production yields for different substrates.

Substrate	Main component of the substrate	Physical state	pH range studied	Optimal pH	Reference
Secondary sludge from a WWTP	Proteins	Slurry-like	7.0-12.0	11.0 for hydrolysis 9.0 for acidification	[95]
Primary sludge from a WWTP	Proteins	Slurry-like	3.0-11.0	10.0	[99]
Slaughterhouse wastewater	Proteins	Liquid	5.5 and 10.0	10.0	[65]
Gelatin-rich wastewater	Proteins	Liquid	4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 5.0 and 7.0	6.0 7.0	[100] [101,102]
Mushroom compost	Carbohydrates	Solid	4.0-12.0	10.0	[73]
OFMSW	Carbohydrates	Slurry-like	5.5 and 10.0	10.0	[65]
Maize silage	Carbohydrates	Slurry-like	5.0 and 11.0	11.0	[69]
Food waste	Carbohydrates	Slurry-like	5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 5.0, 7.0, 9.0 and 11.0 4.0, 5.0, 6.0	10.0 7.0 6.0	[97] [98] [76]
Cheese whey	Carbohydrates	Liquid	3.5-6.0 5.0 and 11.0	5.3-5.5 5.0	[66] [69]
Paper mill wastewater	Carbohydrates	Liquid	5.0-6.0 5.5 and 10.0	5.5-6.0 10.0	[66] [65]
Winery wastewater	Carbohydrates	Liquid	5.5 and 10.0	10.0	[65]
Citrus wastewater	Carbohydrates	Liquid	4.0, 5.0, 6.0 and 7.0	7.0	[71]
Crude glycerol	Lipid derived product	Liquid	5.5 and 10.0	10.0 at mesophilic conditions 5.5 at thermophilic conditions	[65]
Tuna waste	Proteins and carbohydrates	Solid	5.0-10.0	8.0	[72]
Microalgae biomass	Proteins, carbohydrates and lipids	Slurry-like	5.0 and 11-0	11.0	[69]
Olive mill solid waste	Carbohydrates and lipids	Slurry-like	5.0 and 9.0	9.0	[103]

Regarding liquid wastes, the optimal pH varies depending on its organic composition. Hydrolysis and acidification of lipids and proteins need a different pH from simple carbohydrates to achieve high SCCA production yields [104]. Fermentation of monomer-rich substrates, which do not require such an extensive hydrolysis, seems to be more favourable at low pHs. Methanogens are active in a pH range between 6.5-8.2 and its activity is inhibited at higher or lower pHs [57]. However, acidogens can grow in a wider range of pH than methanogens. In this way, Table 1.2 shows that carbohydrate-rich liquid wastes, such as cheese whey [66,69] and citrus wastewater [71] lead to higher SCCA production yield at neutral pH in comparison to acidic or basic conditions. When using paper mill wastewater, Bengtsson et al. [66] reported that neutral pH was more convenient, while Garcia-Aguirre et al. [65] found that alkaline conditions result in higher SCCA production. Despite being the same type of waste, they might have a very different composition. Garcia-Aguirre et al. [65] also found that alkaline pH leads to higher SCCA production yields than lower pH for winery wastewater.

From the aforementioned studies, it can be concluded that for liquid wastewaters that are mainly composed of carbohydrates, the optimal pH for SCCA production depends strongly on the complexity of the carbohydrates forming the waste. As a general rule, alkaline pH would be needed when using wastewaters with complex carbohydrates and neutral pHs are more favorable for liquid wastes with simpler carbohydrates that do not need intensive hydrolysis.

There are fewer studies on optimal pH conditions for protein-rich or lipid-rich liquid substrates. Garcia-Aguirre et al. [65] found that the optimal pH when using crude glycerol as a substrate was 10.0 in mesophilic conditions and 5.5 in thermophilic conditions. In the case of protein-rich substrates, one might find different results depending on the type of waste. Slaughterhouse wastewater was reported to yield more SCCAs at alkaline conditions [65], while gelatin-rich wastewater produced better results at neutral pH [100–102]. Probably, these differences are due to the different complexity of the proteins forming the waste. It seems that the higher the complexity of the proteins, the higher the optimal pH.

Recently, novel pH-stepwise control processes are being considered as a promising alternative to enhance SCCA production from a substrate mainly composed by complex proteins [74,95]. In pH-stepwise processes, pH is maintained firstly at alkaline levels to

promote the hydrolysis step, and then, is lowered to a pH level closer to neutrality to perform the acidogenesis. Wang et al. [74] demonstrated that a pH stepwise control strategy that consisted in maintaining pH at 11.0 during the early stage and then keeping pH at 9.0 during the rest of the fermentation led to the higher SCCA production yields from primary sludge. pH 11.0 at the beginning increased the available soluble proteins and carbohydrates while inhibiting methanogenic activity and, subsequently, pH 9.0 increased the abundance and diversity of acidogenic bacteria while keeping high levels of soluble substrates.

During acidogenic fermentation, the accumulation of SCCAs could lead to a severe drop of pH. At low pH levels, acids exist as undissociated molecules, which could pierce the cell membrane and reduce the microbial activity by directing the energy generated to cell maintenance [105]. Therefore, pH control and extraction of the produced acids would serve to maintain a stable operation of the process. On the contrary, pH control would imply higher process costs due to the addition of acids and bases to adjust pH and the automatic control loop installation. In the design and scaling up of a SCCA production process, it would be necessary to study if the increment of SCCAs produced after implementing a pH control loop compensates the costs derived from pH control.

1.3.3 Temperature and organic composition of the waste

Temperature is another relevant operational parameter since it influences the metabolic rates, enzymatic activities and microorganisms' growth and decay rates [106]. For this reason, the optimal temperature for enhancing SCCA production yield depends on the microbial consortium composition and the organic composition of the waste. Several studies have carried out experiments on SCCA production using different substrates at different temperature ranges, namely psychrophilic (4-20ºC), mesophilic (20-50ºC), thermophilic (50-60ºC) and hyperthermophilic (60-80ºC) conditions [65,67,68,106–109].

Most of the authors agree on the mesophilic range being more favorable in terms of SCCA production yield in comparison to psychrophilic conditions [68,106,110,111]. Fernández-Domínguez et al. [106], for example, found that mesophilic conditions when using OFMSW as substrate led to higher SCCA production yields compared to psychrophilic temperatures. Similarly, if the substrate is WAS, increasing the temperature

from psychrophilic to mesophilic ranges boosts the hydrolysis rate, making carbohydrates and proteins more available and rising the activity of the acid-forming enzymes, enlarging, therefore, SCCA production yield [68,111]. Hydrolytic enzymes also show higher activities and the abundance of acidogenic bacteria was richer at mesophilic conditions than at psychrophilic conditions [111]. Due to all of this, there is a clear agreement on the fact that mesophilic conditions are more favorable to SCCA production rather than psychrophilic conditions.

When comparing the effect of mesophilic temperatures with thermophilic and hyperthermophilic temperatures on SCCA production yield from a solid or a slurry-like waste, the more convenient conditions are dependent on the organic composition of the waste. On one hand, some studies found that mesophilic conditions were more favorable than thermophilic conditions for the acidogenic fermentation of different carbohydrate-rich substrates such as: food waste [107,109], OFMSW [65,106] and cow manure mixed with maize silage [112]. Nevertheless, several studies found higher SCCA production yields at thermophilic conditions than at mesophilic conditions when using protein-rich substrates such as: WAS [65,67,113,114] and meat and bone meal [65]. Based on the above analysis, it can be concluded that mesophilic temperatures boost SCCA production yields with carbohydrate-rich solid substrates rather than thermophilic conditions; and thermophilic temperatures enhance SCCA production with protein-rich substrates in comparison with mesophilic conditions. The reason for the difference is that increasing the temperature from mesophilic to thermophilic range boosts the hydrolysis of the substrates by rising the key hydrolases activities [67], despite the key acid-forming enzymes have been reported to have higher activities at mesophilic conditions rather than thermophilic conditions [68]. Proteins are characterized by their low biodegradability because of their complex structure (in comparison to carbohydrates) so the hydrolysis is considered the rate-limiting step in SCCA biosynthesis from proteins [83]. Thus, carbohydrate-rich solid substrates that do not need such extensive hydrolysis as protein substrates would have higher SCCA production yields at mesophilic conditions than at thermophilic conditions.

Less research is reported regarding the effect of temperature on the acidogenic fermentation of liquid substrates. Most of the studies of SCCA synthesis are carried out in the mesophilic range [25,66,69,104,115]. Nevertheless, thermophilic conditions have been reported to be more optimal in terms of SCCA production from several substrates

such as gelatin-rich wastewater (protein-rich substrate) [100], slaughterhouse wastewater (protein-rich substrate) [65], paper mill wastewater (carbohydrate-rich substrate) [65] and winery wastewater (carbohydrate-rich substrate) [65]. Conversely, mesophilic temperatures are more convenient when employing glycerol as substrate (lipid-rich substrate) [65]. Further research about the influence of temperature on SCCA production from liquid wastes is needed.

Besides the substrate, the optimal temperature could also depend on the microbial population. Some bacterial consortia are more sensitive to temperature changes than others are [116]. Thus, inconsistent results between studies that use substrates with similar organic composition could be caused by the biomass present in each case [117], specifically when the acidogenesis is the rate-limiting step. In addition, synergic effects of the temperature and other process parameters on the SCCA production yield have been described, namely pH [65] and substrate composition [77].

1.3.4 Volumetric organic loading rate, hydraulic retention time and organic composition of the waste

The volumetric organic loading rate (OLRv), usually expressed as g COD L^{-1} d^{-1}, is a parameter that affects SCCA production yield since it represents the amount of substrate available per liter of reactor and per day to be transformed into SCCAs. However, to consider the effect of the amount of biomass over the SCCA production yield, a specific organic loading rate (OLRs) (expressed as g COD g^{-1} VSS d^{-1}) should be used.

For a fixed working volume of reactor and a fixed substrate concentration in the influent, the OLRv is inversely proportional to the HRT. OLRv can be increased in two ways: by increasing the influent substrate concentration or by lowering the HRT. Since this introduction is dealing with waste streams as substrate in acidogenic fermentation, its concentration cannot easily be changed, so OLRv is normally regulated by tuning the HRT. Therefore, the impact of both OLRv and HRT is evaluated together in this section, focusing on the effect of OLRv on the SCCA production yield.

In general terms, increasing the OLRv rises SCCA production, as there is more substrate available for the microorganisms. Furthermore, methanogen growth is slower than acidogen growth, so raising the OLRv is also a way to wash out the methanogens

from the reactor [118,119]. However, at very high OLRv, the reactor operation could become unstable due to a sharp drop of pH caused by a high SCCA production that can cease the activity of acidogenic microorganisms [120–122].

The optimal OLRv is more dependent on the complexity of each one of the components of the waste rather than the proportion of the different fractions (carbohydrates, proteins, lipids) on it. When working with complex substrates (such as WAS) or complex carbohydrates (like food waste), hydrolysis stage would be the rate-limiting step, so a larger HRT of fermentation is needed. Thus, OLRv must be keep low when working with rather complex proteins or carbohydrates to avoid the increase in the broth viscosity produced by the accumulation of non-hydrolyzed components, which would lead to a low SCCA production due to mass transfer limitations [70,123]. Jankowska et al. [69] compared the SCCA production yield obtained at different HRT and pH from four different substrates, complex/simple carbohydrate-rich substrates (maize silage/glucose), complex/simple protein-rich substrate (microalgae biomass/whey). They concluded that SCCA production yield strongly depends on the complexity of each one of the components of the waste and that an alkaline pH enhances SCCA yield more than an increase in the HRT [69]. Alternately, several pretreatments of wastes described in the literature allow to apply larger OLRv by enhancing the hydrolysis step efficiency [124].

There is no doubt that the application of high OLRv would help to wash out the methanogens, but if this OLRv is high enough, some acidogenic microorganisms would be also washed out the reactor. Therefore, the OLRv affects, at the end, all the microbial communities present in the reactor [116,118] and, in consequence, it could modify the SCCA composition distribution, which is discussed in the next section.

1.4 Operational parameters affecting SCCA composition distribution

In the previous section, the influence of different operational parameters together with the organic composition of the waste on the SCCA production yield was analyzed. However, the impact of these parameters on the production of a particular SCCA or a mixture of SCCAs is even more difficult to determine. As previously mentioned, acidogenic fermentation is a complex process where numerous biological reactions take place. Moreover, when using MMC, the different microorganisms present in the reactor could follow different metabolic pathways and could also vary with applied operational

conditions. Thus, the spectrum of obtained products from acidogenic fermentation by MMC could be very wide. Therefore, more knowledge is needed to understand how to drive the acidogenic fermentation process to obtain a specific SCCA or a mixture of SCCAs.

1.4.1 Organic composition of the waste

Some authors studied the influence of the organic composition of the waste on the SCCA composition of the effluent form acidogenic fermentation without considering any other operational conditions. For example, Alibardi and Cossu [54] performed batch fermentation experiments using mixtures of four fractions of different organic wastes and analyzed the produced SCCAs. They only found a correlation between butyric acid production and the chemical composition of the wastes (in terms of percentage of carbohydrates, lipids, and proteins). Carbohydrate content seemed to be the main factor influencing butyric acid concentration. Nevertheless, the correlation was weak, since R^2 value was 0.809 [54]. Ma et al. [89] obtained similar results by carrying out fermentation batch experiments using mixtures of WAS and potato peel or food waste in different ratios. In all the trials, acetic acid was the main product and when the carbohydrate content in the waste was higher than that of proteins, butyric acid and ethanol concentrations increased while propionic and valeric acid decreased. They also correlated propionic, butyric and valeric acid concentrations with lipid, starch, and protein consumption but, again, R^2 values were quite low (between 0.62-0.81) [89]. Yin et al. [64] performed batch tests using glucose, peptone and glycerol as model compounds of simple carbohydrates, proteins and lipids respectively, to elucidate more clearly which component of organic matter is responsible for the formation of each SCCA. It was observed that butyric acid was the predominant compound produced from glucose, acetic acid was the main product from peptone and propionic acid the main one when using glycerol. Additionally, when a mixture of the three components was used, the SCCA composition of the effluent was not the result of an additive effect of each component since propionic acid proportion would be expected to be the 32% of the total SCCAs (in a COD basis) and it was actually the 40% of the total SCCAs [64]. Once again, these studies suggest that the organic composition of the waste is the main factor that influences the SCCA production yield and the composition of the obtained SCCAs, but other parameters may also affect. Therefore, in the following sections, the interactional effect of pH, temperature and

bacterial composition with the organic composition of waste on the SCCA composition is revised. Furthermore, more studies using model compounds of carbohydrates, proteins, and lipids as substrates, but working in continuous or semicontinuous conditions are needed to evaluate the effect of organic composition of the waste on SCCA composition in long term processes.

1.4.2 pH and organic composition of the waste

The effect of pH on SCCA composition is by far the most studied among the different operational parameters. In this section, the joint impact of pH and organic composition of the waste on the composition of the produced SCCA rich stream is analyzed. Table 1.3a and Table 1.3b collect SCCA distributions obtained in the fermentation of wastes with different organic composition.

According to the reviewed studies, there is agreement on the SCCAs obtained from protein-rich substrates at different pH. Thus, pH lower than 5.0 leads to propionic acid while a pH between 5.0 and 11.0 leads to acetic acid [100,125,126]. Liu et al. [125] reported that a pH of 12.0 produced butyric acid as a main component. The rest of the SCCA components with lower presence vary among the different studies [100,125]. In any case, it can be affirmed that pH is an appropriate tool to change SCCA composition when using protein-rich substrates.

Nevertheless, carbohydrate-rich substrates yielded very different SCCA compositions in the different studies shown in Table 1.3a. On the one hand, Atasoy et al. [115] performed experiments using glucose as substrate (carbohydrate) and determined the SCCA composition obtained using different types of inoculums. The main SCCA component was butyric acid independently of the inoculum used for a wide range of pH conditions (pH between 5.0 and 10.0) [115]. On the other hand, pH has a strong effect when using substrates mainly composed of complex carbohydrates, namely paper mill wastewater and cheese whey [66]. In paper mill wastewater fermentation experiments, an increase of pH from 4.9 to 6.0 changed the main SCCA components from acetic acid (49%), butyric acid (18%) and propionic acid (13%); to butyric acid (33%), propionic acid (23%) and acetic acid (22%). An increment of pH from 5.3 to 6.0 in cheese whey acidogenic fermentation experiments, shifted the main SCCA components from acetic

acid (51%), butyric acid (24%) and propionic acid (19%); to propionic acid (41%), acetic acid (31%) and butyric acid (10%) [66].

Table 1.3b summarizes studies of SCCA compositions obtained from fermentation of wastes that are formed of carbohydrates and proteins [52,109,127,128] and carbohydrates and lipids mixtures [103,104]. In these cases, the substrate heterogeneity makes it even more complicated to link the organic composition and the pH with the final SCCA composition obtained. Luo et al. [127] performed experiments of different pH of a mixture of WAS (protein-rich substrate) and wine vinasse (carbohydrate-rich substrate) (1:1, in COD proportions) and obtained a very different SCCA distribution with respect to the protein rich substrates reported in Table 1.3a [100,125–127]. The addition of wine vinasse changed the main SCCA component at different pH ranges: acetic acid (from pH 3.0 to 5.0), acetic and propionic acids (from pH 6.0 to 9.0) and acetic acid (pH 10.0) [127]. Food waste (carbohydrate-rich and protein-rich substrate) is one of the most studied substrates. Different studies arrived at different SCCA distributions at similar pH conditions [52,109,128]. Feng et al. [52] discussed the different pathways that can take place at different pH conditions and would explain the SCCA compositions obtained. Homolactic fermentation (following EMP and PPP pathways) takes place at pH 3.2-4.5 leading to lactate production. Heterolactic fermentation (following PK pathway) occurs at pH 3.2-5.0 leading to lactate and ethanol production. Ethanol type fermentation (following EMP pathway) takes place at pH 4.5 and directs fermentation to ethanol, acetate and hydrogen. Ethanol fermentation (following Entner-Doudoroff pathway) takes place at pH 4.4-6.0 and yields ethanol. Heterolactic fermentation (following Bifidus pathway) occurs at pH 4.5-5.0 leading to acetate and lactate. Acetone-butanol-ethanol fermentation (following EMP pathway) takes place at pH 4.7-4.9 leading to acetone, butanol, ethanol and hydrogen. Butyrate fermentation (following EMP and PPP pathways) occurs at pH 5.0 and leads to butyrate, acetate and hydrogen. Finally, mixed acid fermentation (following EMP and PPP pathways) takes place at pH 6.0 and yields acetate, propionate, butyrate, valerate and hydrogen [52]. Since several pathways can take place at the same pH conditions, the differences between organic composition of the food waste used in the reported studies might explain the different SCCA distributions obtained [52,109,128].

Table 1.3a. Effect of pH on SCCA distribution by using wastes with different organic composition.

Substrate	Main component of the substrate	Inoculum used	pH	Main products (in descending order) (% of the total SCCA)	Reference
Gelatin-rich wastewater	Proteins	Methanogenic anaerobic sludge	4.0 4.5 5.0 5.5 6.0 6.5 7.0	Propionic acid (32%) and acetic acid (15%) Propionic acid (27%) and acetic acid (18%) Acetic acid (23%) and propionic acid (20%) Acetic acid (25%), isobutyric acid (13%), butyric acid (13%), isovaleric acid (13%), propionic acid (12%) and valeric acid (12%) Acetic acid (28%), butyric acid (16%), isobutyric acid (13%) and propionic acid (13%) Acetic acid (32%) and butyric acid (21%) Acetic acid (35%) and butyric acid (22%)	[100]
Sewage sludge from a brewery	Proteins	Pretreated sewage sludge (thermal pretreatment to inactivate methanogens)	3.0 5.0 7.0 9.0 11.0 12.0	Propionic acid (56%) and acetic acid (44%)* Acetic acid (31%), butyric acid (24%) and valeric acid (20%)* Acetic acid (26%), propionic acid (21%), butyric acid (16%) and valeric acid (15%)* Acetic acid (44%) and propionic acid (19%)* Acetic acid (44%) and propionic acid (19%)* Butyric acid (37%) and acetic acid (31%)*	[125]
Secondary sludge from a WWTP	Proteins	Slurry anaerobic biomass	7.0 10.0	Acetic acid (47%) Acetic acid (56%)	[126]
Heat-alkaline pretreated secondary sludge	Proteins	Slurry anaerobic biomass	7.0 10.0	Acetic acid (57%) Acetic acid (65%)	[126]
Glucose solution	Carbohydrates	Small granular anaerobic biomass	5.0 8.0 10.0	Butyric acid (58%) and acetic acid (24%)* Butyric acid (47%) and acetic acid (35%)* Butyric acid (56%) and acetic acid (42%)*	[115]
		Large granular anaerobic biomass	5.0 8.0 10.0	Butyric acid (52%) and acetic acid (39%)* Butyric acid (50%) and acetic acid (33%)* Butyric acid (58%) and acetic acid (39%)*	
		Slurry anaerobic biomass	5.0 8.0 10.0	Butyric acid (46%) and acetic acid (14%)* Butyric acid (55%) and acetic acid (15%)* Butyric acid (47%) and acetic acid (44%)*	
Paper mill wastewater	Carbohydrates	Acidogenic biomass from a reactor treating paper mill wastewater	4.9 6.0	Acetic acid (49%), butyric acid (18%) and propionic acid (13%) Butyric acid (33%), propionic acid (23%) and acetic acid (22%)	[66]
Cheese whey	Carbohydrates	Acidogenic biomass from a reactor treating paper mill wastewater	5.3 6.0	Acetic acid (51%), butyric acid (24%) and propionic acid (19%) Propionic acid (41%), acetic acid (31%) and butyric acid (10%)	[66]

* The SCCA compositions were obtained (and calculated in some cases) from graphically presented results.

Table 1.3b. Effect of pH on SCCA distribution by using wastes with different organic composition.

Substrate	Main component of the substrate	Inoculum used	pH	Main products (in descending order) (% of the total SCCA)	Reference
Secondary sludge from a WWTP + wine vinasse	Proteins and carbohydrates	No inoculum added (endogenous microorganisms from the substrate)	3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0	Acetic acid (52%) and butyric acid (36%)* Acetic acid (41%), butyric acid (24%) and propionic acid (21%)* Acetic acid (37%), propionic acid (31%) and butyric acid (19%)* Propionic acid (40%) and acetic acid (38%)* Propionic acid (45%) and acetic acid (38%)* Propionic acid (42%) and acetic acid (40%)* Acetic acid (43%) and propionic acid (40%)* Acetic acid (38%) and propionic acid (33%)*	[127]
Food waste	Carbohydrates and proteins	Mesophilic anaerobic digested sludge	5.0 6.0 7.0	Acetic acid (60%) and butyric acid (31 %) Butyric acid (53%) and acetic acid (24%) Butyric acid (43%) and acetic acid (34%)	[109]
	Carbohydrates and proteins	Sludge from anaerobic digester	3.2 4.0 4.2 4.5 4.7 5.0 6.0	Lactic acid (87%) Lactic acid (81%) Lactic acid (81%) Lactic acid (57%) and acetic acid (25%) Butyric acid (56%) and acetic acid (19%) Butyric acid (40%) and acetic acid (40%) Butyric acid (40%) and valeric acid (29%)	[52]
Food waste + mature compost	Carbohydrates and proteins	Biomass from anaerobic digester treating food waste	6.0 7.0	Caproic acid (43-47%), butyric acid (21-25%) and acetic acid (22-23%) Acetic acid (36-38%), caproic acid (24-26%) and butyric acid (18-20%)	[128]
Olive oil mil waste	Carbohydrates and lipids	Anaerobic biomass acclimatized to secondary sludge	5.0 9.0	Acetic acid (60%) and propionic acid (23%) Acetic acid (79%)	[103]
Dairy wastewater	Lipids and carbohydrates	Anaerobic sludge treating influent simulating dairy wastewater	4.0 4.5 5.0 5.5 6.0 6.5	Propionic acid (38%) and acetic acid (18%) Propionic acid (34%) and acetic acid (20%) Propionic acid (28%) and acetic acid (26%) Acetic acid (28%), propionic acid (18%) and butyric acid (13%) Acetic acid (33%), butyric acid (14%) and propionic acid (13%) Acetic acid (34%), butyric acid (14%) and propionic acid (12%)	[104]

* The SCCA compositions were obtained (and calculated in some cases) from graphically presented results.

Moreover, the pH effect on the SCCA composition also depends on the microbial population present and, at the same time, pH influences the microbial community structure. Thus, the pH could yield different SCCA composition depending on the culture history. As an example, Mohd-Zaki et al. [129] carried out glucose fermentation experiments using two different pH regulation modes (progressive mode and reset mode) which led to non-identical SCCA compositions at the same pH levels. Progressive pH regulation led to a gradual change from butyric and acetic acids to acetic acid and ethanol as main components as pH increased, while resetting pH regulation caused a clearly defined change from acetic and butyric acids to acetic acid and ethanol as main components when pH exceeded 6.5 [129].

1.4.3 Temperature and organic composition of the waste

Less studies have been published about the effect of the temperature on the SCCA distribution. Each SCCA can be produced by different strains, and each of these species can have a different optimal growth temperature. Thus, the proportions of each SCCA produced can vary with temperature.

From the results collected in Table 1.4, it can be gathered that SCCA distribution for most of the substrates is not as dependent on temperature as it is on pH. The reported results show that the main SCCA component does not vary with the temperature used, from psychrophilic to thermophilic ranges, for both protein-rich substrates [67,68,111] and carbohydrate-rich substrates [106,130]. The composition of the second major and subsequent SCCAs exhibited more variations with temperature in some studies [111,130]. Nonetheless, several studies that used food waste as substrate, reported different SCCA compositions depending on the temperature. Lim et al. [70] carried out food waste fermentation in a semicontinuous reactor at different temperatures and found that the main SCCAs produced were propionic, acetic and valeric acids at 25ºC; acetic and propionic acids at 35ºC and acetic and caproic acids at 45ºC. Later, Jiang et al. [109] performed batch experiments of food waste fermentation at different temperatures and pointed out that the main SCCAs produced were acetic and propionic acids at 35ºC; propionic and acetic acids at 45ºC and butyric acid at 55ºC.

Table 1.4. Effect of temperature on SCCA distribution by using wastes with different organic composition.

Substrate	Main component of the substrate	Inoculum used	Temperature (ºC)	Main products (in descending order) (% of the total SCCA)	Reference
Ultrasonic-pretreated secondary sludge from a WWTP	Proteins	No inoculum added (endogenous microorganisms from the substrate)	10, 20, 37, 55	Acetic acid (47-49%), isovaleric acid (16-23%) and propionic acid (9-15%)	[68]
Secondary sludge from a WWTP	Proteins	No inoculum added (endogenous microorganisms from the substrate)	15 30	Acetic acid (41%) and propionic acid (22%) Acetic acid (49%), isovaleric acid (14%) and propionic acid (13%)	[111]
Dewatered sludge	Proteins	No inoculum added (endogenous microorganisms from the substrate)	35 55	Acetic acid (45%) and isovaleric acid (20%) Acetic acid (51%) and isovaleric acid (22%)	[67]
Palm mill oil effluent	Carbohydrates	Sludge from the treatment of palm oil mill effluent	30 40 55	Acetic acid (37%) and propionic acid (26%) * Acetic acid (43%) and propionic acid (25%) * Acetic acid (58%) and propionic acid (14%) *	[130]
OFMSW	Carbohydrates	No inoculum added (endogenous microorganisms from the substrate)	20, 35, 45, 55, 70	Acetic acid (28-33%), butyric acid (25-29%) and propionic acid (22-26%)	[106]
Food waste	Carbohydrates and proteins	Anaerobic sludge	25 35 45	Propionic acid (44-46%), acetic acid (20-22%) and valeric acid (18-19%) Acetic acid (30-33%) and propionic acid (25-28%) Acetic acid (49-50%) and caproic acid (23-25%)	[70]
	Carbohydrates and proteins	Mesophilic anaerobic sludge	35 45 55	Acetic acid and (37%) and propionic acid (31%) Propionic acid (38%) and acetic acid (33%) Butyric acid (81%)	[109]

* The SCCA compositions were graphically obtained from presented results.

1.4.4 Bacterial composition in the anaerobic reactor and organic composition of the waste

Regarding bacterial composition, numerous studies analyzed the bacteria present in the anaerobic reactors to link them to the production of different SCCAs. Despite the fact that some species of bacteria are known to be responsible for the production of specific types of SCCAs [18], the number of known acidogenic microorganisms is still limited [78].

Nevertheless, the bacterial communities present in a MMC strongly depend on the organic composition of the substrate [131–133]. Moreover, the bacterial composition could also change with pH [52,91,129,134] as well as other operational parameters such as temperature [135]. Some authors analyzed the combined effect of the pH and the bacterial community composition and structure on the SCCA compositions obtained [23,115]. Atasoy et al. [115] evaluated the SCCA composition obtained from glucose fermentation at different pHs when using three different inoculum types. They found that inoculum structure did not affect SCCA composition while pH was determinant, as discussed before. At the same time, the dominant microbial community affected SCCA production more than SCCA composition [115]. Wang et al. [23] performed experiments of food waste fermentation at different pH conditions with two types of inoculums. pH did not affect SCCA composition in the reactors working with anaerobic activated sludge, since acetic and butyric acids where the main components in all pH conditions. However, when the inoculum was aerobic activated sludge, pH 4 and uncontrolled pH yielded acetic and propionic acids, while pH 5 and 6 led to butyric and acetic acids [22].

Numerous studies carried out acidogenic fermentation experiments of different substrates and analyzed the SCCA compositions obtained as well as the bacterial communities at phylum, class, order, family, and genus level [69,78,91,93,115,133]. Nevertheless, the link of each SCCA produced with the microorganisms responsible for its synthesis is still not clear. Further work is needed to understand the relationship of microbial communities and SCCA composition.

1.4.5 Prediction of SCCA distribution by energy-based metabolic models

As explained above, the SCCA composition of the effluents of an acidogenic fermentation process could depend on multiple factors, making it very difficult to predict what composition can be obtained depending on the experimental conditions applied. Against this background, mathematical modelling of the fermentation processes could be a powerful tool to predict SCCA production. The aim of this section in not to support the interpretations gathered in the previous sections for each operational parameter and its correlation with organic composition. Here, the energy-based metabolic models are presented as the best mathematical tool available up to date to predict the SCCA composition. To the best of our knowledge, the energy-based metabolic models developed so far only consider simple molecules as substrate and pH as the variables that affect SCCA composition.

In this sense, several types of models have been proposed to achieve this objective. The first proposed models were based on the stoichiometry of the fermentation reactions, considering it constant and not considering the possible variations due to changes in operational conditions [136]. From that point, several corrections have been applied to consider operational conditions by using variable stoichiometry models [137,138]. However, these models do not have enough predictive capacity. They are very similar to ASM-family models that were created to describe aerobic processes, which are usually controlled by kinetics [139]. Nevertheless, anaerobic processes supply low energy, and their conversion is limited by the thermodynamic equilibrium [140]. Therefore, energy-metabolic modelling seems to be, nowadays, the best way to predict SCCA composition in fermentation processes.

Energy-based metabolic models assume that in anaerobic fermentation processes, microorganisms would follow those pathways that return more net energy from the substrate and consequently lead to biomass growth. Thus, the SCCAs linked to a higher ATP yield would be the dominant products of the fermentation [33]. Some studies developed models to predict fermentation product spectrum of glucose [141], proteins [142] and co-fermentation of carbohydrates and proteins [33] by MMC.

González-Cabaleiro et al. [141] elaborated a model considering that one single hypothetical microbial population was capable of carry out all the most important metabolic pathways from glucose. It was the first model capable of predicting the effect

of pH on the product spectrum by considering the role of the different electron carriers (ferredoxin, NAD(H) and FAD(H_2)) and a model for the transport of solutes across the cell membrane. The model predicted high yields of butyric acid at low pH, high yields of acetic acid and ethanol at high pH and acetic acid and propionic acid as secondary product at neutral pH [141]. The main difference with the experimental study they took as reference is the presence of acetic and butyric acids as main products at low pH, not only butyric acid [141,143]. Later, Regueira et al. [144] improved this model by including electron bifurcation in the butyrate synthesis pathway and homoacetogenesis that consumed part of the hydrogen produced. After this modification, they succeed predicting both acetic and butyric acids at low pH [144].

Subsequently, Regueira et al. [142] built an energy-based metabolic model for predicting SCCA formation from proteinic substrates. In that study [142], they considered the same approach that González-Cabaleiro et al. [141] held previously. Then, it is again assumed that a virtual microorganism can perform all the pathways and that protein consumption can be limited in case it is not thermodynamically favorable. Moreover, the different amino acids could interact among them limiting the degradation of others, mainly provoked by NADH competition. Net NADH balance must be neutral since in absence of oxygen, there is no electron acceptor. This balance can be affected by external pH, so this model is able to predict the preferred pathways that lead to different SCCAs for the fermentation of a substrate with a defined amino acid composition at different pH [142].

Since wastes are made up of more than one component, Regueira et al. [33] proposed a model for fermentation of a mix with different ratios of carbohydrates and proteins by using glucose and gelatin as model substrates. The addition of glucose to casein fermentation adds a source of reductive power, changing the NADH balance and the pathways that maximize ATP yield. In this case, protein consumption rate is assumed to be lower than glucose consumption rate based on experimental results. One of the main limitations of this model is the little information available about the amino acid profile of the gelatin. If it is used as a design tool, the amino acid composition of the proteins used must be previously determined [33].

In view of all the previously mentioned research, energy-based metabolic models seem to be the best method to mechanistically explain fermentation processes.

Experimental data can be used to prove the predictable capacity of these models, but do not allow us to extrapolate to operational conditions different to those investigated. Nevertheless, mechanistic models allow us to explore SCCA distribution under different operational conditions by changing those defined as environmental conditions. Future work is needed to build models for more complex substrates and introduce the hydrolysis step in the metabolic networks.

1.5 Examples of application of bioproduced SCCAs

1.5.1 Polyhydroxyalkanoates (PHAs) synthesis starting from SCCAs

Polyhydroxyalkanoates (PHAs) are some of the multiple products that can be obtained from SCCAs. PHAs can be defined as biodegradable polyesters, composed of hydroxyalkanoic acids, and synthesized by bacteria [145,146]. There are more than 150 different hydroxyalkanoic acids that can be integrated into the PHAs' molecular structure. Consequently, the properties of the resulting polymers depend on their composition [145]. PHAs are usually classified into two different groups depending on the number of carbon atoms of their monomers. Short chain length PHAs (scl-PHAs) are composed of monomers with 3-5 carbon atoms while medium chain length PHAs (mcl-PHAs) contain monomers with 6-14 carbon atoms [147]. scl-PHAs are characterized for having a high degree of crystallinity and high melting and low glass transition temperatures, making them fragile and difficult-to-deform materials. Conversely, mcl-PHAs are less crystalline so they are elastomeric materials that can be used in high value-added applications [147]. Their glass transition temperature is below zero and their melting point is lower than scl-PHAs, so it is easier to mold them [148]. Thus, PHAs with a wide range of properties can be produced and a great number of potential applications can be found for these polymers [149]. Nevertheless, PHAs have gained attention mainly because they are biocompatible and fully biodegradable, and their mechanical properties are comparable to petroleum-based plastics such as polyethylene and polypropylene [150].

Today, PHA production at an industrial scale is carried out by using pure cultures or genetically modified strains. The main disadvantage of PHAs production processes with pure cultures is that they usually require highly pure substrates [147]. Therefore, the PHAs obtained are not commercially competitive against fossil-fuel-derived plastics since their production costs are between 2.0 and 4.6 times higher than the conventional

plastics costs [151]. Besides, pure cultures need large quantities of co-substrates to obtain polymers containing a relatively low fraction of monomers different from 3-hydroxybutyrate (3HB) [152]. It is also possible to produce PHAs by chemical synthesis or by employing genetically modified plants, but these processes have been considered less interesting for the industry.

On the one hand, PHAs can be chemically synthesized from substituted propiolactones, but this process will never be competitive with bacterial fermentation since lactone monomers are very costly [153]. Furthermore, chemical synthesis results in polymers with lower molecular weights in comparison to the ones obtained by microbial synthesis [154]. On the other hand, PHA synthesis using genetically modified plants is a process that still needs to deal with several limitations, namely control of monomer composition, plant transformation processes, expression of the transformed genes in the following generations and the complex extraction of the polymer from the intracellular compartments [155].

Consequently, the current tendency in research is to develop processes carried out by MMC processes. PHA production by MMC follows natural selection principles since microorganisms with PHA storage ability compete with those microorganisms that do not accumulate PHAs. MMC based processes can be carried out in non-sterile conditions and can use organic waste as a carbon source since MMC are more adaptable. Thus, these systems are considered more robust than pure culture based processes, and simultaneously, more cost-efficient [147,152]. As an example, Crutchik et al. [151] estimated that the minimum PHA cost obtained from sewage sludge were 1.26 and 2.26 US$ kg^{-1} PHA for large and small WWTPs, respectively. These values are quite similar to the fossil-fuel derived plastics cost (1.2 US$ kg^{-1} plastic) and considerably lower to cost the PHA obtained by using pure cultures (between 2.4-5.5 US$ kg^{-1} PHA) [151]. Furthermore, MMC based processes can produce a wide range of copolymers with a different composition depending on the feedstock used [152]. Since MMC contain a high variety of bacteria, different pathways can be followed. Copolymers based on 3HB synthesized by MMC can also contain monomers of 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx), 3-hydroxy-2-methylvalerate (3H2MV) and 3-hydroxy-2-methylbutyrate (3H2MB) [156]. Therefore, in MMC based processes, one of the easiest ways to modify the final properties is by changing the carbon source used as substrate. In the literature, up to four natural PHA synthetic routes starting from

different carbon sources have been described (Figure 1.2). Another ten engineered pathways have also been developed [157] but in this introduction, engineered pathways were not considered for PHA production by MMC.

Pathway I starts with the conversion of a sugar to acetyl-CoA by glycolysis and proceeds with the formation of acetoacetyl-CoA from the condensation of two acetyl-CoA molecules by β-ketothiolase (PhaA), which is then transformed into 3-hydroxybutyryl-CoA by NADPH-dependent reductase (PhaB). Finally, the polymerization of 3-hydroxybutyryl-CoA by PHA synthase (PhaC) leads to the formation of P(3HB) monomers. This pathway is the most well-known and is typically used by *Ralstonia eutropha* [158]. Concurrently, PHA depolymerase (PhaZ) catalyzes PHA degradation to form 3-hydroxybutyrate, 3-hydroxybutyrate is transformed to acetoacetic acid by 3-hydroxybutyrate dehydrogenase and acetoacetyl-CoA is regenerated from acetoacetic acid by acetoacetyl-CoA synthetase [154].

Pathway II uses fatty acids as substrates, which are converted to acyl-CoA, 3-ketoacyl-CoA, enoyl-CoA and S-3-hydroxyacyl-CoA by the β-oxidation cycle. These intermediates are used to synthesize R-3-hydroxyacyl-CoA by the action of acyl-CoA oxidase and enoyl-CoA hydratase, 3-ketoacyl-CoA reductase (FabG), (R)-enoyl-CoA hydratase/enoyl-CoA hydratase I (PhaJ) and epimerase, respectively. Polymerization of R-3-hydroxyacyl-CoA leads to mcl-PHAs by specific PhaZ [152,159].

Pathway III starts from the commonly acknowledged ‘unrelated’ carbon sources, which can be defined as carbon sources with a molecular structure completely different to the resultant PHA monomers [159]. Some examples of unrelated carbon sources are glucose, fructose, glycerol, gluconate, ethanol, and acetate [160]. Through this pathway, also named *de novo* fatty acid synthesis, carbon sources are firstly transformed into acetyl-CoA, acetyl-CoA into malonyl-CoA, and malonyl-CoA into malonyl-ACP. Malonyl-ACP is converted to R-3-hydroxyacyl-ACP, which is then transformed into R-3-hydroxyacyl-CoA by 3-Hydroxyacyl-ACP-CoA transferase (PhaG). Finally, R-3-hydroxyacyl-CoA polymerization gives rise to mcl-PHAs. Both Pathways II and III have been described for most of the *Pseudomonas* spp. [158].

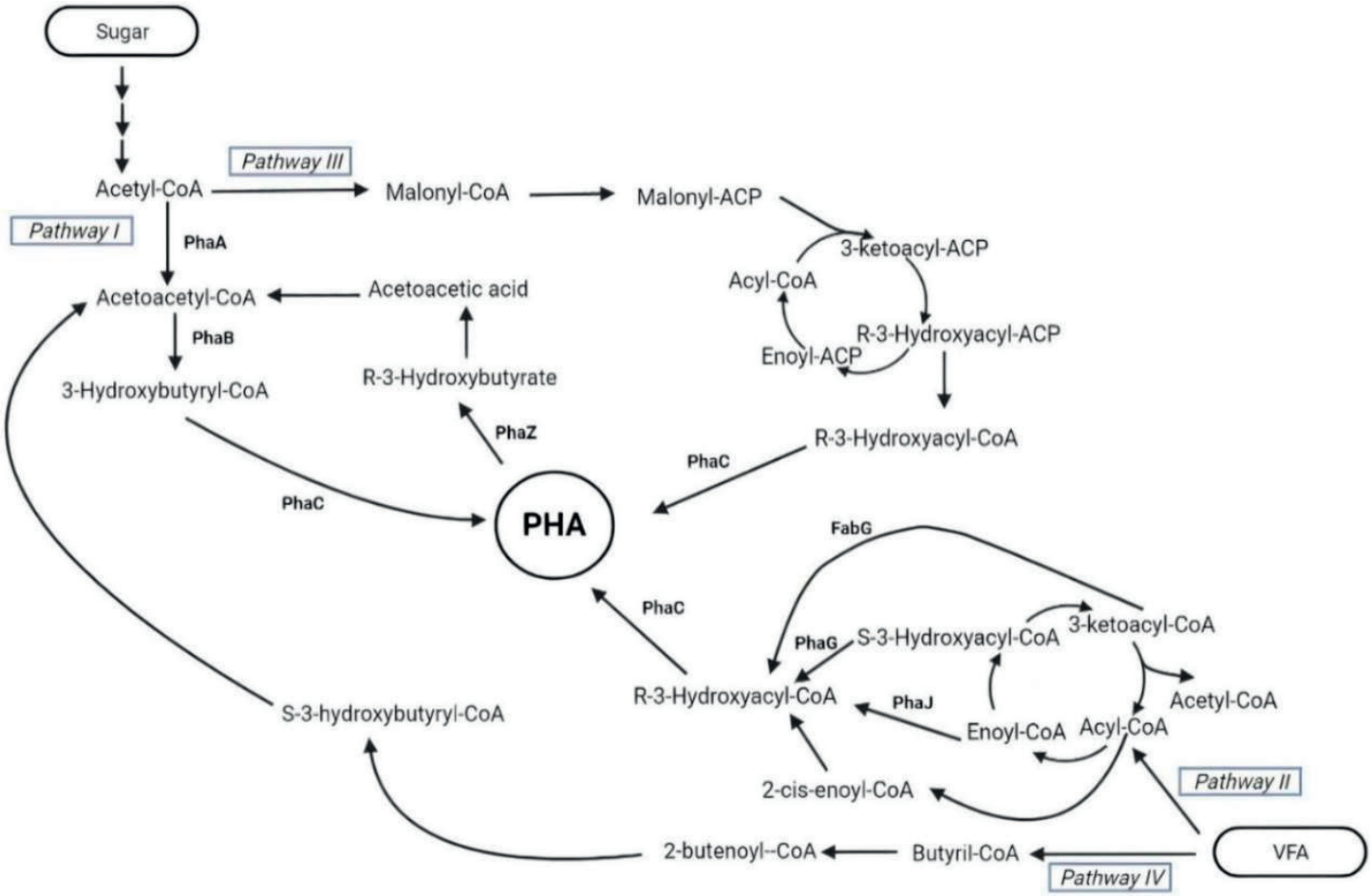


Figure 1.2. Metabolic pathways for PHA synthesis (adapted from Meng et al. [157]). PhaA, β-ketothiolase; PhaB, NADPH-dependent reductase; PhaC, PHA synthase; PhaG, 3-Hydroxyacyl-ACP-CoA transferase; PhaJ, (R)-enoyl-CoA hydratase/enoyl-CoA hydratase I; PhaZ, PHA depolymerase; FabG, 3-ketoacyl-CoA reductase. Created with BioRender.com.

Pathway IV is specific for butyric acid, which could be substrate in PHA synthesis without entering the β-oxidation cycle. Butyric acid is transformed into S-3-hydroxybutyryl-CoA, which is then converted into acetoacetyl-CoA that would follow the same steps of Pathway I to produce P(3HB) monomers. This fourth pathway was observed in Rhizobium (Cicer) sp. Strain CC 1192 [154].

Traditionally, the most widely used substrate in PHA synthesis with pure cultures has been glucose (Pathway I) [147]. However, PHA production by a MMC based process usually starts from SCCAs (Pathway II and IV). On the one hand, SCCAs are preferred with respect to other substrates, such as glycerol or carbohydrates, since the last two have the tendency to synthetize glycogen instead of PHAs [161]. Moreover, the synthesis starting from SCCAs is energetically more favourable because their complete β-oxidation produces more energy in the form of ATP molecules than the oxidation of a molar equivalent of glucose [162]. However, when using bioproduced SCCAs as substrate, the properties and monomer composition of the resultant PHAs would depend on the composition of the SCCA-rich-stream [163–165] used as raw material. It has been demonstrated that when using SCCAs with an even number of carbons, polymers rich in 3HB monomers are formed. On the contrary, feeding SCCAs with an odd number of carbons lead to 3HV monomers [166]. In this sense, the odd-to-even ratio is defined as the sum of odd-equivalent SCCAs such as propionic or valeric acids, divided by the sum of even-equivalent SCCAs, such as acetic or butyric acids [29] and it can be used as a parameter to evaluate the quality of the effluents obtained from the acidogenic fermentation to be used as feedstocks in PHA production. Pure polyhydroxybutyrate (PHB) is brittle and stiff, but the incorporation of 3HV in the polymer (as in PHB-co-PHV) enhances elasticity and flexibility [167]. Thus, high odd-to-even ratios are desired in order to enhance polymer mechanical properties [61]. Table 1.5 sums the composition and properties of the PHAs obtained when using different SCCAs or mixtures of SCCAs in MMC based processes. Since the polymerization and crystallization have a great influence on the final properties of PHAs and they take place inside the cell cytoplasm, the properties of the PHAs produced by MMC based processes are not necessarily the same as the properties of PHAs produced by pure cultures [152].

Table 1.5. Thermal and mechanical properties of PHAs produced by MMC cultures. HAc, acetic acid; HPr, propionic acid; HBt, butyric acid; HV, valeric acid; T_m, melting temperature; ΔH_m, melting enthalpy; T_g, glass transition temperature; PDI, polydispersity index; M_w, weight average molecular weight. NA denotes no available information.

Substrate	SCCA composition (% mol) (HAc:HPr:HBt: HV:other)	PHA composition (% mol) (3HB:3H2MB:3H V:3H2MV:3HHx)	PHA content (% gPHA g^{-1} VSS)	Thermal properties						Molecular weight	Reference
				$T_{m(1)}$ (ºC)	$T_{m(2)}$ (ºC)	Total ΔH_m (J g^{-1})	T_{g1} (ºC)	T_{g2} (ºC)	PDI	M_w (x 10^5)	
Acetate	100:0:0:0:0	90:4:4:1:1	-	171	-	77	5	-	2.0	8.1	[156]
Acetate	100:0:0:0:0	66:4:28:2:0	-	96/109	146/161	9	-6	-	1.4	5.5	[168]
Acetate	100:0:0:0:0	100:0:0:0:0	21	171	-	64	-	-	1.3	32	[169]
Acetate	100:0:0:0:0	96:0:4:0:0	52	-	-	-	-	-	2.2	0.9	[170]
Acetate	100:0:0:0:0	NA	26	93	150	32	-6	-	2.3	2.2	[171]
Propionate	0:100:0:0:0	12:6:63:14:6	-	89	-	18	-14	-	2.0	4.5	[156]
Propionate	0:100:0:0:0	11:13:35:41:0	-	84	20	-	-0.2	-	1.5	5.6	[168]
Propionate	0:100:0:0:0	NA	30	92	38	104	-18	-	2.8	4.3	[171]
Butyrate	0:0:100:0:0	83:5:7:2:2	-	137	150	50	3	-	1.7	9.0	[156]
Valerate	0:0:0:100:0	12:5:78:4:1	-	98	-	2	-12	-	3.9	6.2	[156]
Acetate+ propionate	82:18:0:0:0	55:5:30:9:0	-	124	156	632	−8	-	3.1	3.9	[168]
Acetate+ propionate	54:47:0:0:0	46:8:32:15:0	-	70	97	19	-2	-	1.7	5.0	[168]
Acetate+ propionate	22:78:0:0:0	20:9:39:32:0	-	119	155	10	-6	-	1.4	5.5	[168]
Acetate+ propionate	60:40:0:0:0	94:0:6:0:0	25	157	-	54	1	-	1.3	33	[169]
Acetate+ propionate	84:16:0:0:0	60.3:0:39.7:0:0	54	-	-	-	-	-	3.2	1.4	[170]
Acetate+ propionate	64:36:0:0:0	NA	32	82	24	93	-19	-	1.7	2.3	[171]
SCCA mix	47:18:22:13	61:0:39:0:0	77	113	138	-	-16	-	2.3	2.1	[165]
SCCA mix	73:13:12:0.02	79:0:21:0:0	68	121	137	-	-10	-	2.7	3.9	[165]
SCCA mix	74:7:16:3	85:0:15:0:0	56	134	147	-	-1	-	2.3	6.5	[165]

Furthermore, the cost of the carbon source for PHA biosynthesis can reach 50% of the final production cost [148], so the use of waste-derived SCCAs would clearly reduce the price of the final product and make it competitive with fossil-fuel-derived polymers. Also, it would be possible to integrate PHA production and wastewater or organic waste treatments [161]. In a MMC based process using waste or wastewater as substrate, PHA synthesis takes place in four steps:

1) Acidogenic fermentation of the waste material to produce a SCCA-rich stream.
2) Enrichment of a PHA-accumulating population from aerobic activated sludge by feast-famine feeding strategies.
3) Maximization of the accumulation of PHAs in a batch process with nutrient limitation.
4) Extraction of the PHAs from the biomass.

Factors influencing PHA production in the enrichment and accumulation steps have been previously reviewed [161,172]. Also, extraction methods for PHA recovery have been extensively evaluated [149,173]. For an industrial application, it would be necessary to produce PHAs with a fixed composition to maintain the quality and properties of the bioplastics. It has been demonstrated that PHA composition can be controlled by shifting the SCCA composition in the feeding stream [156,174,175]. Therefore, to obtain a PHA with a constant composition over time, the SCCA composition of the feeding stream must also be constant. Then, for developing a commercial PHA production from organic wastes, more knowledge is needed in the control of SCCA composition obtained in the acidogenic fermentation. Furthermore, in both PHA enrichment and accumulation steps, the SCCA concentration in the SCCA-rich stream used as substrate is also a key parameter.

1.5.2 Medium chain carboxylic acids (MCCAs) synthesis by chain elongation reactions starting from SCCAs

Another application of SCCAs that is gaining interest in the last decade is the biosynthesis of medium chain carboxylic acids (MCCAs) through the chain elongation process. MCCAs are defined as saturated fatty acids with 6 to 12 carbon atoms comprising one carboxylic group, including compounds such as caproic acid, enanthic acid or caprylic acid [11,176]. MCCAs are more hydrophobic than SCCAs, which are totally

miscible with water excepting for valeric acid [177–179]. Hence, MCCAs can be easily separated since they form an oily layer with water at a pH below or around their pKa [11,178]. Furthermore, MCCAs are regarded as more valuable that the common anaerobic products such as SCCAs, ethanol or lactic acid since they contain higher energy density [178]. MCCAs can be used in the synthesis of numerous products such as feed additives [180,181], antimicrobials [182], biopolymers [183], flavor additives, fragrances, pharmaceuticals, dyes and lubricants [11]. Concretely, caproic acid has been reported as a precursor in biofuel production [184].

Nowadays, MCCAs are obtained by extraction from vegetable oils such as palm oil [179] or from animal fat [178]. Other possibility is to produce them from fossil fuels by chemical synthesis. For instance, caproic acid can be obtained by carbonylation of ethanol with carbon monoxide and water or by oxidizing propanal or hydrocarbons [185] and caprylic acid can be synthesized by oxidizing the octanal or dehydrogenation of octanol [178]. Despite MCCA chemical synthesis has good yields, this kind of processes require a huge initial investment for the facilities and the petroleum costs are being increased [185]. So, MCCA production by fermentation is seen as a more economically and environmentally friendly alternative [178].

Similarly to SCCA synthesis, MMC are more convenient to produce MCCAs than pure or engineered cultures since a consortium of microorganisms has more potential to deal with complex organic matter as substrate and with alterations in feedstocks characteristics [177,179]. The use of MMC is considered as a potentially viable system to produce MCCAs at industrial scale since MCCA do not require so energy intensive downstream processes as in SCCA production due to their higher hydrophobicity [178]. On the contrary, pure cultures require strict sterilization conditions and high-purity substrates and makes the operation more complicated [178]. Hence, bacterial isolates use is currently limited to the study of the metabolic pathways [179]. There are two different chain elongation pathways: reverse β-oxidation (RBO) cycle and fatty acid biosynthesis (FAB) cycle [186].

By the RBO cycle, acetate is elongated to butyrate in a first cycle, and butyrate is transformed into caproate in a second cycle [186]. Figure 1.3 represents the chain elongation pathway by the RBO cycle starting from lactate or ethanol as electron donor (ED). RBO cycle starts after the oxidation of the compound used as ED to acetyl-CoA

[178]. Lactate and ethanol have been pointed out as the most convenient ED since they could generate a number of reducing equivalents (NADH) that can support MCCA synthesis by the RBO cycle, they provide the required energy (ATP) to perform the following reactions and they are easily transformed into acetyl-CoA [178,187]. When the ED is ethanol, this compound is oxidated to acetaldehyde, and subsequently to acetyl-CoA, by ethanol dehydrogenase and acetaldehyde dehydrogenase, respectively [178]. Afterwards, approximately 1/6 of the acetyl-CoA is transformed into acetate, yielding energy in form of ATP and the rest of the acetyl-CoA starts the RBO cycle [178]. In each of these cycles, one acetyl-CoA is added to an acyl-CoA, incrementing the chain length by 2 carbon atoms [187]. In the first cycle, acetyl-CoA is linked to another acetyl-CoA synthetizing acetoacetyl-CoA catalyzed by acetoacetyl-CoA thiolase. Next, the acetoacetyl-CoA is transformed to butyryl-CoA in several reactions under the catalysis of 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase and butyryl-CoA dehydrogenase [178]. Finally, butyrate is released by transferring the CoA of the butyryl-CoA to the acetate used as electron acceptor (EA) by the catalysis of acetate CoA transferase [178]. The produced acetyl-CoA is linked to another acetyl-CoA obtained from the ethanol oxidation to restart the first RBO cycle again, while the butyryl-CoA obtained enters the second elongation cycle where is linked to an acetyl-CoA to obtain caproic acid in different steps [178].

When lactate was the ED, it was first oxidized to pyruvate by lactate dehydrogenase; and subsequently transformed into acetyl-CoA by pyruvate dehydrogenase [178]. Similarly to ethanol acting as ED, part of the acetyl-CoA was used to produce acetate and release energy in form of ATP and the remaining acetyl-CoA entered the RBO cycle [178]. Nevertheless, it was reported lactate conversion into propionate by the acrylate pathway as a competing pathway to the chain elongation reactions [187]. Hence, propionate could act as EA instead of acetate or butyrate and lead to valerate, heptylate and nonanoate by chain elongation [178]. However, there is no evidence until now of carboxylic acid production of more than 8 carbon atoms by chain elongation, possibly due to enzymatic limitations [187].

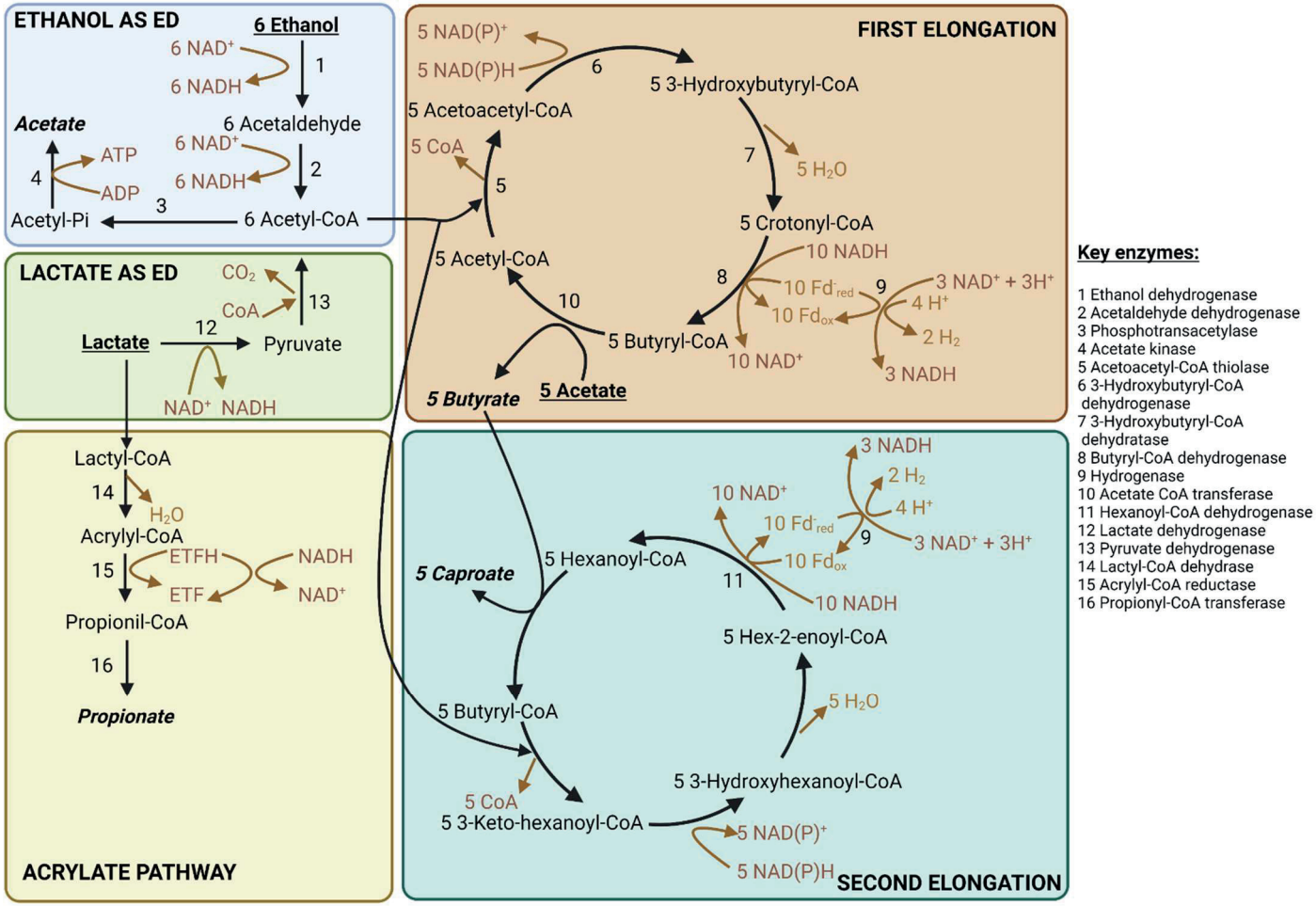


Figure 1.3. Reverse β-oxidation pathway for MCCA synthesis by chain elongation using ethanol or lactate as electron donor (ED) (adapted from [178]). Substrates are marked as underlined and bold text and products are marked as italic and bold text. CoA, coenzyme A; ETF, electron transfer flavoprotein; ETFH, reduced electron-transfer flavoprotein; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin. Created with BioRender.com.

Apart from ethanol or lactic acid, other compounds can act as ED, namely methanol [188], n-propanol [189], amino acids [190] and D-galactitol [191]. The product spectrum is modified when the ED is changed. For example, when using methanol as ED, isobutyrate formation is observed due to the conversion of butyrate by isomerization [188]. Regarding the EAs, other compounds besides acetate could be used: propionate, butyrate, malonate or succinate [192]. Nevertheless, the highest growth rate with *Clostridium kluyveri*, which is the microorganism usually chosen to study the RBO cycle pathway, was achieved with ethanol as ED and acetate as EA. This fact might indicate that *Clostridium kluyveri* might prefer EAs and EDs with short carbon chains [192]. However, since acetate is synthetized from ethanol to generate ATP (Figure 1.3), it is not necessary to provide acetate as EA. Even so, caproic acid production by chain elongation using ethanol as ED without acetic acid yields less energy (0.333 mol ATP per mol ethanol consumed) than using ethanol as ED and acetic acid as EA (0.5 mol ATP per mol ethanol consumed) [179] .

An alternative pathway to the RBO cycle that has been less studied is the FAB cycle (Figure 1.4). In contrast to RBO cycle, the ED in the FAB cycle must be malonyl-ACP, so previous conversion of acetyl-CoA (obtained from ethanol or lactate) to malonyl-ACP is performed aided by the enzymes acetyl-CoA carboxylase, malonyl transferase and ketoacetyl-ACP synthase [186]. Once the malonyl-ACP is formed, it will enter the FAB cycle by incorporating itself to an acyl-ACP catalyzed by ketoacyl-ACP synthase, resulting in a carbon chain length increment of 2 carbon atoms [186]. The obtained β-ketoacyl-ACP starts a transformation in several steps to acyl-ACP (with 2 carbon atoms more than the one that starts the cycle) in which the enzymes ketoacyl-ACP reductase, hydroxyacyl-ACP reductase and enoyl-ACP reductase take part [193]. Finally, the acyl-ACP with 2 extra carbon atoms is converted to fatty acid by the action of a thioesterase [193]. FAB cycle pathway is less efficient than RBO cycle since acetyl-CoA needs to be converted to malonyl-ACP and it also requires more ATP [186,194]. Nevertheless, several authors found higher abundance of enzymes involved in the FAB cycle pathway than those participating in the RBO cycle when using substrates such as waste activated sludge alkaline fermentation liquor [194] or xylose [195].

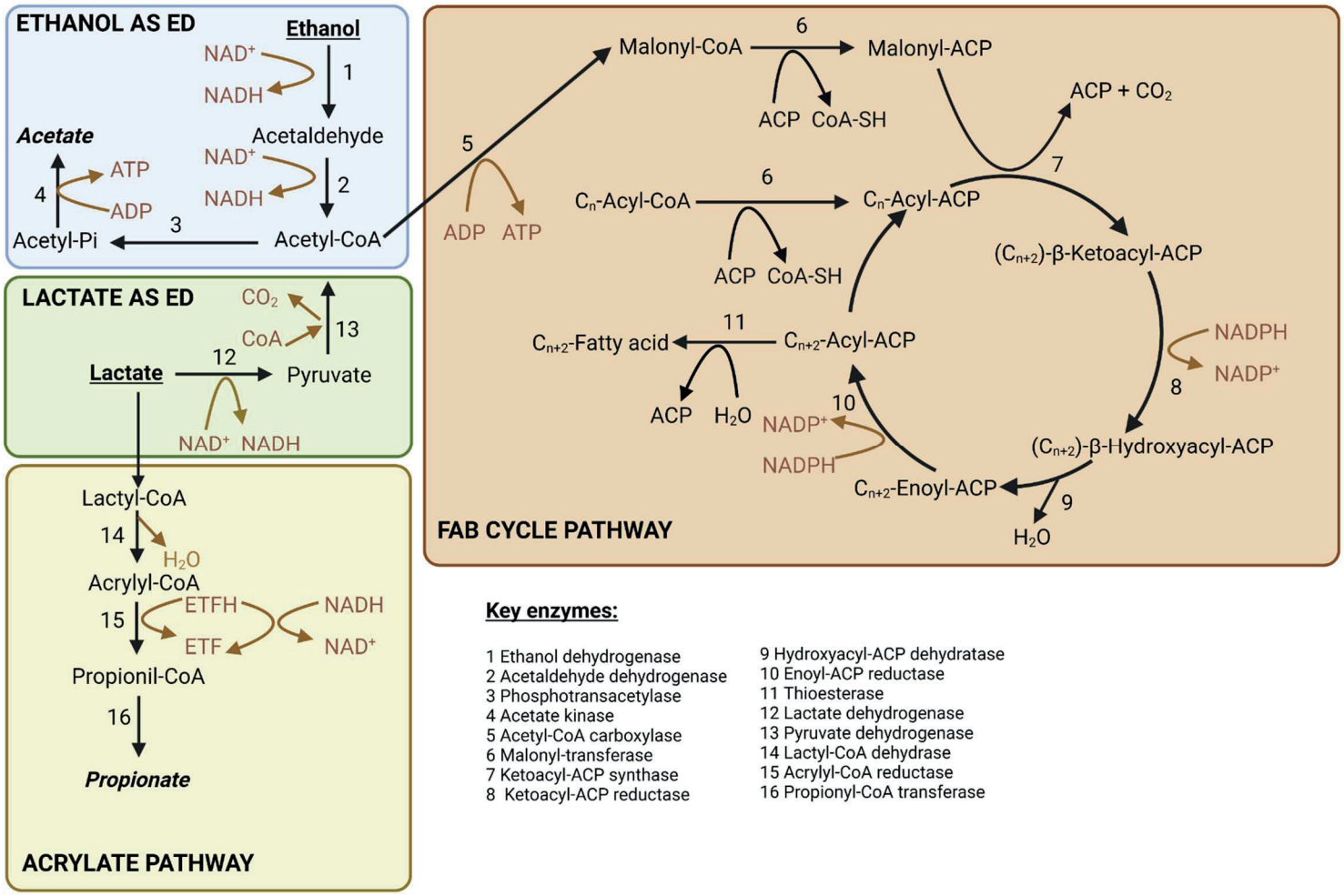


Figure 1.4. Fatty acids biosynthesis (FAB) pathway for MCCA synthesis by chain elongation using ethanol or lactate as electron donor (ED) (adapted from [186]). Substrates are marked as underlined and bold text and products are marked as italic and bold text. ACP, acyl carrier protein; CoA, coenzyme A; ETF, electron transfer flavoprotein; ETFH, reduced electron-transfer flavoprotein. Created with BioRender.com.

Large-scale MCCA production by MMC systems aims to use waste or wastewater as substrate. This process takes place in three steps:

1) Pretreatment of the waste/wastewater.
2) MCCA biosynthesis.
3) Extraction of the obtained MCCA.

Pretreatment of the substrate can comprise different processes, such as homogenization, removal of impurities, disintegration, concentration or dilution [178,179]. After the pretreatment, two fermentation steps are needed to transform the substrate into MCCAs: acidogenesis of the substrate and chain elongation of the SCCAs obtained from the acidogenesis [178]. These two steps can be carried out in a single reactor or in two different reactors: one to perform the hydrolysis and acidification of the substrate and the second to perform the MCCA synthesis [178]. Several works have discussed the optimal operational conditions [11,177,186,187] and the strategies to enhance MCCA production [178,187], which would be different if the fermentation takes place in a one or two stage reactor. Finally, there are two main methods to extract the produced MCCAs when produced by MMC: in-line pertraction [196,197] and electrodialysis [176,198,199].

Among substrates that have already been tested, one can find the following wastes: OFMSW [200,201], manure [202], food waste [203,204], lignocellulosic biomass [205,206], dairy processing wastes [207–209] or brewing wastes [196,197,210]. Substrates for MCCA synthesis are usually divided in three different classes according to their composition: 1) substrates containing both ED and EA, 2) substrates that can generate the EA in situ and do not contain ED, so it must be supplied externally and 3) substrates that can be used to produce EA and ED so no external supply is needed. The ideal option would be a process in which only the waste is provided since the addition of an ED increases the economic costs [179]. However, it is quite common that the ED must be provided externally. Substrates containing easily degradable compounds, such as dairy processing wastes, might not need external ED supplementation, since they contain high concentrations of sugars that can be transformed into ethanol or lactic acid [179]. Other substrates, such as brewing wastes, might be composed by a sufficient quantity of an ED, such as lactic acid or ethanol [179]. Regarding the EA, in general, the even-carbon SCCAs would lead to even-carbon MCCAs and odd-carbon SCCAs would lead to odd-carbon

MCCAs [186]. There are exceptions to this rule. On the one side, as seen in Figure 1.3 and Figure 1.4, starting from acetate as EA, if lactate is the ED, propionic acid might be produced by the acrylate pathway and be used as precursor in odd-carbon MCCAs formation [186]. On the other side, some even-carbon carboxylic acids will be always produced in an odd-carbon short chain carboxylic acid elongation process if ethanol is the ED due to the acetic acid production (Figures 1.3 and 1.4) [186]. Apart, branched medium chain carboxylic acids are normally obtained from branched SCCAs [211]. In conclusion, substrate organic composition notably affects the chain elongation process and the medium chain carboxylic acids obtained. Therefore for developing a MCCA production process at industrial level, it is essential to acquire knowledge related to the products formed by the acidogenic fermentation of the organic wastes or wastewaters.

1.6 Conclusions and research gaps to be solved

Short chain carboxylic acids are value added products and their synthesis from organic wastes or wastewaters by MMC based processes is a promising technology that would avoid the use of fossil fuels. However, further knowledge about how to control SCCA production yield and composition is needed to scale up bioprocesses based on the use of waste streams as feedstock. This introduction rounds up the main metabolic routes that lead to SCCA synthesis by MMC. Next, the interactional effect of pH, temperature, OLRv, HRT and the organic composition of the waste/wastewater on SCCA production yield is discussed. From the aforementioned analysis, it can be gathered that organic composition of waste/wastewater, in terms of carbohydrates, proteins and lipids, is the main factor that affects the SCCA production yield, even though the operational parameters, like pH, temperature, OLRv and HRT also play a role. In this sense, Table 1.6 sums up the operational conditions that lead to a high SCCA production yield depending on the organic composition of the waste/wastewater. Likewise, the effect of pH, temperature and bacterial composition on SCCA composition was analyzed together with substrate composition. The effect of the aforementioned factors on SCCA composition might be interconnected and is still not clear. More research is needed to predict and control SCCA composition of the effluent of an acidogenic fermentation reactor. Finally, PHA and MCCA biosynthesis by MMC based processes were presented as examples of two interesting applications of the waste-based bioproduced SCCAs. Despite all the research performed in the last years in the SCCA fermentation platform,

there is still limited knowledge in some areas that should be studied deeply. Further studies need to cover the following gaps:

1) *Establishment of standard parameters to quantify both SCCA production yield and SCCA composition.* In this introduction, studies of SCCA fermentation of solid, slurry-like, and liquid wastes (mostly wastewaters) have been analyzed together. Nevertheless, the SCCA production yields are expressed differently, depending on the type of waste (solid and/or liquid waste) research field. Moreover, some studies examine the operational parameters that maximize SCCA production, quantifying it as the degree of acidification or the SCCA concentration obtained instead of the SCCA production yield. Additionally, SCCA compositions are sometimes related to the sum of all the SCCAs obtained and on other occasions, related to the total soluble organic matter. The use of standard parameters would facilitate the comparison between different studies.
2) *Study of the specific organic loading rate (OLRs) as another parameter that can affect both SCCA production yield and SCCA composition.* Unless in some studies of solid waste fermentation, where the parameter inoculum-to-substrate ratio is regarded, the amount of biomass present in the reactor is rarely considered. We found little data that allow us to analyse this parameter. On some occasions, authors only detail the origin of the inoculum, but they do not specify the amount added or the biomass concentration in the acidogenic fermentation reactor. We consider that the amount of available substrate for a certain amount of biomass might affect the SCCA yield or composition. OLRs is a parameter that could serve to analyse this effect.

Table 1.6. Operational conditions that lead to high SCCA production yields depending on the organic composition of the substrate.

Operational conditions that lead to high SCCA production yields				
Parameter		**Main component of the substrate**		
		Carbohydrates	**Proteins**	**Lipids**
pH	*Solid waste*	Alkaline pH		
	Slurry-like waste	Complex carbohydrates: alkaline pH Simple carbohydrates: neutral pH	Alkaline pH	
	Liquid waste	Complex carbohydrates: alkaline pH Simple carbohydrates: neutral pH	Complex proteins: alkaline pH Simple proteins: neutral pH	Mesophilic conditions: alkaline pH Thermophilic conditions: neutral pH
Temperature	*Solid or slurry-like waste*	Mesophilic conditions	Thermophilic conditions	NA
	Liquid waste	More research is needed	More research is needed	Mesophilic conditions
OLRv	*Solid, slurry-like or liquid waste*	Simple substrates: high OLRv to inhibit methanogenesis Complex substrates: maximum OLRv restricted by hydrolysis		

*NA denotes no available information.

3) *Further study of the SCCA composition obtained from different substrates regarding their organic components rather than considering it only as a concrete type of substrate.* As emphasized in this introduction, organic composition of the substrate, in terms of carbohydrates, proteins and lipids, has a strong influence on both SCCA production yield and the type of SCCA produced. Additionally, attention should be paid when reviewing literature as substrates with different organic compositions and complexities have been grouped under the same substrate name, even when its composition, origin and seasonality is different. Also, the substrate consumption needs to be specifically analysed in terms of carbohydrate, protein and lipid amounts apart from soluble COD amounts.
4) *Further analysis of the microbial communities to link the different genera to the different SCCAs obtained.* There are still many unidentified species among the acidogenic bacteria. Systematic studies of microbial communities in an acidogenic reactor will help to develop a better knowledge of the bacteria responsible for the production of each SCCA as well as their metabolic interactions.

1.7 Thesis overview

This thesis aims to cover the research gaps founded from the literature review presented in this introduction section. As mentioned previously, standard process effectiveness parameters must be stablished to quantify the conversion of the substrate into SCCAs or other products as well as the composition of the effluent. Standard process effectiveness parameters would allow to compare results between different works. In **Chapter 3**, besides the description of the simulated wastewater preparation and the inoculum used, the reactor configuration and operation, the analytical methods, the microbial communities' characterization and the statistical analyses, the process effectiveness parameters that will be used in the whole document are defined. These parameters could be used in any acidogenic fermentation process starting from liquid waste.

The rest of the research gaps which were pointed out in the anterior section were encompassed in the experimental part of this thesis (**Chapters 4, 5 and 6**). The main goal of this study is to achieve a deeper knowledge related to the biological transformations

that take part during the acidogenic fermentation to be able to predict effluent composition and scale up this process. Therefore, one of the objectives is to link the fermentation products obtained to the organic components of the substrate and the different bacterial communities present. To this end, acidogenic fermentation experiments were performed using a simulated wastewater containing simple organic model compounds, namely carbohydrates and proteins, either alone or mixed in different proportions. Lipids are less desirable substrates as their hydrolysis is slower and they are transformed to LCFA, which could inhibit anaerobic bacteria [54,64]. For that reason, lipids were excluded from this study. In all the experiments, the effluent composition and the bacterial communities present were analyzed systematically in each of the conditions tested. From the results found and the literature, it was intended to identify possible connections between the substrate organic components, the effluent components and the microbial communities.

Given that it is pretended to develop a process as similar as possible to an industrial continuous process, the experiments were carried out a lab-scale semicontinuous reactor with biomass retention. This way, the effluent would be able to be used directly in a posterior application, such as the ones already mentioned. Two operational parameters, OLRv and OLRs, were selected to study their effect over the substrate conversion and the effluent composition. In previous research, the effect of the OLRv on the substrate conversion was normally analyzed only to find the OLRv that lead to the highest SCCA production yield, when a high OLRv could lead to hydrolysis limitations due to the complexity of the substrate. Additionally, as stated before, it is very rare to found in literature any consideration to the ratio between the amount of substrate and biomass concentration, specially in wastewaters studies. In order to determine how the ratio of substrate to biomass affected substrate conversion and effluent composition, the OLRs was chosen as another parameter to consider. The study of the OLRv and OLRs was performed in those experiments where the substrate was made up of only one organic component (carbohydrates or proteins).

Following, the experimental work carried out in the different chapters of this thesis is presented. In **Chapter 4,** acidogenic fermentation experiments starting from simulated wastewater containing food-grade sugar as carbohydrate model compound were performed to assess the effect of OLRv, the OLRs and the influent carbon-to-nitrogen ratio on the carbohydrate conversion to SCCAs and other products, on the effluent composition and on the bacterial communities present. Similar experiments were carried

out in **Chapter 5** but using simulated wastewater containing whey protein isolate as protein model compound, to evaluate the effect of OLRv and the OLRs on the protein conversion to SCCAs and other products, on the effluent composition and on the microbial composition of the biomass. Next, in **Chapter 6**, fermentation experiments employing simulated wastewater containing different proportions of food-grade sugar and whey protein isolate under fixed conditions of OLRv and OLRs were conducted to analyze the effect of the organic composition of the substrate on the substrate conversion to SCCAs and other products, on the effluent composition and on the microbial communities present.

Finally, **Chapter 7** summarize the main conclusions deduced from this thesis.

Chapter 2:

Objectives

The main objective of this thesis is to gain insight into the acidogenic fermentation process. From the research gaps found after the literature review, it is aimed to explore the effect of the volumetric organic loading rate (OLRv), the specific organic loading rate (OLRs) and the substrate organic composition on the process performance, the effluent composition and the biomass composition by performing long-term experiments in a sequencing batch reactor.

The specific objectives of this thesis are the following:

i. To study the impact of the OLRv, OLRs and influent carbon-to-nitrogen ratio on the process effectiveness parameters and the resultant effluent composition in an acidogenic sequencing batch reactor fed by a simulated wastewater formed only by a model carbohydrate compound.
ii. To assess the impact of the OLRv and OLRs on the process effectiveness parameters and the resultant effluent composition in an acidogenic sequencing batch reactor by a simulated wastewater composed only by a model protein compound.
iii. To evaluate the impact of the organic composition of the substrate on the process effectiveness parameters and the resultant effluent composition in an acidogenic sequencing batch reactor fed by a simulated wastewater containing different ratios of carbohydrate and protein model compounds under fixed conditions of OLRv and OLRs.
iv. To analyze the different microbial communities present in the reactor under the different conditions tested to link them to the different effluent components produced.

Chapter 3:

Materials and methods

3.1 Simulated wastewater preparation and inoculum used

The substrate of the experiments was a simulated wastewater containing food-grade sugar and/or whey protein isolate (HSN®) as model compounds for carbohydrate and protein, respectively. Particularly, in the experiments with food-grade sugar as the solely carbon source (described in Chapter 4), the feeding solution contained 12 g O_2 L^{-1} in terms of Chemical Oxygen Demand (COD). In the experiments with whey protein isolate as unique carbon source (detailed in Chapter 5), the feeding solution contained 10 g O_2 L^{-1} in terms of COD during the start-up period and 14 g O_2 L^{-1} in terms of COD during the rest of the experiment. When the substrate was a mixture of food-grade sugar and whey protein isolate (Chapter 6), the feeding solution contained a total of 14 g O_2 L^{-1} of COD composed by different ratios of food-grade sugar (C, model carbohydrate) and whey protein isolate (P, model protein) (25%C/75%P, 50%C/50%P and 75%C/25%P, % in terms COD). The feeding solution also contained mineral salts considered essential for biomass growth, which are summarized for each of the experiments in Table 3.1. In addition, in the experiment where mixtures of carbohydrate and protein (Chapter 6) were used, NH_4Cl was added to adjust the nitrogen content (0-4 g L^{-1} NH_4Cl, depending on the condition tested) and NaOH was added to adjust the pH to 9 from the 19th day of operation till the end. After adding the mineral salts and the food-grade sugar (where appropriated), the feeding solution was autoclaved to avoid any contamination by fungi. Whey protein isolate (where appropriated) was added in a laminar flow cabinet after autoclaving the feeding solution to avoid any protein alteration during the autoclaving process.

The inoculum used in the experiments with carbohydrate and protein as solely carbon source (Chapter 4 and Chapter 5, respectively) was anaerobic granular sludge obtained from an anaerobic digester of a papermill wastewater treatment plant (UIPSA, La Pobla del Claramunt, Spain). In the experiment with mixtures of carbohydrate and protein as substrate (Chapter 6), the inoculum used was a mixture in equal proportions (in mass basis) of the biomass from the reactor after performing the experiments with only carbohydrate (Chapter 4) and the biomass from the reactor after the operation with only protein (Chapter 5).

Table 3.1. Composition of the mineral salts in the feeding solution.

Carbohydrate as solely carbon source (adapted from Tamis et al. [212])		**Protein as solely carbon source** (adapted from Duong et al. [101])		**Mixtures of carbohydrate and protein** (adapted from Duong et al. [101])	
Salt	Concentration (mg L^{-1})	Salt	Concentration (mg L^{-1})	Salt	Concentration (mg L^{-1})
NH_4Cl	1356*	Na_2HPO_4	2180	$NaHCO_3$	6000
KH_2PO_4	254	KH_2PO_4	1060	Na_2HPO_4	2180
$MgSO_4 \cdot 7H_2O$	118	$CaCl_2 \cdot 2H_2O$	48	KH_2PO_4	1060
KCl	40	$MgSO_4 \cdot 7H_2O$	54	$CaCl_2 \cdot 2H_2O$	48
EDTA	72	$FeCl_2 \cdot 4H_2O$	7.2	$MgSO_4 \cdot 7H_2O$	54
$ZnSO_4 \cdot 7\ H_2O$	24	$CoCl_2 \cdot 6H_2O$	1.2	$FeCl_2 \cdot 4H_2O$	7.2
$CoCl_2 \cdot 6\ H_2O$	1.8	$MnCl_2 \cdot 4H_2O$	0.3	$CoCl_2 \cdot 6H_2O$	1.2
$MnCl_2.4\ H_2O$	5.8	$CuCl_2 \cdot 2H_2O$	0.018	$MnCl_2 \cdot 4H_2O$	0.3
$CuSO_4.5\ H_2O$	1.8	$ZnCl_2$	0.03	$CuCl_2 \cdot 2H_2O$	0.018
$FeSO_4.7\ H_2O$	5.6	HBO3	0.03	$ZnCl_2$	0.03
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	1.2	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.054	HBO3	0.03
$CaCl_2.H_2O$	8.2	$Na_2SeO_3 \cdot 5H_2O$	0.06	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.054
		$NiCl_2 \cdot 6H_2O$	0.03	$Na_2SeO_3 \cdot 5H_2O$	0.06
		EDTA	0.6	$NiCl_2 \cdot 6H_2O$	0.03
				EDTA	0.6

*When the influent carbon-to-nitrogen ratio was increased to 25, NH_4Cl concentration in the feeding solution was 634 mg L^{-1}.

3.2 Reactor configuration and operation

The experiments were carried out in a stirred reactor with an effective working volume of 2.65 L and a headspace of 1.66 L (Figure 3.1). The reactor was operated as a sequencing batch reactor (SBR) to retain biomass. The exchange volume ratio was set at the 45% of the working volume. Each cycle consisted of the following phases: settling (60 min), effluent discharge (27 min), feeding (60 min) and reaction (variable time length depending on the experiment). During the feeding and reaction phases, the reactor was stirred at 80 rpm. In this work, the reactor was operated under different operational conditions by modifying operational parameters such as the volumetric Organic Loading Rate (OLRv) and the specific Organic Loading Rate (OLRs) or the composition of the feeding solution (influent carbon-to-nitrogen ratio or carbohydrate and protein ratio). The operational conditions tested in each of the experiments are detailed in the corresponding chapter.

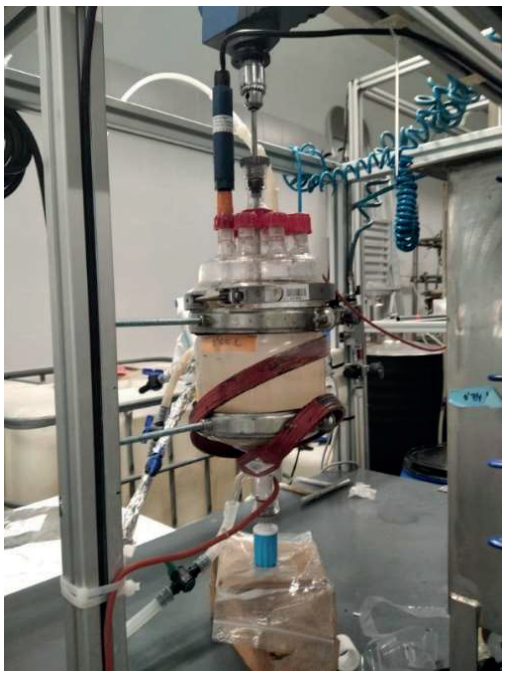


Figure 3.1. Picture of the reactor used in this study.

After a start-up period, each experiment was performed by varying only one operational parameter at a time until reaching steady-state conditions. Steady state conditions were deemed to have been achieved when the composition of the components analyzed in the effluent fluctuated by less than 10% of the total COD of the effluent. In

all the experiments, the targeted OLRv was applied by changing the total cycle duration (which oscillated from 8 to 24h, accordingly, the reaction phase length). After choosing the OLRv, the desired OLRs was achieved by keeping the biomass concentration at an appropriate value by purging when necessary. Consequently, the Sludge Retention Time (SRT) and the biomass concentration were different depending on the experiment and the conditions applied. In the experiment using food-grade sugar as substrate, the SRT varied from 4 to 14 days and the biomass concentration ranged between 2.8 and 7.3 g VSS L^{-1}. In the experiment utilizing whey protein isolate as substrate, the SRT varied from 7 to 13 days and the biomass concentration ranged between 3.4 and 9.1 g VSS L^{-1}. In the experiment employing mixtures of food-grade sugar and whey protein isolate as substrate, the SRT varied from 5 to 12 days and the biomass concentration ranged between 3.2 and 6.6 g VSS L^{-1}.

The temperature was measured on-line using a submersible sensor (Axiomatic Pt100) and controlled at 37ºC by using a silicone heating belt (HORST GmbH, HBSI1, 5m). The pH was registered on-line by utilizing a pH-probe (Prominent®, PHES-112-SE SLg225). All the sensors and the actuators were connected to a PLC system.

3.3 Analytical methods

Total soluble COD was determined after filtering effluent samples with 0.45 μm pore filters. 200 μL of the filtered sample were added to the COD Mercury-free kits 0-15000 mg L^{-1} (Lovibond). Total soluble COD analyses were performed in triplicate.

Carboxylic Acids, including SCCAs and some MCCAs, (CAs), ethanol, glucose, lactic acid, sucrose, protein and ammonium concentrations were determined after filtering effluent samples with 0.22 μm pore filters to remove the biomass present.

For CAs (including acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids) and ethanol analysis, 800 μL of each sample were transferred to a chromatography vial to which 200 μL of a stabilizing solution composed of crotonic acid (2 g L^{-1}, internal standard) and phosphoric acid (2%) was subsequently added. The resulting samples were analyzed by gas chromatography (GC) in the GC 7820A (Agilent Technologies) equipped with a DB FFAP column (30 m x 0.25 mm x 0.25 μm) and a flame ionization detector (FID). A sample of 5 μL was injected at 260ºC using helium as carrier gas with a split ratio of 10:1 at 4 mL min^{-1}. The initial oven

temperature was set at 60 °C and held for 1 min, followed by a temperature ramp of 5 °C min^{-1} until reaching 100 °C. Subsequently, a second ramp of 10 °C min^{-1} was applied until reaching 210 °C. FID was set at 280 °C.

Food-grade sugar was measured as sucrose equivalents. Sucrose was analyzed by HPLC analysis (HPLC Dionex Ultimate3000, Dionex) using an ionic exchange column ICSep ICE-COREGEL 87H3 (Transgenomic Inc., NE, USA). The mobile phase was 6 mM sulfuric acid at a flow rate of 0.6 mL min^{-1} and the volume of injection was 20 µL. Chromeleon software (Dionex) was used for data treatment.

Glucose and lactic acid were measured in a YSI 2950 Biochemistry Analyzer (Xylem Inc.). Whey protein isolate concentration was determined using the Bradford method in a spectrophotometer at 595 nm [213]. Ammonium concentrations were measured by a gas selective electrode (GSE) (AMTAX sc, Hach Lange, Germany).

Mixed liquor samples were taken from the reactor to determine VSS and calculate the OLRs. Moreover, TSS and VSS were determined periodically in effluent samples. TSS were calculated using the method 2540D [214]. VSS were measured following the method 2540E [214].

3.4 Bacterial communities' analysis

The microbial community composition was determined by Illumina amplicon sequencing of the 16S rRNA gene. Samples of biomass were taken from the reactor when steady state conditions were established in each experiment. Until the extraction process, the samples were stored at -20ºC after being washed with PBS and centrifuged three times. The DNA extraction process was performed using the Soil DNA isolation plus kits (Norgen Biotek CORP, Canada) following the manufacturer protocol. The concentration and the quality of the DNA extracted were determined by using the NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, USA). A 260/280 ratio of 1.8±0.1 was selected as a good quality indicator and a DNA concentration of 5 ng μL^{-1} was required as the minimum concentration needed for sequencing. Prior to further analyses, DNA samples were stored at -20ºC. Sequencing analyses were performed by the Genomics Service (SG) of the Universitat Autònoma de Barcelona (UAB, Spain). The primers 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA ATCC were used to target the variable regions V3 and V4 of the 16S rRNA of bacteria. The organisms were categorized using the RefSeq+RDP database [215] and the sequence reads were examined using Usearch software. Each sample taken from the reactor was extracted and sequenced twice, so the results exhibited in this work correspond to the mean of two sequencing analyses from the same sample.

3.5 Calculations

3.5.1 Organic compounds concentrations (in terms of COD)

The COD conversion factor for the organic compounds analyzed in this study were the following: acetic acid: 1.07 g O_2 g^{-1}; propionic acid: 1.51 g O_2 g^{-1}; isobutyric acid: 1.82 g O_2 g^{-1}; butyric acid: 1.82 g O_2 g^{-1}; isovaleric acid: 2.04 g O_2 g^{-1}; valeric acid: 2.04 g O_2 g^{-1}; isocaproic acid: 2.20 g O_2 g^{-1}; caproic acid: 2.20 g O_2 g^{-1}; ethanol: 2.08 g O_2 g^{-1}; glucose: 1.07 g O_2 g^{-1}; lactic acid: 1.07 g O_2 g^{-1}; sucrose: 1.12 g O_2 g^{-1} and protein: 1.51 g O_2 g^{-1}.

3.5.2 Specific Organic Loading Rate (OLRs)

As stated previously, the OLRs is one of the operational parameters studied in this study. The OLRs is defined as the ratio between the volumetric organic loading rate (OLRv), expressed as g COD L^{-1} d^{-1}, and the biomass concentration in the reactor, expressed as g VSS L^{-1} (Eq 3.1.).

$$OLRs\left(\frac{g\ COD}{g\ VSS\cdot d}\right)=\frac{OLRv\left(\frac{g\ COD}{L\cdot d}\right)}{[Biomass\ in\ the\ reactor]\left(\frac{g\ VSS}{L}\right)} \quad \text{(Eq. 3.1)}$$

3.5.3 Process effectiveness parameters to quantify both CA production yield and CA composition

Next, the process effectiveness parameters are defined and the utility of each of them is specified.

3.5.3.1 Bioconversion

Bioconversion was determined using the Eq 3.2.

$$Bioconversion\ (\%) = \frac{COD_{TOTAL,\ INF} - COD_{SUBSTRATE,\ EFF}}{COD_{TOTAL,\ INF}} \cdot 100 \qquad \text{(Eq. 3.2)}$$

where $COD_{TOTAL,\ INF}$ is the total COD of the influent and $COD_{SUBSTRATE,\ EFF}$ is the substrate concentration (expressed as COD equivalents) in the effluent. In all the experiments, the total COD of the influent corresponds to the substrate COD measured in the influent.

Bioconversion quantifies the global substrate conversion into fermentation products in the liquid phase, new biomass or biogas. High bioconversion values implies that there were no substrate consumption limitations. Low bioconversion values could be caused by hydrolysis limitations or low activity of the biomass.

3.5.3.2 Degree of acidification (DA)

The degree of acidification (DA) was calculated using Eq. 3.3.

$$DA(\%) = \frac{\sum COD_{CAs,\ EFF}}{COD_{TOTAL,\ INF}} \cdot 100 \qquad \text{(Eq. 3.3)}$$

being $\sum COD_{CA,\ EFF}$ the sum of the CAs in the effluent (including acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids) expressed as COD equivalents and $COD_{TOTAL,\ INF}$ is the total COD of the influent.

The degree of acidification is a parameter that express the fraction of the substrate in the influent that has been converted to CAs. A high value of the degree of acidification entails that a high proportion of the substrate in the influent has been transformed to CAs. A low value of the degree of acidification implies that a high fraction of the substrate was not converted to CAs due to substrate consumption limitation, or it was used for new biomass formation or for synthesis of other compounds (in the gas or in the liquid phase).

3.5.3.3 Acidified COD

The acidified COD was calculated using Eq. 3.4.

$$Acidified\ COD(\%) = \frac{\sum COD_{CAs,\ EFF}}{COD_{TOTAL,\ EFF}} \cdot 100 \qquad \text{(Eq. 3.4)}$$

being $\sum COD_{CA,\ EFF}$ the sum of the CAs in the effluent (including acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids) expressed in terms of COD and $COD_{TOTAL,\ EFF}$ is the total COD of the effluent.

The acidified COD is a parameter that quantifies the fraction of organic content in the effluent that corresponds to CAs. A high value of the acidified COD implies that a high proportion of effluent is composed by CAs. A low value of the acidified COD entails that a high proportion of the effluent is formed by compounds that are not CAs.

3.5.3.4 CA production yield

The CA production yield is defined as the ratio between the total concentration of the CAs in the effluent (expressed in terms of COD) and the difference between the substrate concentration in the influent and the effluent (expressed in terms of COD) (Eq. 3.5).

$$CA\ production\ yield\ (g\ COD\ g^{-1}\ COD) = \frac{\sum COD_{CAs,\ EFF}}{COD_{TOTAL,\ INF} - COD_{SUBSTRATE,\ EFF}} \qquad \text{(Eq. 3.5)}$$

being $\sum COD_{CA,\ EFF}$ the sum of the CAs in the effluent (including acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids) expressed as COD equivalents; $COD_{TOTAL,\ INF}$ is the total COD of the influent and $COD_{SUBSTRATE,\ EFF}$ is the substrate concentration (expressed in terms of COD) in the effluent. In all the experiments, the total COD of the influent corresponds to the substrate COD measured in the influent.

The CA production yield is a parameter that express the fraction of the consumed substrate that has been converted to CAs. A high CA production yield implies that a high part of the consumed substrate has been transformed into CAs. A low CA production yield entails that a high of the consumed substrate has been converted into fermentation products which are not CAs i.e., ethanol or lactic acid.

3.5.3.5 Odd-to-even ratio

The odd-to-even ratio is defined as the ratio between the amount in the effluent of SCCAs with odd number of carbon atoms and the amount in the effluent of SCCAs with an even number of carbon atoms (Eq. 3.6) [29].

$$odd - to - even\ ratio = \frac{[Propionic] + [n - Valeric]}{[Acetic] + [i - Butyric] + [n - Butyric] + [i - Valeric]} \quad (Eq. 3.6)$$

where [Propionic] is the concentration of propionic acid in the effluent (mg O_2 L^{-1}), [n-Valeric] is the concentration of n-valeric acid in the effluent (mg O_2 L^{-1}), [Acetic] is the concentration of acetic acid in the effluent (mg O_2 L^{-1}), [i-Butyric] is the concentration of isobutyric acid in the effluent (mg O_2 L^{-1}), [n-Butyric] is the concentration of n-butyric acid in the effluent (mg O_2 L^{-1}) and [i-Valeric] is the concentration of isovaleric acid in the effluent (mg O_2 L^{-1}).

This parameter is often used to quantify the quality of the acidified effluents to be used as feedstock for PHA synthesis, since SCCAs with odd number of carbons lead to PHA with a higher proportion of 3HV monomers [166], and consequently, PHA with better mechanical properties [167]. Therefore, a high odd-to-even ratio is sought if its aimed to use the effluent in the production of PHA with a high content of 3HV monomers. Since caproic and isocaproic acids have been not examined deeply as PHA precursors, they were excluded from the odd-to-even ratio calculation.

3.5.4 Statistical analyses of the effectiveness parameters and the effluent organic content and composition

Statistical analyses were performed to evaluate if the different conditions tested produced a significant impact on the reactor performance, including the effectiveness parameters and the organic content of the effluent as well as its composition. In this study, two main statistical analyses were used: t-test and one-way ANOVA test. t-test was applied when only two groups were compared. In case three or more groups need to be contrasted, one-way ANOVA test was used.

3.5.4.1 t-test

Prior to perform the t-test to compare two groups, F-test was used to evaluate if their variances were significantly different. If the variances of both groups were not significantly different, t-test was applied. If the variances were significantly different, t-test with Welch's correction was used. Gaussian distribution was assumed for all the data and the confidence level for both, F-test and t-test, was set at 95%, so $p<0.05$ implies statistical significance. GraphPad Prism version 9.4.1 (GraphPad Software Inc., San Diego, California, USA) was used to carry out t-test analyses.

3.5.4.2 One-way ANOVA test

Before using the one-way ANOVA test, Brown-Forsythe test was used to examine if the variances of the different groups were significantly different. If the variances of the different groups were not significantly different, ordinary ANOVA test was applied. If the variances of the different groups were significantly different, Brown-Forsythe ANOVA test was used. Gaussian distribution was assumed for all the data and the confidence level was set for both Brown-Forsythe test and ANOVA test at 95%, so $p<0.05$ implies statistical significance.

In case the ANOVA test showed significant differences among the groups, Tukey's comparison test was used to contrast the different groups by pairs and find out which groups are significantly different. For Tukey's comparison test, the confidence level was fixed at 95%, so $p <0.05$ implies statistical significance.

GraphPad Prism version 9.4.1 (GraphPad Software Inc., San Diego, California, USA) was used to carry out ANOVA test and Tukey's comparison test.

3.5.5 Statistical analyses of the composition of the bacterial communities

The Statistical Analysis of Metagenomic Profiles (STAMP) program [216] was used to find statistically significant differences between the different species under the different conditions tested based on relative abundances from amplicon sequencing. Following the user's guide recommendations, two samples comparison was performed

with the Fisher's exact test and the confidence interval was calculated with the Newcombe-Wilson method and a confidence level of 95%.

3.5.6 Shannon index and diversity t-test

Shannon index was determined to assess the alpha-diversity of the bacterial communities developed at the different operational conditions tested. Moreover, diversity t-test was performed to identify statistical differences among Shannon indexes obtained for the different conditions examined. Both Shannon index calculation and diversity t-test were carried out using Past 4.11 software [217].

Chapter 4:

Acidogenic fermentation of a carbohydrate model compound: exploring the influence of the volumetric organic loading rate, specific organic loading rate and influent carbon-to-nitrogen ratio on the process performance and biomass composition

4.1 Overview

In this chapter, acidogenic fermentation experiments were performed in a sequencing batch reactor (SBR) starting from simulated wastewater containing food-grade sugar as model compound for carbohydrates. The main goal is to evaluate the influence of the volumetric organic loading rate (OLRv), specific organic loading rate (OLRs) and influent carbon-to-nitrogen ratio on the process effectiveness parameters, the effluent composition and the bacterial communities, at family and genus level, present in the reactor. Therefore, each operational parameter was individually changed while maintaining the other two constant, until attaining the steady state. Statistical methods were used to compare if the results obtained under the different tested conditions are significantly different and, consequently, find out if the OLRv, OLRs and influent carbon-to-nitrogen ratio have significant effect on the acidogenic fermentation. Likewise, microbial communities favoured under each of the tested conditions and indirectly, the product spectrum formed, were evaluated.

Hereinafter, this chapter starts with the description of the results regarding the reactor operation, process effectiveness parameters, effluent composition and bacterial communities. Next, the effect of the OLRv, OLRs and influent carbon-to-nitrogen ratio on the acidogenic fermentation performance, effluent composition and bacterial communities are discussed together. Finally, the potential applications of the obtained effluents obtained were evaluated. Materials and methods corresponding to the experiments of this chapter can be found in Chapter 3.

4.2 Results

4.2.1 Reactor operation

The reactor was operated for 171 days at different conditions of OLRv, OLRs and the influent carbon-to-nitrogen ratio. Table 4.1 gathers the different operational periods and the conditions applied in each one of them. Figure 4.1 shows the profiles against time of the organic matter in terms of total COD (chemical oxygen demand) concentration in the influent, total COD concentration in the effluent and the COD equivalents in the effluent corresponding to the food-grade sugar and the total carboxylic acids (CAs). Three values of OLRv were tested: low OLRv (6.0±0.3 g COD L^{-1} d^{-1}), medium OLRv

(11.0±0.9 g COD L^{-1} d^{-1}) and high OLRv (15.8±0.7 g COD L^{-1} d^{-1}) while maintaining the OLRs at ca. 2 g COD g^{-1} VSS d^{-1} and the influent carbon-to-nitrogen ratio at 12. Then, two values of OLRs were applied: low OLRs (2.3±0.3 g COD g^{-1} VSS d^{-1}) and high OLRs (3.8±0.9 g COD g^{-1} VSS d^{-1}) keeping the OLRv at its highest value (ca. 16 g COD L^{-1} d^{-1}) and the influent carbon-to-nitrogen ratio at 12. Finally, at the highest values of both OLRv and OLRs, the influent carbon-to-nitrogen ratio was changed from 12 to 25. Despite not having a pH automatic control loop, the pH value in each of the achieved steady states remained around a value of 5 (Table 4.1). Consequently, the effect of the OLRv, OLRs and influent carbon-to-nitrogen ratio on the process performance, the effluent composition and the biomass composition was not affected by the variation of the pH value.

Table 4.1. Experimental conditions tested throughout the study of the acidogenic fermentation using food-grade sugar as sole carbon source.

Period	**Duration (d)**	**OLRv (g COD L^{-1} d^{-1})**	**OLRs (g COD g^{-1} VSS d^{-1})**	**Influent carbon-to-nitrogen ratio**	**HRT (d)**	**Average pH in the bulk liquid**
Start-up	19	10.6±0.6 **(medium)**	2.1±0.3 **(low)**	12 **(low)**	1.2±0.1	
I	29	11.0±0.9 **(medium)**	1.8±0.3 **(low)**	12 **(low)**	1.1±0.1	5.2±0.3
II	29	15.8±0.7 **(high)**	2.3±0.3 **(low)**	12 **(low)**	0.8±0.1	5.2±0.2
III	46	17.6±1.1 **(high)**	3.8±0.9 **(high)**	12 **(low)**	0.8±0.1	5.5±0.4
IV	21	17.3±0.8 **(high)**	3.4±0.6 **(high)**	25 **(high)**	0.8±0.1	5.4±0.7
V	27	6.0±0.3 **(low)**	2.2±0.2 **(low)**	12 **(low)**	2.2±0.1	4.9±0.7

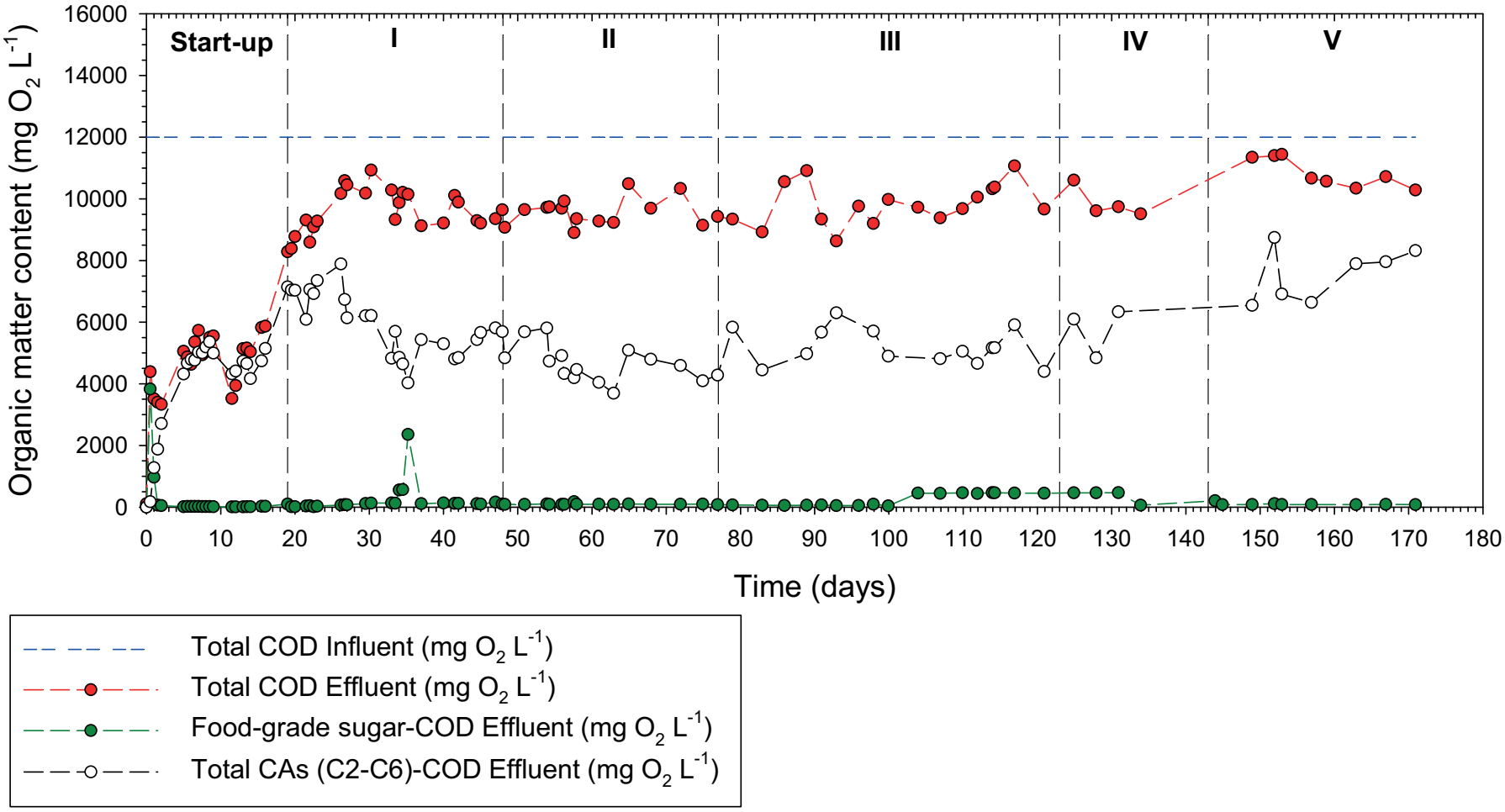


Figure 4.1. Reactor operation during the study of the acidogenic fermentation using food-grade sugar as sole carbon source. Vertical black discontinuous lines indicate the beginning of a new period.

The reactor operation started at the medium level of OLRv (Table 4.1). The biomass used as inoculum came from an anaerobic digester fed with papermill wastewater. The first step needed for CA accumulation is the inhibition and wash-out of the methanogenic microorganisms that use the CAs to produce methane. Several methods are available to inhibit methanogenic activity, namely heat-shock pre-treatment of the biomass, pH control or the use of chemical inhibitors [218]. But also, the application of high OLRv, as the ones applied in this study, should be enough to wash-out the methanogens when there are no hydrolysis limitations due to the complexity of the substrate [177]. If the substrates are simple, by applying high OLRv, SCCA formation is benefited due to the large substrate availability, leading to a decrease of pH and the subsequent inhibition of the methanogenic activity [1]. Since the food-grade sugar is a structurally simple carbohydrate, no hydrolysis limitations were expected and therefore, the application of an OLRv of 10.6 g COD L^{-1} d^{-1} was enough to arrest methanogens [219]. At an OLRv of 10.6 g COD L^{-1} d^{-1}, 19 days were required to achieve steady state values of the total COD and total CAs (in COD equivalents) in the effluent.

During the reactor operation, the food-grade sugar concentration in the effluent was almost zero unless very exceptional times. After the start up period, the average pH in the bulk liquid remained below 5.5 (Table 4.1). Since the optimal pH range for methanogens is 6.5-8.2 [1], the methanogenic activity was almost completely inhibited after the start-up period. Regarding the total COD of the effluent, it oscillated between 9000-11500 mg O_2 L^{-1} during periods I to V, with the following mean values: period I (9644±660 mg O_2 L^{-1}), period II (9577±445 mg O_2 L^{-1}), period III (9825±695 mg O_2 L^{-1}), period IV (9868±500 mg O_2 L^{-1}), period V (10886±502 mg O_2 L^{-1}). With the exception of period V, the total COD in the effluent for the rest of the periods did not show significant differences. The total COD in the effluent in period V (low OLRv value) was significantly higher than the obtained in period I (medium OLRv value) ($p=0.0001$) and period II (high OLRv value) ($p=0.0001$). Hence, decreasing the OLRv to 6.0 g COD L^{-1} d^{-1} lead to higher total COD in the effluent. Nevertheless, no changes were observed in the total COD in the effluent when increasing the OLRs from 2.3 up to 3.8 g COD g^{-1} VSS d^{-1} (periods II and III) and when the influent carbon-to-nitrogen ratio was risen from 12 up to 25 (period III and IV).

Regarding the total CAs in the effluent, there are more clear differences among the different periods. The conditions applied in the different periods yielded the following average total CAs in the effluent: period I (5902±992 mg O_2 L^{-1}), period II (4636±586

mg O_2 L^{-1}), period III (5212±583 mg O_2 L^{-1}), period IV (5755±803 mg O_2 L^{-1}), period V (7574±871 mg O_2 L^{-1}). Periods I, II and V (medium, high and low OLRv values, respectively) showed significant differences among them ($p=0.0001$ for periods I and II; $p<0.0001$ for periods I and V and $p<0.0001$ for periods II and V). From these results, it can be observed that the lower the OLRv applied, the higher total CAs achieved in the effluent. However, increasing the OLRs from 2.3 up to 3.8 g COD g^{-1} VSS d^{-1} did not modify the total CAs in the effluent given that there were no significant differences between periods II and III ($p=0.3157$). Plus, increasing the influent carbon-to-nitrogen ratio from 12 up to 25 did not affect the total CAs in the effluent since periods III and IV did not exhibit significant differences ($p=0.8268$).

Concerning the reactor performance, one of the objectives of this study was to find out if it was possible to operate a semicontinuous reactor with biomass retention where the acidogenic fermentation of a model carbohydrate was carried out. If it is aimed to use the effluent directly for a subsequent application, low VSS concentration in the effluent and therefore, good settleability in the reactor are pursued. The reactor was inoculated with granular anaerobic biomass. Despite this, after the start-up period, the biomass turned into flocculent. On the one hand, the granules were broken due to the shear stress caused by the stirring and washout due to the methane production. On the other hand, during the start-up period part of the granules were washed out from the reactor due to the flotation caused by the biogas bubbles produced. Figure 4.2 shows the difference of the biomass inoculated and the biomass after 171 days of operation. Besides the color change, it can be observed that the biomass taken from the reactor at the end of the operation is totally flocculent.

Despite having a flocculent biomass, it was possible to operate the reactor successfully since the biomass had a good settleability. In this study, the objective was to assess the effect of the OLRv and the OLRs on the acidogenic fermentation process. So, the way of operating the reactor was to fix the OLRv and the OLRs. To select the OLRv, the total cycle duration (and, accordingly the reaction phase length) was modified since the feeding solution concentration was constant. Once the OLRv was fixed, the targeted OLRs was set by keeping the biomass concentration at the corresponding value by purging when necessary. In this experiment, using food-grade sugar as substrate, the biomass grew up very fast, especially at the largest OLRv. Thus, it was necessary to purge very frequently to keep constant the biomass concentration. Moreover, when varying the

OLRv maintaining the same OLRs, the required purge was different for each applied OLRv and this fact caused to have a different sludge retention time (SRT) for each OLRv: 14 days at 6.0 g COD L^{-1} d^{-1}, 11 days at 11.0 g COD L^{-1} d^{-1} and 4 days at 15.8 g COD L^{-1} d^{-1}. When varying the OLRs and the influent carbon-to-nitrogen ratio, the SRT was not altered. In each SBR cycle, the effluent was discharged with a solid concentration of approximately 0.4 g VSS L^{-1} after 60 minutes of settling while the biomass concentration in the reactor ranged between 2.8 and 7.3 g VSS L^{-1} depending on the selected OLRv and OLRs.

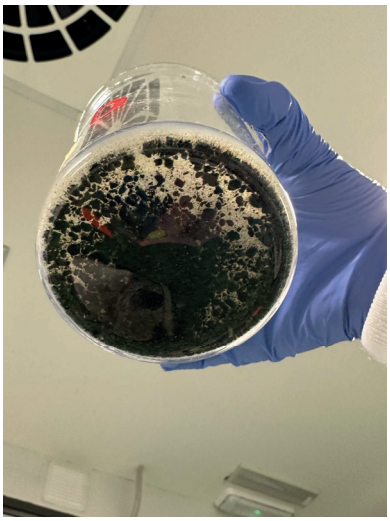
(a)  (b) 

Figure 4.2. a) Sample of the biomass used as inoculum in the acidogenic fermentation study fermentation using food-grade sugar as sole carbon source. b) Sample of the biomass after the reactor operation during the study of the acidogenic fermentation using food-grade sugar as sole carbon source.

4.2.2 Process effectiveness parameters: bioconversion, degree of acidification, acidified COD, CA production yield and odd-to-even ratio

In this section, the effect of the OLRv, OLRs and influent carbon-to-nitrogen ratio on the process effectiveness parameters is presented. Table 4.2 collects the values of these process effectiveness parameters under the different conditions tested during the carbohydrate fermentation experiments. The statistical results of the ANOVA test and Tukey's multiple comparisons test to evaluate the effect of the OLRv (Table S4.2) and the t-test results for comparing the process effectiveness parameter values at the different

OLRs and influent carbon-to-nitrogen ratios (Table S4.3 and Table S4.4) can be found in Appendix I.

Table 4.2. Bioconversion, Degree of Acidification, Acidified COD, Carboxylic Acid production yield and odd-to-even ratio in the different conditions tested during the study of the acidogenic fermentation using food-grade sugar as sole carbon source.

OLRv (g COD L^{-1} d^{-1})	**OLRs (g COD g^{-1} VSS d^{-1})**	**Influent carbon-to-nitrogen ratio**	**Bioconversion (%)**	**Degree of Acidification (%)**	**Acidified COD (%)**	**CA production yield (g COD g^{-1} COD)**	**Odd-to-even ratio**
6.0±0.3 **(low)**	2.2±0.2 **(low)**	12 **(low)**	99.4±0.1	59±7	73±8	0.6±0.1	0.03± 0.01
11.0±0.9 **(medium)**	1.8±0.3 **(low)**	12 **(low)**	99.0±0.2	45±5	56±6	0.5±0.1	0.21± 0.03
15.8±0.7 **(high)**	2.3±0.3 **(low)**	12 **(low)**	99.2±0.1	38±1	45±1	0.4±0.1	0.16± 0.00
17.6±1.1 **(high)**	3.8±0.9 **(high)**	12 **(low)**	96.5±0.2	40±6	50±4	0.4±0.1	0.21± 0.06
17.3±0.8 **(high)**	3.4±0.6 **(high)**	25 **(high)**	96.3±0.2	46±7	58±8	0.5±0.1	0.28± 0.02

Concerning the OLRv, the resultant bioconversion was very similar for all the tested OLRv with values over 99%. Only a significant decrease was produced when the OLRv was risen from 6.0 up to 11.0 g COD L^{-1} d^{-1}, but a clear trend cannot be described since there was no significant difference between 6.0 to 15.8 g COD L^{-1} d^{-1} (Table S4.2 in Appendix I). Besides, the different OLRv values tested lead to a degree of acidification from 38% to 59%. Despite an apparent trend of a decreasing degree of acidification when the OLRv was increased, significant differences were only found between the lowest OLRv (6.0 g COD L^{-1} d^{-1}) and the other two tested OLRv (11.0 and 15.8 g COD L^{-1} d^{-1}) (Table S4.2 in Appendix I). With respect to the acidified COD, lowering the OLRv from 15.8 up to 6.0 g COD L^{-1} d^{-1} increased the acidified COD from 45% up to 73%. In this case, there are significant differences among the three values levels of the tested OLRv (Table S4.2 in Appendix I), so there is a clear increasing trend of the acidified COD when

the OLRv is lowered. The CA production yield showed values among 0.4 and 0.6 g COD g^{-1} COD. Similarly to the degree of acidification, the CA production yield decreased significantly when the OLRv was increased from 6.0 to 11.0-15.8 g COD L^{-1} d^{-1} (Table S4.2 in Appendix I). Finally, the odd-to-even ratio was comprised between 0.03 and 0.21. Once again, the odd-to-even ratio exclusively exhibited significant differences between the lowest OLRv (6.0 g COD L^{-1} d^{-1}) and other two OLRv (11.0 and 15.8 g COD L^{-1} d^{-1}) (Table S4.2 in Appendix I). In conclusion, decreasing the OLRv from 15.8 up to 6.0 g COD L^{-1} d^{-1} conducts to higher acidified COD in the effluent and does not affect notably the bioconversion. Moreover, at an OLRv of 6.0 g COD L^{-1} d^{-1}, the degree of acidification and the CA production yield obtained are higher but the odd-to-even ratio is lower than the achieved with the OLRv of 11.0 and 15.8 g COD L^{-1} d^{-1}.

Next, the effect of the OLRs over the process effectiveness parameters is examined. On the one hand, increasing the OLRs from 2.3 up to 3.8 g COD g^{-1} VSS d^{-1} leads to a significant decrease of bioconversion from 99.2% to 96.5%. On the other hand, no significant changes were observed when rising the OLRs in the rest of the process effectiveness parameters (Table S4.3 in Appendix I). The mean values of the degree of acidification, the acidified COD and odd-to-even ratio ranged between 38%-40%, 45%-50% and 0.16-0.21, respectively. Moreover, the CA production yield was maintained constant at 0.4 g COD g^{-1} COD regardless of the applied OLRs.

Finally, no significant variations were measured in the process effectiveness parameters between the influent carbon-to-nitrogen ratios of 12 and 25 (Table S4.4 in Appendix I). Bioconversion, degree of acidification, acidified COD, CA production yield and odd-to-even ratio ranged between 96.3-96.5%, 40-46%, 50-58%, 0.4-0.5 g COD g^{-1} COD and 0.21-0.28, respectively.

4.2.3 Effluent composition

Following, in this section, the effect of the OLRv, OLRs and influent carbon-to-nitrogen ratio on the effluent composition is presented. Table S4.1 (Appendix I) shows the concentration (mg COD L^{-1}) of the compounds measured in the effluent under the different conditions tested.

The effluent composition (%COD) at the different OLRv applied is represented in Figure 4.3. The statistical difference between the effluent components can be deduced from the ANOVA test and Tukey's multiple comparisons test in Table S4.5 (Appendix I). At an OLRv of 6 g COD L^{-1} d^{-1}, there were two main components in the effluent, namely, butyric acid (47.0%) and acetic acid (28.0%). When increasing the OLRv to 11.0 and 15.8 g COD L^{-1} d^{-1}, acetic acid composition did not vary significantly, yielding 23.0% and 22.4%, respectively. However, at OLRv of 11.0 and 15.8 g COD L^{-1} d^{-1}, butyric acid sharply decreased to 14.0% and 11.0%, respectively; and ethanol rose to 24.0% and 30.0%, respectively, from the 8.0% obtained at 6.0 g COD L^{-1} d^{-1}. Propionic and isobutyric acids increased significantly between the lowest OLRv (2.4% and 0.9%, respectively) and the other two OLRv (4.5-6.9% and 5.2-9.0%, respectively). The only compound showing significant different composition between the two highest OLRv (11.0 and 15.8 g COD L^{-1} d^{-1}) was propionic acid, but this acid accounted for a maximum of the 6.9% of the effluent COD. No CAs of more than four carbons were detected in proportions larger to the 3.0% of the effluent COD. The effluent COD corresponding to non-identified compounds ranged from 12.2% to 23.9%.

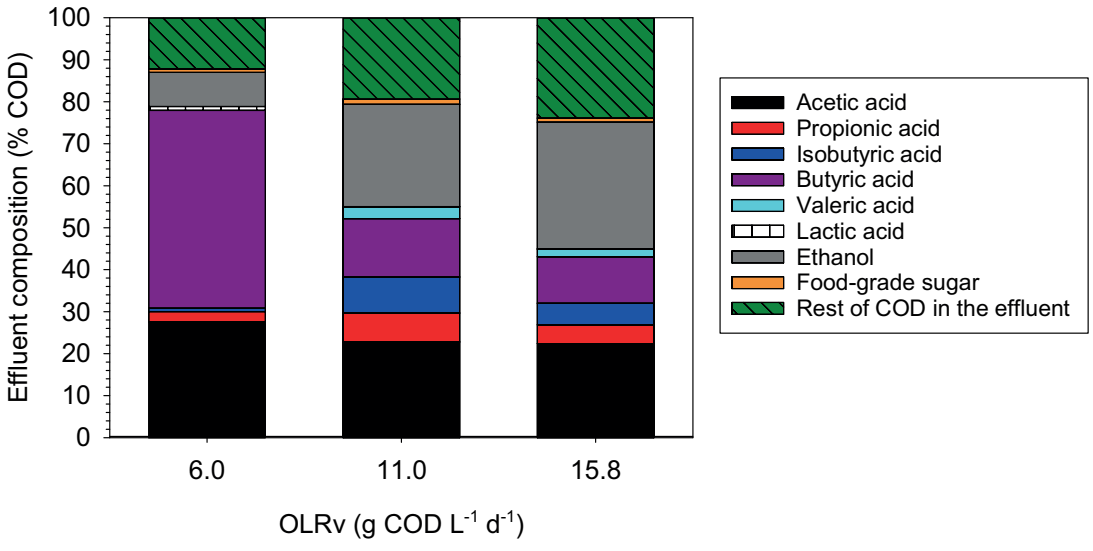


Figure 4.3. Effluent composition at different OLRv tested (6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}) at an OLRs of approximately 2 g COD g^{-1} VSS d^{-1} and an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

The effluent composition (%COD) at the different OLRs applied is plotted in Figure 4.4. The statistical difference between the effluent components can be gathered from the t-test in Table S4.6 (Appendix I). With the rise of the OLRs from 2.3 to 3.8 g COD g^{-1} VSS d^{-1}, the CAs with less than four carbons show no significant differences. In this way, acetic, propionic, isobutyric and butyric acids ranged from 22.4%-20.0%, 4.5%-4.2%, 5.2%-4.1% and 11.0%-12.5%, respectively. The largest difference between both OLRs tested was in the ethanol composition, who decreased significantly from 30.0% to 13.0%, when rising the OLRs from 2.3 to 3.8 g COD g^{-1} VSS d^{-1}. This OLRs increase also conducted to the formation of caproic acid that was not detected at 2.3 g COD g^{-1} VSS d^{-1} and comprised the 6% of the effluent composition at 3.8 g COD g^{-1} VSS d^{-1}. Moreover, food-grade sugar was increased significantly from 0.9% to 4.4% when the OLRs was increased from 2.3 to 3.8 g COD g^{-1} VSS d^{-1}, showing substrate consumption limitation at the highest OLRs. The effluent COD corresponding to non-identified compounds increased from 23.9% to 31.8% when the OLRs was increased from 2.3 to 3.8 g COD g^{-1} VSS d^{-1}.

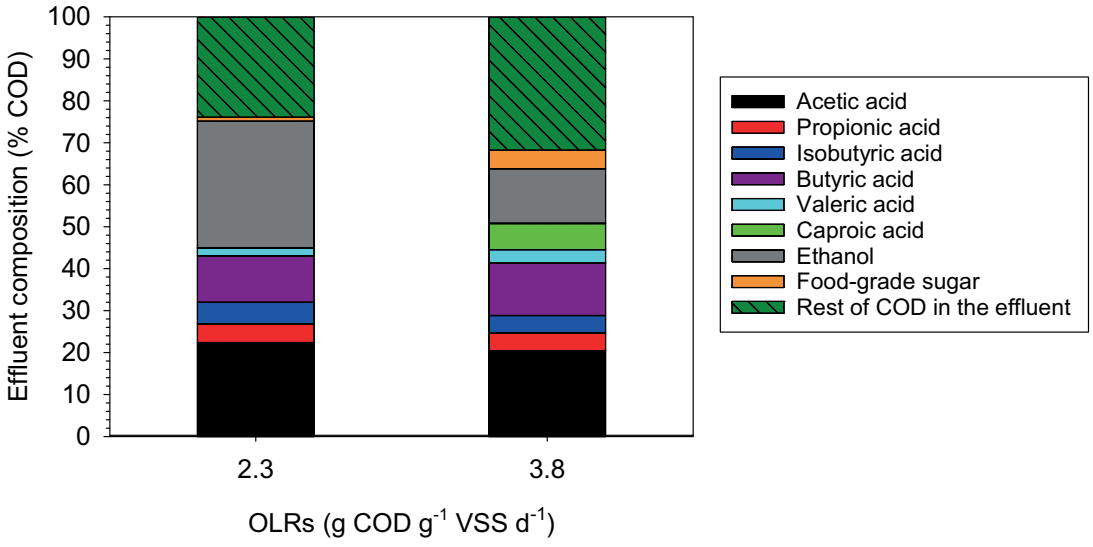


Figure 4.4. Effluent composition at different OLRs tested (2.3 and 3.8 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

The effluent composition (%COD) at the different influent carbon-to-nitrogen ratios tested is depicted in Figure 4.5. The statistical difference between the effluent components

can be gathered from the t-test in Table S4.7 (Appendix I). The effluent composition obtained at the influent carbon-to-nitrogen ratios 12 and 25 is very similar. Only propionic acid increased significantly from 4.2% to 7.3% when the influent carbon-to-nitrogen ratio was increased from 12 up to 25, respectively. The effluent COD corresponding to non-identified compounds decreased from 31.8% to 20.1% when the influent carbon-to-nitrogen ratio was enlarged from 12 to 25, respectively.

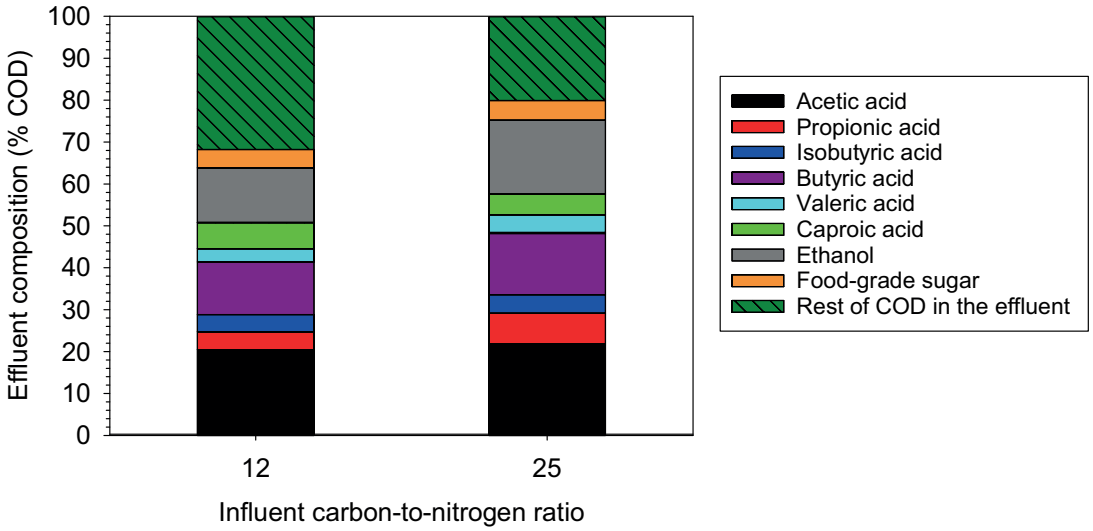


Figure 4.5. Effluent composition at different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source.

4.2.4 Bacterial communities

In this section, the bacterial communities detected under the different tested conditions are presented at family and genus levels. The effect of the OLRv, OLRs and influent carbon-to-nitrogen ratio on the microbial communities are individually analyzed.

4.2.4.1 Effect of OLRv on the bacterial communities

The relative abundances (in %) of the bacterial communities at family level at the different OLRv tested are shown in Figure 4.6. Figure S4.1 (Appendix I) depicts the difference in the proportions including only the families that show significant differences in the relative abundance at the three tested levels of OLRv. Four main families were

detected: *Ruminococcaceae*, *Bifidobacteriaceae*, *Clostridiaceae 1* and *Enterobacteriaceae*. Thus, *Ruminococcaceae* relative abundance raised significantly from 29% to 42% when shifting the OLRv from 6.0 to 11.0 g COD L^{-1} d^{-1} At 15.8 g COD L^{-1} d^{-1}, the relative abundance of *Ruminococcaceae* was 48%, which was significantly higher than the relative abundance at 6.0 g COD L^{-1} d^{-1} but did not show significant differences with the measured one at an OLRv of 11.0 g COD L^{-1} d^{-1} (Figure S4.1 in Appendix I). The relative abundances of *Bifidobacteriaceae* were 38%, 29%, and 18% at 6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}, respectively. According to this, increasing the OLRv up to 15.8 g COD L^{-1} d^{-1} significantly decreases *Bifidobacteriaceae* relative abundance with respect to measured ones at 6.0-11.0 g COD L^{-1} d^{-1}, while there was no significant difference between 6.0 and 11.0 g COD L^{-1} d^{-1} (Figure S4.1 in Appendix I). *Clostridiaceae 1* relative abundances were 10%, 12% and 13% at 6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}, respectively, showing no significant differences among the tested OLRv. Finally, *Enterobacteriaceae* relative abundance was significantly different between 6.0 and 11.0 g COD L^{-1} d^{-1} (12% and 3%, respectively) and between 11.0 g COD L^{-1} d^{-1} and 15.8 g COD L^{-1} d^{-1} (3% and 10%, respectively) (Figure S4.1 in Appendix I). However, since there were no significant differences between 6.0 and 15.8 g COD L^{-1} d^{-1}, a clear trend cannot be described.

Figure 4.7 shows the relative abundances of the bacterial communities at genus level at the different OLRv tested. Figure S4.2 (Appendix I) depicts the difference in the proportions including only the genera that show significant differences in the relative abundance at the three tested levels of OLRv. Four main genera were found: *Bifidobacterium*, *Ethanoligenens*, *Clostridium sensu stricto* and *Clostridium IV*. The relative abundances of *Bifidobacterium* were 38%, 29%, and 18% at 6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}, respectively. Like this, increasing the OLRv up to 15.8 g COD L^{-1} d^{-1} significantly decreased *Bifidobacterium* relative abundance with respect to 6.0-11.0 g COD L^{-1} d^{-1}, while there was no significant difference between 6.0 and 11.0 g COD L^{-1} d^{-1} (Figure S4.2 in Appendix I). At the same time, increasing the OLRv up to 15.8 g COD L^{-1} d^{-1} significantly rose the relative abundance of *Ethanoligenens* up to 42% with respect to the relative abundances at 6.0 and 11.0 g COD L^{-1} d^{-1} (22% and 29%, respectively) (Figure S4.2 in Appendix I). However, no significant difference was detected in *Ethanoligenens* relative abundance at 6.0 and 11.0 g COD L^{-1} d^{-1}. *Clostridium sensu stricto* relative abundances were 10%, 12% and 13% at 6.0, 11.0 and 15.8 g COD L^{-1} d^{-1},

respectively, showing no significant differences among the tested OLRv. Lastly, the relative abundances of *Clostridium IV* were 6%, 12% and 5% at 6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}, respectively. According to this, there was only significant difference in the relative abundances measured at 11.0 and 15.8 g COD L^{-1} d^{-1}.

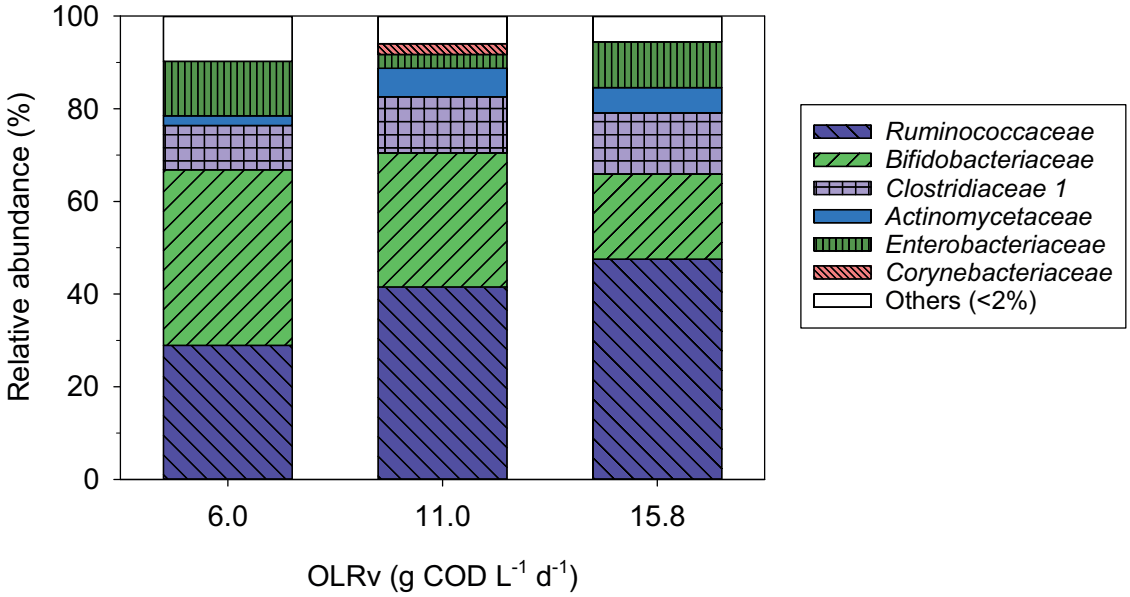


Figure 4.6. Bacterial communities at family level at different OLRv tested (6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}) at an OLRs of approximately 2 g COD g^{-1} VSS d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

No significant differences were found among the Shannon Index calculated for quantifying the biodiversity obtained at the different OLRv tested, for both family and genus levels (Table S4.8 in Appendix I).

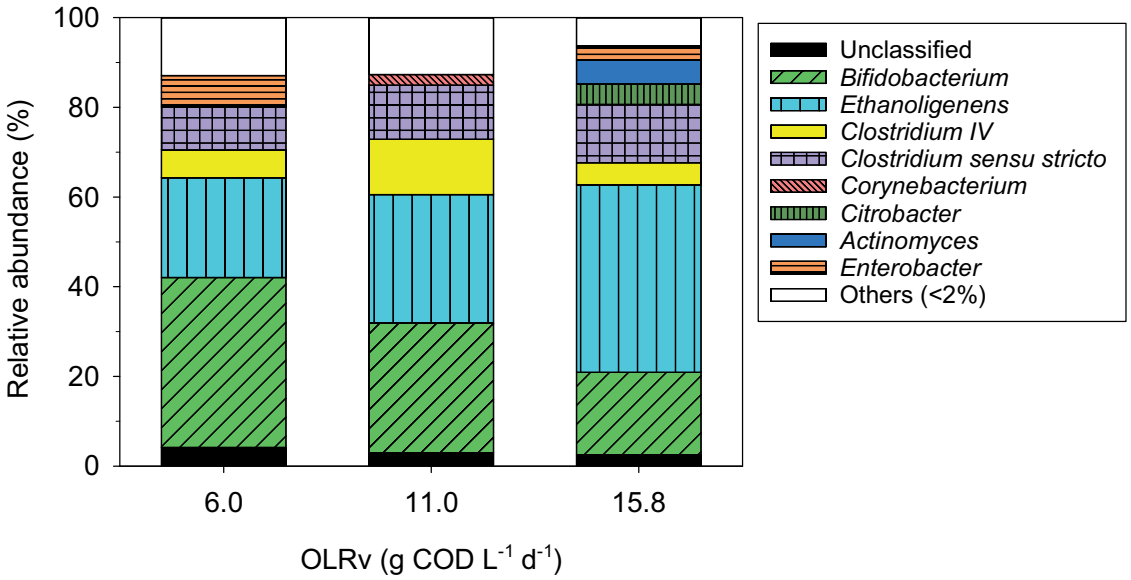


Figure 4.7. Bacterial communities at genus level at different OLRv tested (6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}) at an OLRs of approximately 2 g COD g^{-1} VSS d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

4.2.4.2 Effect of OLRs on the bacterial communities

The relative abundances (in %) of the bacterial communities at family level at the different tested OLRs are shown in Figure 4.8. No significant differences were found between the families with a relative abundance over 2% at the applied OLRs. The relative abundances detected of the main families were the following: *Ruminococcaceae*, 48% and 45%; *Bifidobacteriaceae*, 18% and 24%; *Clostridiaceae 1*, 13% and 19%; and *Enterobacteriaceae*, 10% and 4% for OLRs 2.3 and 3.8 g COD g^{-1} VSS d^{-1}, respectively.

The relative abundances (in %) of the bacterial communities at genus level at the different tested OLRs are shown in Figure 4.9. Figure S4.3 (Appendix I) represents the difference in the proportions including only the genera that show significant differences in the relative abundance at the two tested levels of OLRs. The relative abundances detected of the main genera were the following: *Ethanoligenens*, 42% and 40%; *Bifidobacterium*, 18% and 24%; *Clostridium sensu stricto*, 13% and 19%; *Actinomyces*, 5% and 3%; and *Clostridium IV*, 5% and 4% for OLRs 2.3 and 3.8 g COD g^{-1} VSS d^{-1}, respectively. No significant differences were found for the genera with the highest relative abundances at the two applied OLRs.

No significant differences were found among the Shannon Index at the different OLRs tested, for both family and genus levels (Table S4.9 in Appendix I).

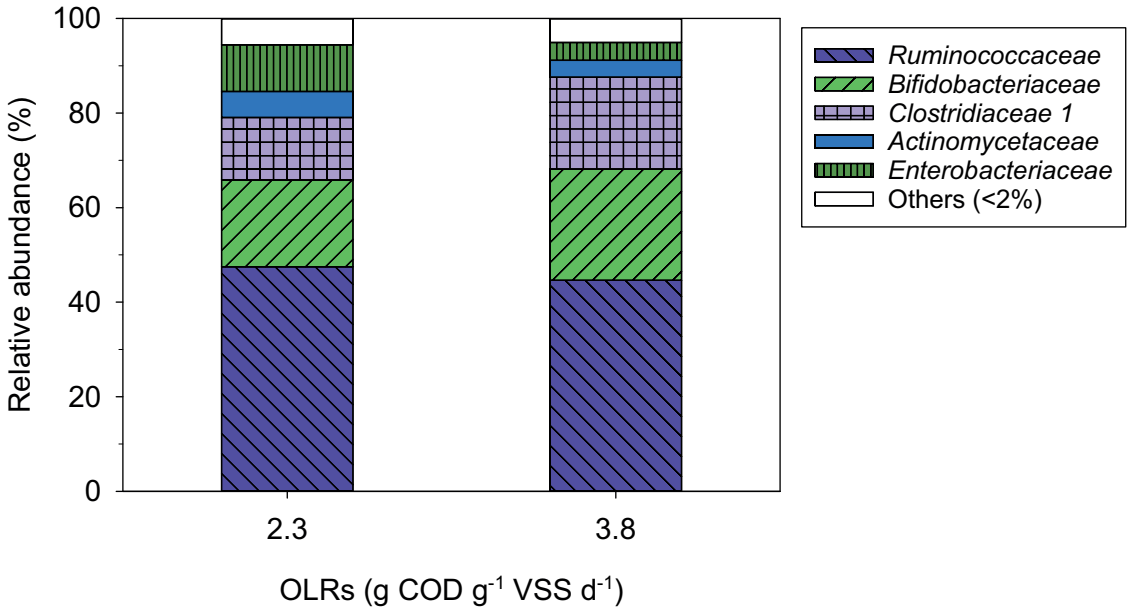


Figure 4.8. Bacterial communities at family level at different OLRs tested (2.3 and 3.8 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

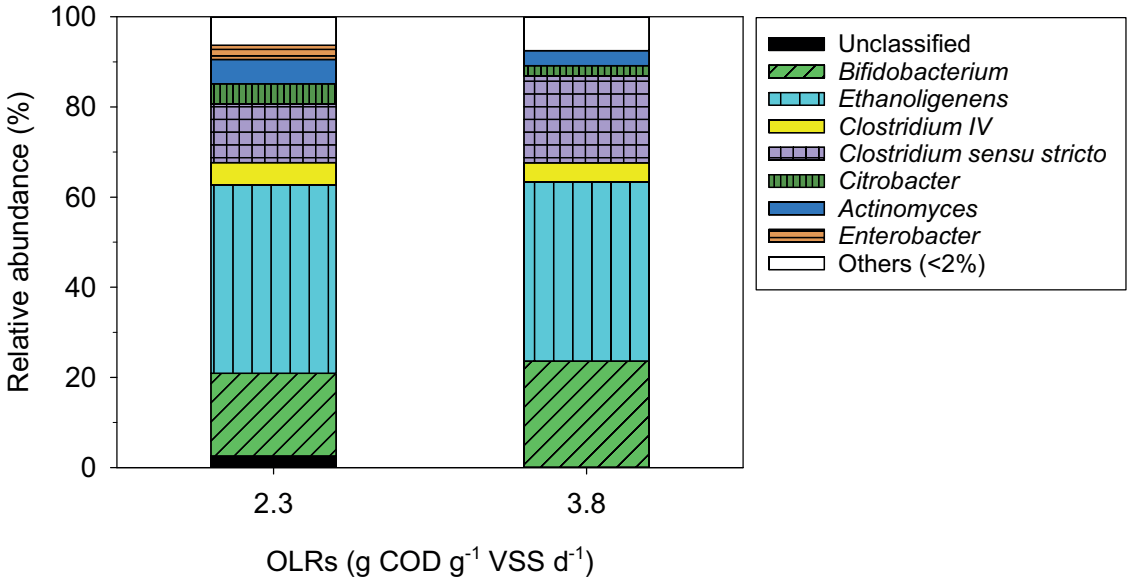


Figure 4.9. Bacterial communities at genus level at different OLRs tested (2.3 and 3.8 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

4.2.4.3 Effect of influent carbon-to-nitrogen ratio on the bacterial communities

Figure 4.10 plots the relative abundances (%) of the bacterial communities at family level at the two-influent carbon-to-nitrogen ratios examined. Figure S4.4 (Appendix I) presents the difference in the proportions including only the families that show significant differences in the relative abundance at the two tested levels of influent carbon-to-nitrogen ratio. Four main families were detected: *Ruminococcaceae*, *Bifidobacteriaceae*, *Clostridiaceae 1* and *Enterobacteriaceae*. Only *Clostridiaceae 1* exhibited significantly different relative abundances of 19% and 31% at influent carbon-to-nitrogen ratios of 12 and 25, respectively (Figure S4.4 in Appendix I). The relative abundances of the rest of the main families were the following: *Ruminococcaceae*, 45% and 34%; *Bifidobacteriaceae*, 24% and 19% and *Enterobacteriaceae*, 4% and 6% for influent carbon-to-nitrogen ratios of 12 and 25, respectively.

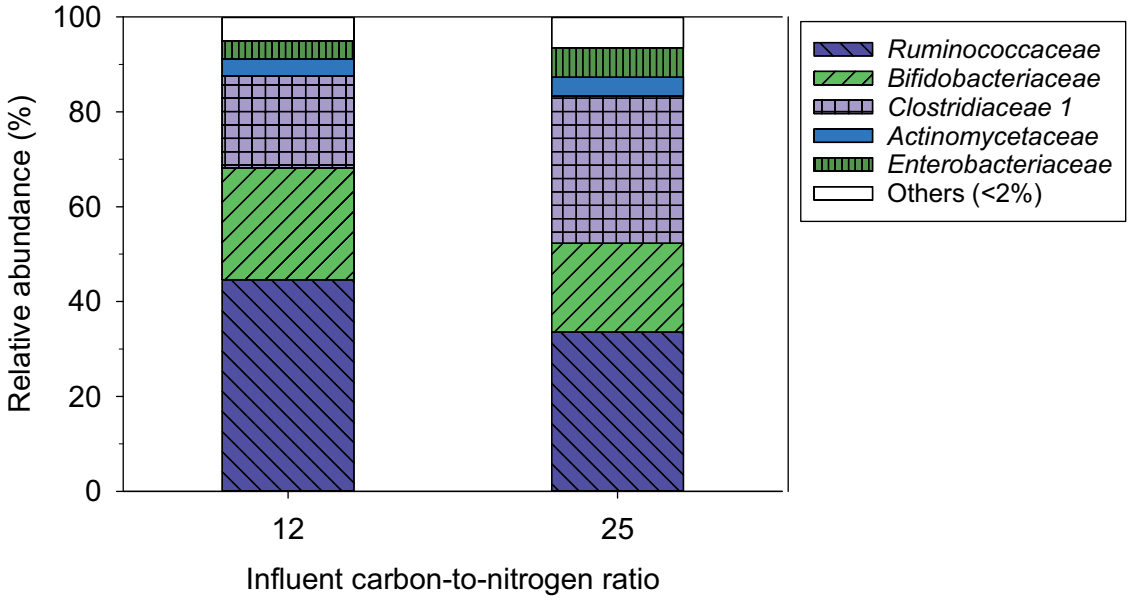


Figure 4.10. Bacterial communities at family level at different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source.

Figure 4.11 represents the relative abundances (in %) of the bacterial communities at genus level at the two influent carbon-to-nitrogen ratios examined. Figure S4.5 (Appendix I) depicts the difference in the proportions including only the genera that show significant differences in the relative abundance at the two tested levels of influent

carbon-to-nitrogen ratio. Three main genera were detected: *Ethanoligenens*, *Clostridium sensu stricto* and *Bifidobacterium*. Among the predominant genera, only *Clostridium sensu stricto* exhibited significantly different relative abundances of 19% and 31% at influent carbon-to-nitrogen ratios of 12 and 25, respectively (Figure S4.5 in Appendix I). At influent carbon-to-nitrogen ratios of 12 and 25, *Ethanoligenens* had relative abundances of 45% and 34%, respectively, while *Bifidobacterium* had relative abundances of 24% and 19% (Figure S4.5 in Appendix I).

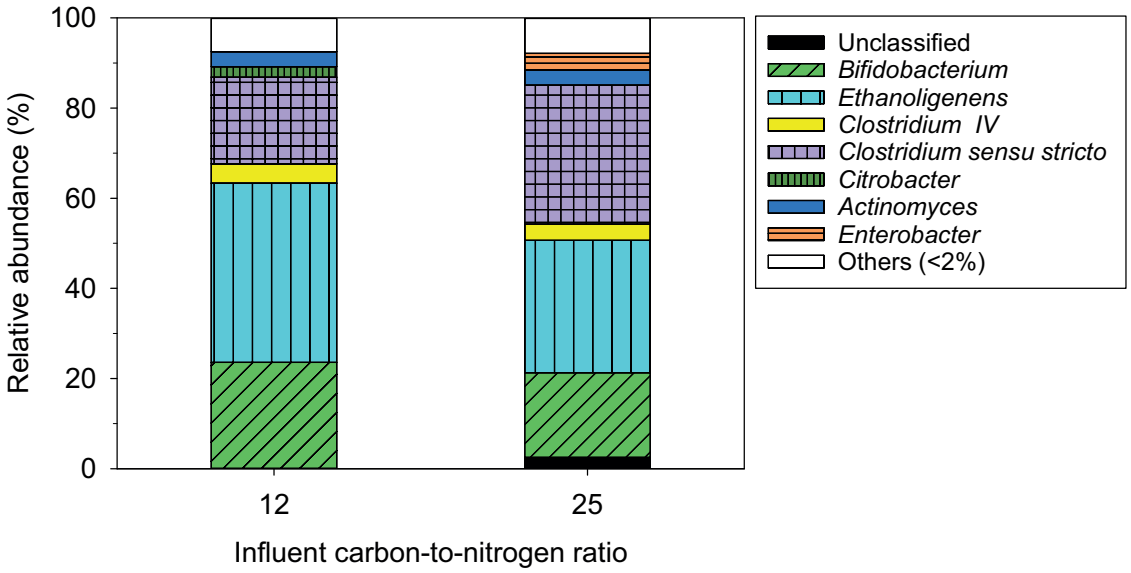


Figure 4.11. Bacterial communities at genus level at different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source.

No significant differences were found among the Shannon Index at the different influent carbon-to-nitrogen ratios tested, for both family and genus levels (Table S4.10 in Appendix I).

4.3 Discussion

In the following section, the results presented in the previous section are discussed.

4.3.1 Effect of the OLRv

The results presented in the previous section clearly show that the OLRv impacts the acidogenic fermentation process when the substrate is only formed by carbohydrates. First, the decrease of the OLRv from medium-high values (11.0-15.8 g COD L^{-1} d^{-1}) to low values (6.0 g COD L^{-1} d^{-1}) leads to a higher COD concentration in the effluent (Figure 4.1). The difference between the total COD of the influent and of the effluent can only be related to organic matter either used for biomass growth or transformed into biogas. The new biomass formed depends on the applied SRT: the lower the SRT, the higher biomass formation since biomass yield is higher. The resultant SRTs at the OLRv of 6.0, 11.0 and 15.8 g COD L^{-1} d^{-1} were 14, 11 and 5 days, respectively. So, it could already be expected that at the OLRv of 6.0 g COD L^{-1} d^{-1}, which corresponds to the highest SRT applied, less organic matter was removed from the effluent for biomass growth. Besides, the observed growth yield (Yobs) was calculated for each applied OLRv, and all the values were close to the expected biomass growth yields for this kind of biological reaction. Therefore, the removed organic matter derived to biogas production might be negligible or very much lower than the one used for biomass growth.

Regarding the total CA concentration in the effluent, it can be concluded that the lower the applied OLRv, the higher the total CAs achieved in the effluent (Figure 4.1). The lower CA concentration obtained at higher OLRv is not related to a lower carbohydrate consumption, but to a higher conversion of the food-grade sugar to ethanol instead of CAs. As shown in Table 4.2, the bioconversion at the three tested levels of OLRv is over 99% and it is not affected by the changes of OLRv. Food-grade sugar is a simple carbohydrate, so these high values of bioconversion were foreseen since hydrolysis limitations were not expected. Nevertheless, when changing the OLRv from 6.0 to 11.0-15.8 g COD L^{-1} d^{-1}, the effluent composition is sharply modified. At the lowest OLRv (6.0 g COD L^{-1} d^{-1}), the effluent was mainly composed by butyric (47%) and acetic (28%) acids while the ethanol only comprised the 8% (Figure 4.3). When the OLRv is increased up to 11.0 and 15.8 g COD L^{-1} d^{-1}, the butyric acid significantly decreases to 14% and 11%, respectively while the ethanol significantly rises to 24% and 30%, respectively (Figure 4.3, Table S4.5 in Appendix I). These changes in the effluent composition affected the degree of acidification, the acidified COD, the CA production yield and the odd-to-even ratio. The significantly lower ethanol proportion in the effluent at 6.0 g COD L^{-1} d^{-1} with respect to 11.0-15.8 g COD L^{-1} d^{-1} (Figure 4.3, Table S4.5 in

Appendix I), conducted to a significantly higher degree of acidification and CA production yield (Table 4.2, Table S4.2 in Appendix I). Simultaneously, it was shown that decreasing the OLRv conducted to a significantly higher acidified COD (Table 4.2, Table S4.2 in Appendix I). Regarding the odd-to-even ratio, it was significantly increased from 0.03 at 6.0 g COD L^{-1} d^{-1} up to 0.16-0.21 at 11.0-15.8 g COD L^{-1} d^{-1} (Table 4.2, Table S4.2 in Appendix I). The odd-to-even ratio increase was caused by the notable decrease of the butyric acid and, in a lesser extent, to the increase of the propionic acid.

By analyzing the bacterial communities present, one might expect to explain the obtained effluent composition at the different applied OLRv. Four main genera were detected under all the operational conditions tested using food-grade sugar as substrate: *Bifidobacterium*, *Ethanoligenens*, *Clostridium sensu stricto* and *Clostridium IV*.

Bifidobacterium have been described as microorganisms with a strictly fermentative metabolism, which are able to degrade complex poly or oligosaccharides to fermentable monosaccharides by using glycoside hydrolases [220]. Once hydrolyzed, the resultant monosaccharides are transformed mainly to acetic and lactic acid by fructose-6-phosphate phosphoketolase (F6PPK) pathway, also named the 'bifid shunt' [221,222]. *Ethanoligenens* have been described as fermentative hydrogen-producing bacteria through ethanol-type fermentation (acetate-ethanol) [223]. *Ethanoligenens* has been reported to produce ethanol, acetate, hydrogen and carbon dioxide [224]. *Clostridium sensu stricto* has been reported as a genus formed by species that are often saccharolytic and proteolytic [225]. Many species comprised in this genus have been described as acetic acid producers [226]. Moreover, some members among the genus *Clostridium sensu stricto,* as for example*, Clostridium tyrobutyricum* can produce butyric acid directly from monosaccharides or from lactic acid (in presence of acetic acid) [227]. Finally, the genus *Clostridium sensu stricto* can be responsible for isobutyric acid synthesis. As a member of this genus, *Clostridium luticellarii* was described as a promising species for isocarboxylic acid production [228]. Finally, bacteria comprised in *Clostridium IV* genus have been reported to be acetic and butyric acid-producing microorganisms [229].

Therefore, the effluent composition can be explained by the bacterial communities present in the biomass at the different tested OLRv. First, food-grade sugar hydrolysis to glucose and fructose might be performed either by the genus *Bifidobacterium* or by *Clostridium sensu stricto* [220,225]. These two genera are present at all the tested OLRv.

Once hydrolyzed, the food-grade sugar was converted to different compounds depending on the applied OLRv.

At an OLRv of 6.0 g COD L^{-1} d^{-1}, the effluent had two main components: butyric acid and acetic acid (Figure 4.3). At this condition, the two majoritarian genera were *Bifidobacterium* and *Ethanoligenens* (Figure 4.7). Both together with *Clostridium sensu stricto* and *Clostridium IV* might be responsible of acetic acid production [221,223,226,229]. Regarding the butyric acid, it might be produced by either *Clostridium sensu stricto* or *Clostridium IV* genera [227,229]. As stated before, *Bifidobacterium* might produce acetic acid and lactic acid [221]. Since lactic acid was detected in less than the 1% of the effluent COD, it might be being consumed by the genus *Clostridium sensu stricto* to produce butyric acid from acetic and lactic acids [227].

At an OLRv of 11.0-15.8 g COD L^{-1} d^{-1}, the effluent was mostly composed by ethanol, acetic acid, butyric acid and isobutyric acid (in decreasing order) (Figure 4.3). With respect to 6.0 g COD L^{-1} d^{-1}, ethanol and isobutyric acid increased significantly, acetic acid showed no significant differences and butyric acid significantly decreased (Table S4.5 in Appendix I). At the two highest OLRv, *Ethanoligenens* was the predominant genus (Figure 4.7), so it might be the responsible of the high increase of the ethanol production at these OLRv [224]. Once again, any of the main genera (*Bifidobacterium*, *Ethanoligenens*, *Clostridium sensu stricto* and *Clostridium IV*) might be responsible of acetic acid production [221,223,226,229] and *Clostridium sensu stricto* and *Clostridium* IV might be responsible of butyric acid production [227,229]. Finally, isobutyric acid might be synthesized by species of the *Clostridium sensu stricto* genus [228].

As stated in the Introduction, there are very few previous publications related to the acidogenic fermentation of the different components of the organic matter. Most of the studies starting from simple carbohydrates reported batch experiments, so the OLRv was not an analyzed parameter. There are few previous studies carried out in semicontinuous or continuous reactors starting from substrates only composed by carbohydrates analyzing the effect of the OLRv [230,231]. However, as far as we know, there are no studies considering the effect of the ratio between substrate and biomass. As an example, Rafay et al. (2022) performed acidogenic fermentation experiments from glucose in continuously stirred tank reactors operated in continuous mode at a fixed HRT while

increasing the glucose concentration in the feeding solution only when the glucose was totally consumed in the effluent (so, indirectly increasing the OLRv) to explore the limits of the carbohydrate conversion [231]. In one of the reactors, they tested OLRv between 4.5 and 43.9 g COD L^{-1} d^{-1} at 30ºC and pH controlled at 5.4. In the OLRv range from 4.5 to 23.3 g COD L^{-1} d^{-1} (corresponding to 4 to 17 g O_2 L^{-1} of glucose in the influent), which is similar to the range tested in our study, the effluent composition followed a trend very different to the measured in the present study. In this range, when the OLRv is increased, oppositely to our study, ethanol is decreased and butyric and acetic acids are increased. Also, propionic and valeric acids, that in our study were produced in a lesser extent, are decreased when increasing the OLRv [231]. Nevertheless, the biomass concentration in the reactor is not reported in this range. Considering that the reactor has no biomass retention, the biomass concentration might be considerably lower and the operating OLRs might be higher than the applied ones in our study. Consequently, since they are not fixing the biomass concentration in the reactor, the effluent composition reported might be simultaneously impacted by the OLRv and the OLRs.

4.3.2 Effect of the OLRs

As indicated in the results section, increasing the OLRs from 2.3 to 3.8 g COD g^{-1} VSS d^{-1} did not lead to significant differences in the total COD of the effluent and the total CAs of the effluent (Figure 4.1). Regarding the effluent composition, there is a significant decrease on the ethanol concentration when the OLRs is increased from 2.3 up to 3.8 g COD g^{-1} VSS d^{-1} (Table S4.6 in Appendix I), that was not compensated with an increase of the total CAs. The only acid that it was significantly increased when the OLRs was risen is caproic acid (Table S4.6 in Appendix I). By increasing the OLRs, the ratio between substrate and biomass is increased and this might promote caproic acid synthesis [177]. Actually, a decrease in ethanol concentration could be expected since ethanol is used as electron donor in chain elongation reactions [232], which can also explain the increase in caproic acid concentration. Positive relationship between medium-chain carboxylic acid (MCCA) production and organic overloading by increasing organic loading rates was found when simple substrates were used since it implies more available substrate [233]. Surpassing the capacity of the biological system is usually used to promote carboxylic acids accumulation and inhibit competitive microorganisms such as methanogens. Only in the case of using complex substrates,

increasing the organic loading rate do not necessarily benefit MCCA production, since it does not always mean more biodegradable substrate available [233]. As food-grade sugar is a structurally simple substrate, it could be expected that overloading the system would promote MCCA synthesis. In this case, when the OLRv was risen from 6.0 to 15.8 g COD L^{-1} d^{-1} at the low OLRs, the organic loading was not enough to produce MCCAs. However, at the highest OLRv, by increasing the OLRs from 2.3 to 3.8 g COD g^{-1} VSS d^{-1}, caproic acid concentration increased significantly, probably because the substrate consumption capacity was overcome. In fact, at the highest OLRs applied, food-grade sugar concentration in the effluent is significantly higher in comparison to the lowest tested OLRs (Figure 4.4, Table S4.6 in Appendix I). In this case, when decreasing the ratio between substrate and biomass, food-grade sugar consumption might be limited. Consequently, the bioconversion is significantly lower when the OLRs was increased (Table S4.3 in Appendix I). As it was expected, the rest of the process effectiveness parameters (degree of acidification, the acidified COD, the CA production yield and the odd-to-even ratio) did not vary significantly since the total CA in the effluent did not change and caproic acid was the only acid that increased at a higher OLRs.

Regarding the bacterial communities present, at the two tested levels of OLRs the four main genera previously mentioned were present: *Bifidobacterium*, *Ethanoligenens*, *Clostridium sensu stricto* and *Clostridium IV*. No significant differences were found in these four genera at the two OLRs applied. The only genera that showed significant difference was *Enterobacter* (Figure S4.3 in Appendix I), but it just accounted to a maximum relative abundance of 3% at an OLRs of 2.3 g COD g^{-1} VSS d^{-1}. This similarity between the bacterial genera present in the reactor is in accordance with the few differences found in the effluent composition at the two OLRs conditions (Table S4.6 in Appendix I).

The significant decrease of ethanol when the OLRs is increased from 2.3 to 3.8 g COD g^{-1} VSS d^{-1} can not be explained by the *Ethanoligenens* relative abundance which showed no significant differences between both conditions (42% and 40%, respectively). However, ethanol decrease might be the result of chain-elongation reactions leading to caproic acid production [232], which was significantly higher under 3.8 g COD g^{-1} VSS d^{-1} with respect to 2.3 g COD g^{-1} VSS d^{-1} (Table S4.6 in Appendix I). Most of the reported caproic acid-producing bacteria are comprised in the families *Ruminococcaceae* and

Clostridiaceae 1 [234], which account for the 45% and 19%, respectively at an OLRs of 3.8 g COD g^{-1} VSS d^{-1} (Figure 4.8).

4.3.3 Effect of the influent carbon-to-nitrogen ratio

Finally, the influent carbon-to-nitrogen ratio was the parameter that less impacted the acidogenic fermentation. Increasing the influent carbon-to-nitrogen ratio from 12 to 25 did not conduct to significant differences in the total COD of the effluent and the total CAs of the effluent (Figure 4.1). Regarding the effluent components, only propionic acid showed a significant increase when increasing the influent carbon-to-nitrogen ratio from 12 up to 25 (Table S4.7 in Appendix I), but it just accounted for a maximum of the 7.3% of the effluent COD at influent carbon-to-nitrogen ratio of 25. Consequently, no significant changes were observed in the process effectiveness parameters when modifying the influent carbon-to-nitrogen ratio (Table S4.4 in Appendix I).

As expected, the bacterial populations at both influent carbon-to-nitrogen ratios, were very similar. Among the main genus, only *Clostridium sensu stricto* showed significant differences (Figure S4.5 in Appendix I). When increasing the influent carbon-to-nitrogen ratio from 12 to 25, *Clostridium sensu stricto* increased from 19% to 31%. *Clostridium sensu stricto* has been reported as potential propionate producer [235], so the higher concentration of propionic acid at an influent carbon-to-nitrogen ratio of 25 might be linked to the increase of *Clostridium sensu stricto* relative abundance.

4.4 Potential application of the acidogenic fermentation effluents

In this section, the applicability of the effluents obtained at the different operational conditions tested is discussed. Firstly, the effluent obtained at low OLRv (in this case 6.0 g COD L^{-1} d^{-1}) was a mixture mainly composed by butyric and acetic acid. Therefore, this effluent would be appropriate for polyhydroxyalkanoate (PHA) production, since it has a large proportion of butyric acid, which has been reported as one of the preferred substrates for PHA production since it is very favourable energetically [24,236,237]. Moreover, this effluent contains a low proportion of unidentified compounds (Figure 4.3). Nevertheless, its low odd-to-even ratio would lead to polyhydroxybutyrate (PHB) production, which is less desirable in comparison to poly(hydroxybutyrate-co-hydroxyvalerate)

(PHB-co-PHV) [29]. As mentioned in the introduction section, a polymer containing 3-hydroxyvalerate (as PHB-co-PHV) has better elasticity and flexibility than a polymer only formed by 3-hydroxybutyrate (such as PHB) [1]. Hence, PHB-co-PHV is more convenient for most of the applications than PHB [61].

If the OLRv is increased (in this case up to 11.0-15.8 g COD L^{-1} d^{-1}), the effluent obtained is mainly composed by acetic acid and ethanol, being suitable for medium chain carboxylic acids (MCCA) production in a separate reactor. In this subsequent rector, ethanol acts as electron donor (ED) which provides the required energy for the reverse β-oxidation (RBO) cycle reactions in which acetic acid acts as electron acceptor (EA) [11,238]. As explained in detail in the introduction section, acetic acid is converted into butyric acid in a first RBO cycle and then, butyric acid is transformed into caproic acid in a second RBO cycle [186]. Under the OLRv of 11.0 and 15.8 g COD L^{-1} d^{-1}, the ED:EA carbon molar ratio were 0.72 and 0.89, respectively. Several authors have studied the optimal ED:EA in terms of MCCA production yield and product selectivity when the ED is ethanol. Wu et al. (2020) tested different ED:EA ratios using acetic acid as EA and ethanol as ED and found that the optimal in terms of MCCA production yield was 1:1 [194]. On the contrary, other authors have reported an ED:EA ratio of 4:1 as the optimal in terms of MCCA production and selectivity with ethanol as ED [239,240]. Despite the optimal ED:EA ratio (with ethanol as ED) is still being discussed, the ED:EA ratios obtained in the effluents under the OLRv of 11.0 and 15.8 g COD L^{-1} d^{-1} are quite low, so additional ethanol might have to be externally provided. At the highest OLRv, when increasing the OLRs from 2.3 to 3.8 g COD g^{-1} VSS d^{-1}, caproic acid, which is the MCCA with less number of carbon atoms, is directly synthesized in the acidogenic fermentation reactor. That means that the increase of the OLRs in an acidogenic fermentation reactor could be an interesting operational strategy to produce MCCAs from effluents composed by carbohydrates. Finally, at the higher OLRv and OLRs, the effect of increasing the influent carbon-to-nitrogen ratio from 12 to 25 results in lower non-identified fraction of the effluent while caproic acid composition remains constant.

4.5 Conclusions

In this study, a SBR was used to perform acidogenic fermentation experiments starting from a substrate only composed by food-grade sugar as a model compound of

carbohydrates. From the experimental work, the effect of the OLRv, OLRs and the influent carbon-to-nitrogen ratio on the process performance was assessed.

The OLRv was the parameter with more impact on the acidogenic fermentation process. When it was increased from 6.0 to 15.8 g COD L^{-1} d^{-1} (at an around 2 g COD g^{-1} VSS d^{-1} and the influent carbon-to-nitrogen ratio at 12), it was observed that the lower the OLRv, the higher the acidified COD. The bioconversion remains unchanged at a value over 99%. However, the process performance was mostly changed when increasing the OLRv from 6.0 to 11.0-15.8 g COD L^{-1} d^{-1}, showing that:

- The degree of acidification and the CA production yield significantly decreased.
- The predominant effluent components changed from a mixture of butyric and acetic acid to a mixture of acetic acid and ethanol.
- The odd-to-even ratio significantly increased since butyric acid decreased and propionic acid increased.

When the OLRs was increased from 2.3 up to 3.8 g COD g^{-1} VSS d^{-1} (at an OLRv of 16 g COD L^{-1} d^{-1} and the influent carbon-to-nitrogen ratio of 12), it was observed the following:

- The unique effectiveness parameter that changed significantly was bioconversion, which decreased due to food-grade sugar consumption limitation.
- Caproic acid concentration was significantly increased while ethanol was significantly decreased. Ethanol might be used in caproic acid synthesis as electron donor.

Finally, the influent carbon-to-nitrogen ratio was increased from 12 to 25 while keeping the OLRv and the OLRs at their highest values, observing no significant changes in the effectiveness parameters. Propionic acid was the only effluent that was significantly modified since it increased when the carbon-to-nitrogen ratio was increased.

In relation to the bacterial communities present in the reactor, under all the conditions tested, four main genera were detected: *Bifidobacterium*, *Ethanoligenens*, *Clostridium IV* and *Clostridium sensu stricto*. For all the conditions tested, it was possible to link all the main effluent components to the different genera detected in the reactor.

The effluent yielded at an OLRv of 6.0 g COD L^{-1} d^{-1} would be appropriate for PHB production due its high butyric acid content. Besides, the effluents corresponding to the OLRv of 11.0-15.8 g COD L^{-1} d^{-1} (OLRs around 2 g COD g^{-1} VSS d^{-1} and the influent

carbon-to-nitrogen ratio of 12) would be adequate to be feed to a subsequent chain-elongation reactor where the conditions should be optimized to the bioproduction of MCCAs.

Chapter 5:

Acidogenic fermentation of a protein model compound: Exploring the influence of volumetric and specific organic loading rates on process performance and biomass composition

5.1 Overview

In this chapter, a sequencing batch reactor (SBR) was used to conduct acidogenic fermentation experiments using a simulated wastewater containing whey protein isolate as protein-model compound. The objective is to assess the influence of the volumetric and the specific organic loading rate (OLRv and OLRs, respectively) on the process effectiveness parameters, effluent composition and biomass composition at family and genus level. For this purpose, first the OLRv was tuned while keeping constant the OLRs and then, two values of OLRs where tested while the OLRv remained unchanged. For each of the conditions tested, no parameters were changed until the steady state was reached. Statistical methods were used to compare if the results obtained at the different conditions tested were significantly different. Like this, we evaluated the effect of the tested parameters on the acidogenic fermentation performance based on which microbial communities were favoured under each condition tested and indirectly, on the product spectrum obtained.

Below, this chapter starts with the description of the results regarding the reactor operation, process effectiveness parameters, effluent composition and bacterial communities. Then, the effect of the OLRv and the OLRs on the process performance, effluent composition and bacterial communities are discussed together. Finally, the potential applications of the obtained effluents obtained were evaluated. Materials and methods corresponding to the experiments of this chapter can be found in Chapter 3.

5.2 Results

5.2.1 Reactor operation

The reactor was operated during 144 days at different conditions of OLRv and OLRs. Table 5.1 gathers the different operational periods and the conditions applied in each one of them. Figure 5.1 shows the profiles over time for the organic matter in terms of total COD (chemical oxygen demand) concentration in the influent, total COD concentration in the effluent, the COD equivalents in the effluent corresponding to the whey protein isolate and the total carboxylic acids (CAs) and the ammonium concentration in the effluent. Initially, the start-up of the reactor was performed at an OLRv of 4.6±0.3 g COD L^{-1} d^{-1} and an OLRs of 0.8±0.3 g COD g^{-1} VSS d^{-1}. After the start-up period, similar values

of OLRv and OLRs to the ones tested with food-grade sugar in the Chapter 4 were evaluated. Like this, three values of OLRv were tested: low OLRv (6.5±0.6 g COD L^{-1} d^{-1}), medium OLRv (12.5±0.6 g COD L^{-1} d^{-1}) and high OLRv (18.5±0.3 g COD L^{-1} d^{-1}) while maintaining the OLRs at approximately 1.9 g COD g^{-1} VSS d^{-1}. Then, two values of OLRs were applied: low OLRs (1.9±0.1 g COD g^{-1} VSS d^{-1}) and high OLRs (3.3±0.3 g COD g^{-1} VSS d^{-1}) while keeping the OLRv at its highest value (approximately 18.5 g COD L^{-1} d^{-1}). Despite not having a pH automatic control loop, the pH value in each of the achieved steady states remained around a value of 7 (Table 5.1). Consequently, the effect of the OLRv and OLRs on the process performance, the effluent composition and the biomass composition was not affected by the variation of the pH value.

Table 5.1. Experimental conditions tested throughout the study of the acidogenic fermentation using whey protein isolate as sole carbon source.

Period	**Duration (d)**	**OLRv (g COD L^{-1} d^{-1})**	**OLRs (g COD g^{-1} VSS d^{-1})**	**HRT (d)**	**Average pH in the bulk liquid**
Start-up	29	4.6±0.3	0.8±0.3	2.2±0.1	7.3±0.2
I	38	6.5±0.6 **(low)**	1.9±0.4 **(low)**	2.2±0.2	6.8±0.1
II	30	12.5±0.6 **(medium)**	1.8±0.4 **(low)**	1.1±0.1	6.8±0.1
III	26	18.5±0.3 **(high)**	1.9±0.1 **(low)**	0.6±0.2	6.9±0.1
IV	21	18.6±0.4 **(high)**	3.3±0.3 **(high)**	0.7±0.2	6.9±0.2

It should be mentioned that the biomass used as inoculum was anaerobic granular biomass from a digester producing methane from a papermill wastewater, as in Chapter 4. So, the inhibition and wash-out of the methanogens were necessary to be able to accumulate carboxylic acids. The operational strategy used to inhibit the methanogenic microorganisms was the application of OLRv exceeding capacity of the system, i.e. no other physical pretreatments of the biomass or chemical inhibitors were used. Despite the substrate was composed of a protein-model compound, this is a more complex substrate than the food-grade sugar used in Chapter 4. Therefore, to prevent possible hydrolysis limitations, the start-up of this SBR was done with an OLRv of 4.6 g COD L^{-1} d^{-1} and an OLRs of 0.8 g COD g^{-1} VSS d^{-1}, which are lower values than the ones used for starting-up the acidogenic fermentation of food-grade sugar (10.6±0.6 g COD L^{-1} d^{-1} and 2.1±0.3 g

COD g^{-1} VSS d^{-1}, see Section 4.2.1). However, after 29 days of operation, it was observed that the whey protein isolate was totally consumed but the total COD and the total CAs of the effluent were not being accumulated (start-up period, Figure 5.1). This indicated that methanogens were not completely inhibited. Hence, it was decided to increase both OLRv and OLRs to 6.5 g COD L^{-1} d^{-1} and 1.9 g COD g^{-1} VSS d^{-1}, respectively (period I, Figure 5.1). At these conditions, 11 more days were needed to observe total COD and total CA accumulation in the effluent (Figure 5.1).

During the whole operation, the concentration of the whey protein isolate in the effluent was almost zero, with the exception of some days in which the OLRv or the OLRs were incremented. As the increase of the whey protein isolate in the effluent was only transitional and during the rest of the operation the concentration was zero, it can considered that there were no hydrolysis limitations in the acidogenic fermentation of the whey protein isolate. After the start up period, the total COD in the effluent varied between 10000 and 12000 mg O_2 L^{-1} with the following mean values in the different periods: period I (11376±539 mg O_2 L^{-1}), period II (11209±550 mg O_2 L^{-1}), period III (10916±526 mg O_2 L^{-1}) and period IV (10093±737 mg O_2 L^{-1}). Periods I, II and III did not show significant differences among them ($p=0.9341$ for periods I and II; $p=0.4368$ for periods I and III and $p=0.7516$ for periods II and III). Thus, increasing the OLRv from 6.5 to 18.5 g COD L^{-1} d^{-1} did not produce significant changes in the total COD of the effluent. Besides, periods III and IV did not show significant differences ($p=0.0767$), meaning that increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1} did not lead to significant changes in the total COD concentration of the effluent.

With respect to the total CAs in the effluent, the mean concentration for each of the periods was the following: period I (9232±481 mg O_2 L^{-1}), period II (8261±1318 mg O_2 L^{-1}), period III (8823±616 mg O_2 L^{-1}) and period IV (8705±825 mg O_2 L^{-1}). Since there were no significant differences among periods I, II and III ($p=0.5140$ for periods I and II; $p=0.8165$ for periods I and III and $p=0.9703$ for periods II and III), it can be concluded, therefore, that increasing the OLRv from 6.5 to 18.5 g COD L^{-1} d^{-1} did not produce significant changes in the total CA concentration in the effluent. In addition, there was no significant difference between periods III and IV ($p=0.9980$), implying that increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1} did not affect the total CA concentration in the effluent.

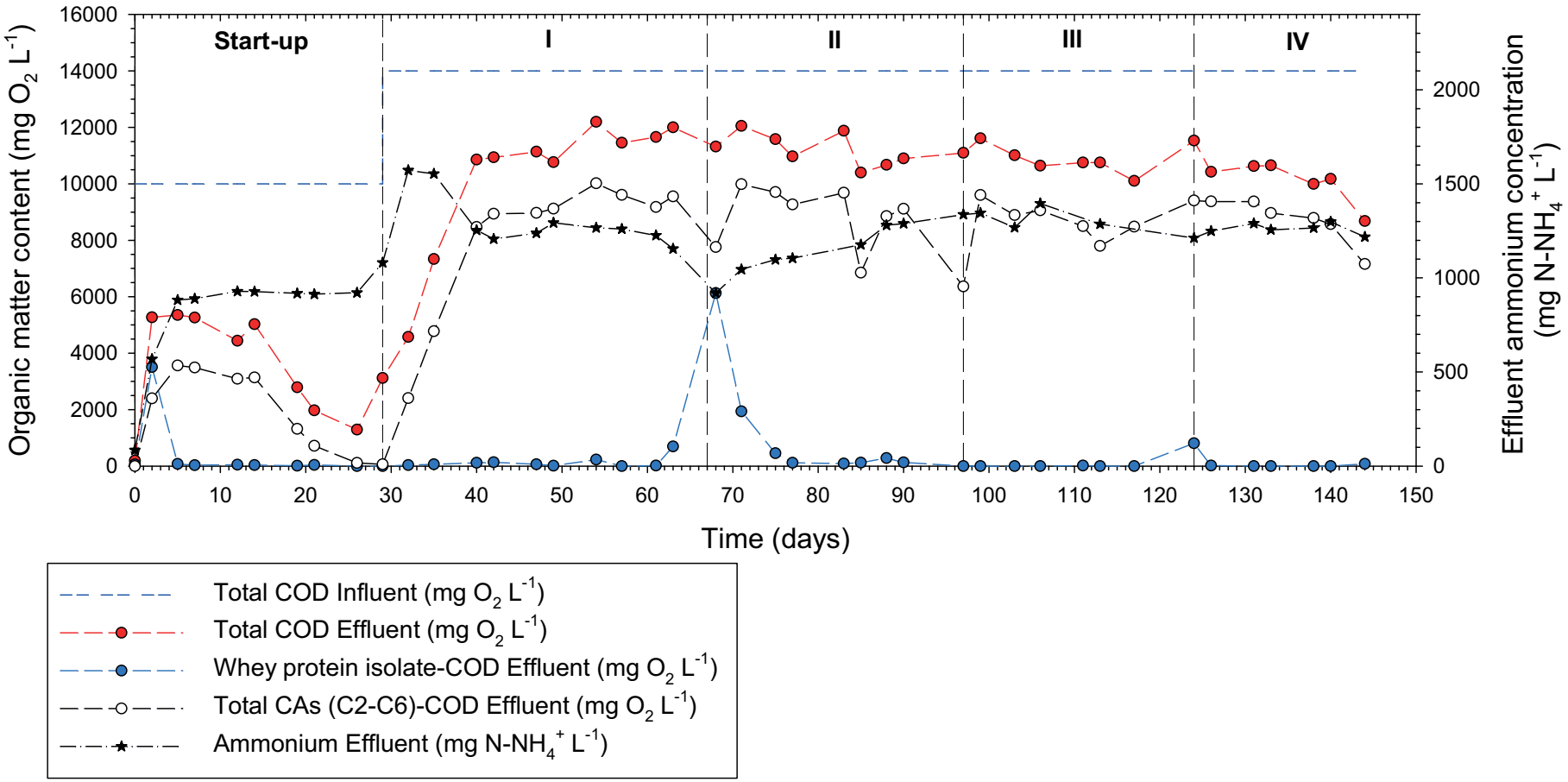


Figure 5.1. Reactor operation during the study of the acidogenic fermentation using whey protein isolate as sole carbon source. Vertical black discontinuous lines indicate the beginning of a new period.

Lastly, the concentration of ammonium in the effluent was also assessed, as proteins typically release ammonium during fermentation. The ammonium concentrations in the effluent of the different operational periods were the following: period I (1238±43 mg $N\text{-}NH_4^+$ L^{-1}), period II (1156±143 mg $N\text{-}NH_4^+$ L^{-1}), period III (1302±71 mg $N\text{-}NH_4^+$ L^{-1}) and period IV (1263±30 mg $N\text{-}NH_4^+$ L^{-1}). The statistical analysis showed that there were no significant differences among periods I, II and III ($p=0.5695$ for periods I and II; $p=0.4413$ for periods I and III and $p=0.1584$ for periods II and III), meaning that increasing the OLRv from 6.5 to 18.5 g COD L^{-1} d^{-1} did not lead to a significantly higher ammonia release. Furthermore, increasing the OLRs did not produce significant changes in the ammonium concentration in the effluent between periods III and IV ($p=0.3091$). The ammonium concentration during the whole reaction operation was lower than 1700-1800 mg $N\text{-}NH_4^+$ L^{-1} which were previously reported to inhibit the acidogenesis reactions with unacclimated inoculum [84].

Another objective of this study was to assess the feasibility of operating a semicontinuous reactor with biomass retention where the acidogenic fermentation of a model protein was carried out. Since the objective is to produce an effluent to be used directly in a posterior application, good settleability in the reactor and low solid concentration in the effluent are attempted. In these experiments, the reactor was inoculated with granular anaerobic biomass. As in the experiments carried out with food-grade sugar as carbon source (Chapter 4), most of the granular biomass also turned into flocculent due to the stirring stress and the washout of the granules by flotation during the start-up period. Figure 5.2 depicts the biomass the biomass inoculated and the biomass after the 144 days of operation. It can be noticed that the biomass taken from the reactor at the end of the operation was predominantly flocculent.

Even with a flocculent biomass, the reactor was successfully operated since the biomass had a good settleability. As in the experiments with food-grade sugar (Chapter 4), the OLRv was selected by tuning the total cycle duration (and, accordingly the reaction phase length) as the feeding solution concentration was constant. Then, the desired OLRs was set by keeping the biomass concentration at the corresponding value by purging when necessary. Despite the carbon source used in this experiment was a simple protein, the growth of the biomass was not as fast as the growth of the biomass when using food-grade sugar as carbon source. Therefore, increasing the OLRv did not affect notably the SRT, which was 10, 9 and 9 days at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively. However,

enlarging the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1}, decreased the resultant SRT from 9 to 7 days due to the purges performed to keep the biomass concentration at the proper level. Like in the experiment in Chapter 4, in each cycle, the effluent was discharged after a settling step of 60 min. The solid concentration in the effluent during the whole operation was 0.4±0.1 g VSS L^{-1} while the biomass concentration in the reactor varied among 3.4 and 9.1 g VSS L^{-1} depending on the selected OLRv and OLRs.

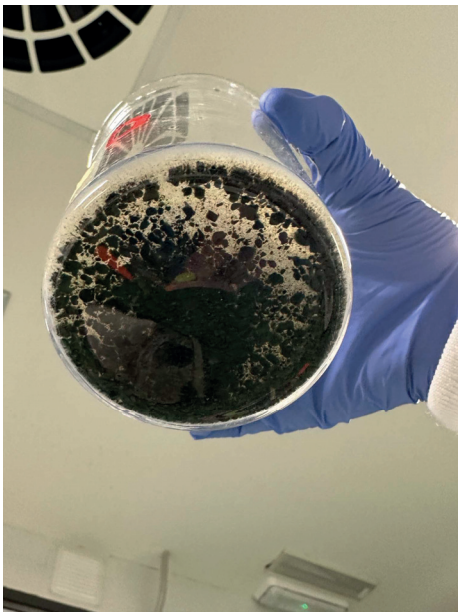
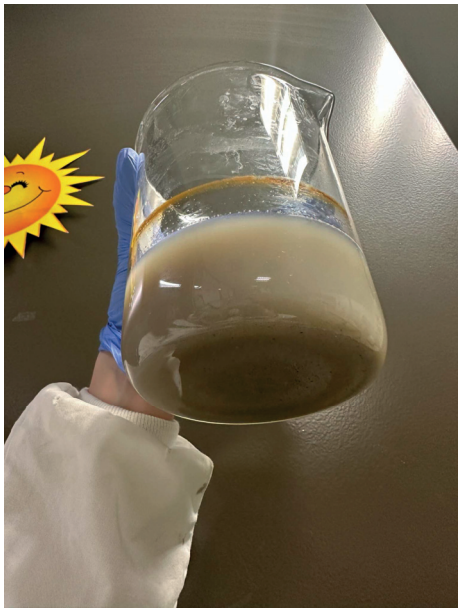
a)  b) 

Figure 5.2. a) Sample of the biomass used as inoculum in the acidogenic fermentation study fermentation using whey protein isolate as sole carbon source. b) Sample of the biomass after the reactor operation during the study of the acidogenic fermentation using whey protein isolate as sole carbon source.

5.2.2 Process effectiveness parameters: Bioconversion, degree of acidification, acidified COD, CA production yield and odd-to-even ratio

Below, the effect of the OLRv and OLRs on the bioconversion, degree of acidification, acidified COD, CA production yield and odd-to-even ratio is presented. Table 5.2 gathers the values of these process effectiveness parameters under the different tested conditions during the fermentation experiments using whey protein isolate as carbon source. The results of the ANOVA test and Tukey's multiple comparisons test to evaluate the effect of the OLRv (Table S5.2) and the t-test results for comparing the

process effectiveness parameter values at different OLRs (Table S5.3) can be found in the Appendix II.

Table 5.2. Bioconversion, Degree of Acidification, Acidified COD, Carboxylic Acid yield and odd-to-even ratio in the different conditions tested during the study of the acidogenic fermentation using whey protein isolate as sole carbon source.

OLRv (g COD L^{-1} d^{-1})	**OLRs (g COD g^{-1} VSS d^{-1})**	**Bioconversion (%)**	**Degree of Acidification (%)**	**Acidified COD (%)**	**CA production yield (g COD g^{-1} COD)**	**Odd-to-even ratio**
6.5±0.6 **(low)**	1.9±0.4 **(low)**	98.9±1.9	63±3	82±3	0.6±0.1	0.26± 0.03
12.5±0.6 **(medium)**	1.8±0.4 **(low)**	99.0±1.1	63.5±1.1	83.3±0.6	0.6±0.2	0.32± 0.02
18.5±0.3 **(high)**	1.9±0.1 **(low)**	100.0±0.1	63±4	82±3	0.6±0.1	0.25± 0.02
18.6±0.4 **(high)**	3.3±0.3 **(high)**	99.8±0.4	63±2	85±3	0.6±0.1	0.35± 0.04

When the OLRv was increased from 6.5 to 18.5 g COD L^{-1} d^{-1}, the bioconversion, the degree of acidification, the acidified COD and the CA production yield were not significantly modified (Table S5.2 in Appendix II), showing values around 99%, 63%, 82% and 0.6 g COD g^{-1} COD, respectively. Finally, the odd-to-even ratio showed significant differences between 6.5 and 12.5 g COD L^{-1} d^{-1} (low and medium levels) and between 12.5 and 18.5 g COD L^{-1} d^{-1} (medium and high levels) (Table S5.2 in Appendix II). Since there were no significant differences between the low and the high level of OLRv, a tendency of the odd-to-even ratio when increasing the OLRv from 6.5 to 18.5 g COD L^{-1} d^{-1} cannot be described. In conclusion, varying the OLRv from 6.5 to 18.5 g COD L^{-1} d^{-1} did not have a strong effect on any of the process effectiveness parameters when the substrate is only composed by proteins.

Below, the effect of the OLRs over the process effectiveness parameters is examined (Table 5.2). Once again, by increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1}, no significant changes were detected in the bioconversion, the degree of acidification, the acidified COD and the CA production yield (Table S5.3 in Appendix II). However, the odd-to-even ratio showed a significant increase when rising the OLRs from 1.9 to 3.3 g

COD g^{-1} VSS d^{-1} (Table S5.3 in Appendix II). To sum up, enlarging the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1} only increases slightly the odd-to-even ratio.

5.2.3 Effluent composition

In this section, the effect of the OLRv and OLRs on the effluent composition is presented. Table S5.1 (Appendix II) shows the concentration (in mg COD L^{-1}) of the compounds measured in the effluent under the different conditions tested.

The effluent composition (% COD) at the different OLRv applied is represented in Figure 5.3. The statistical difference between the effluent components can be deduced from the ANOVA test and Tukey's multiple comparisons test in Table S5.4 (Appendix II). Under the three tested values of OLRv, the effluent was composed of six main acids (in decreasing order): acetic acid (15.4-19.0%), isovaleric acid (15.0-17.0%), propionic acid (11.5-15.0%), butyric acid (11.9-14.8%), isocaproic acid (7.2-11.2%) and isobutyric acid (7.2-9.0%) (Figure 5.3). It is noticeable that the composition of these six acids was quite similar and none of them was clearly majoritarian. The only component showing significant changes when varying the OLRv was the isocaproic acid (Table S5.4 in Appendix II). When increasing the OLRv up to 18.5 g COD L^{-1} d^{-1}, isocaproic acid increased to 11.2% with respect to the 7.2% and 7.5% obtained at 6.5 and 12.5 g COD L^{-1} d^{-1}, respectively. The rest of the majoritarian components did not show significant changes when the OLRv was increased from 6.5 to 18.5 g COD L^{-1} d^{-1}. Besides the majoritarian components, the rest of the components detected in the effluent accounted for less than the 3% of the effluent COD. The effluent COD corresponding to non-identified compounds fluctuated between 14.9% to 19.0%.

The effluent composition (% COD) at the different OLRs applied is plotted in Figure 5.4. The statistical difference between the effluent components can be gathered from the t-test in Table S5.5 in Appendix II. Once again, under both tested OLRs, six main components were found. In decreasing order, the majoritarian components of the effluent were: acetic, isovaleric, butyric, propionic, isocaproic and isobutyric acids. However, unlike the OLRv, it was observed that the OLRs variation from 1.9 to 3.3 g COD g^{-1} VSS d^{-1} led to more significant changes in the effluent composition. With the increase of the OLRs from 1.9 up to 3.3 g COD g^{-1} VSS d^{-1}, butyric, propionic and isobutyric acids increased significatively from 11.9%, 11.5% and 7.5% to 15.8%, 15.5% and 9.1%,

respectively. The rest of the main components showed no significant differences when increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1}. The rest of the components detected in the effluent accounted for less than the 3.5% of the effluent COD. The effluent COD corresponding to non-identified compounds decreased from 19.0% to 8.4% when the OLRs was increased from 1.9 up to 3.3 g COD g^{-1} VSS d^{-1} since three of the main components were increased.

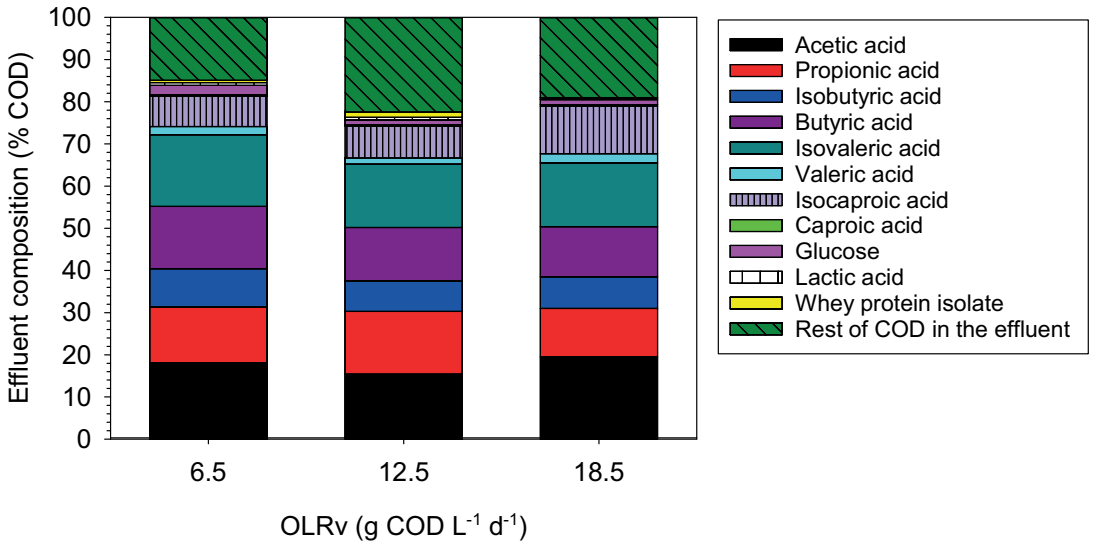


Figure 5.3. Effluent composition at different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) and at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein isolate as sole carbon source.

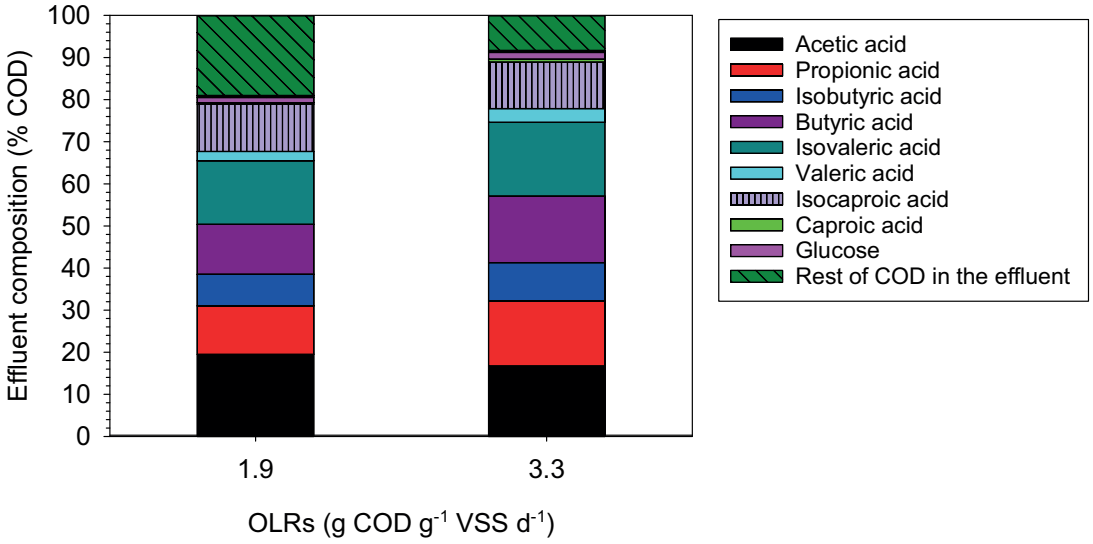


Figure 5.4. Effluent composition at different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) and at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein isolate as sole carbon source.

5.2.4 Bacterial communities

In this section, the bacterial communities detected under the different conditions tested are presented at family and genus levels. The effect of the OLRv and the OLRs on the microbial communities are individually analyzed.

5.2.4.1 Effect of the OLRv on the bacterial communities

The relative abundances (in %) of the bacterial communities at family level at the different OLRv tested are shown in Figure 5.5. Figure S5.1 (Appendix II) depicts the difference in the proportions including only the families that show significant differences in the relative abundance at the three tested levels of OLRv.

Three main families were detected at the three values of OLRv tested: *Rikenellaceae*, *Synergistaceae* and *Dysgomonadaceae*. The relative abundance of *Rikenellaceae* was 35%, 10%, and 14% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively. This way, decreasing the OLRv below 12.5 g COD L^{-1} d^{-1}, significantly increased the relative abundance of *Rikenellaceae* since there were significant differences between the low and the medium and high values of OLRv (Figure S5.1 in Appendix II). The relative abundance of *Synergistaceae* described the opposite trend to the relative abundance of *Rikenellaceae*. Thus, decreasing the OLRv below 12.5 g COD L^{-1} d^{-1}, significantly decreased the relative abundance of *Synergistaceae* since there were significant differences between the low and the medium and high values of OLRv (Figure S5.1 in Appendix II). Specifically, the relative abundance of *Synergistaceae* was 7%, 29%, and 31% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively. *Dysgomonadaceae* relative abundance was 19%, 26% and 17% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, showing no significant differences at the three tested OLRv (Figure S5.1 in Appendix II).

Apart from these three main families, other four families were present with a relative abundance larger than 5%. *Ruminococcaceae* relative abundance was 10%, 3%, and 5% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, exhibiting significant differences only between the low and the medium values of OLRv (Figure S5.1 in Appendix II). *Clostridiales Incertae Sedis XI* relative abundance was 5%, 8%, and 3% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, revealing significant differences only between the medium and the high values of OLRv (Figure S5.1 in Appendix II).

Peptostreptococcaceae and *Porphyromonadaceae* relative abundances were 4%, 4%, 8% and 7%, 7%, 4% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, showing no significant differences under the three tested levels of OLRv (Figure S5.1 in Appendix II). Finally, the fraction corresponding to families with a relative abundance lower than the 2% (denoted as 'Others (<2%)' in Figure 5.5) was 6%, 7%, and 10% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, showing no significant differences among them (Figure S5.1 in Appendix II).

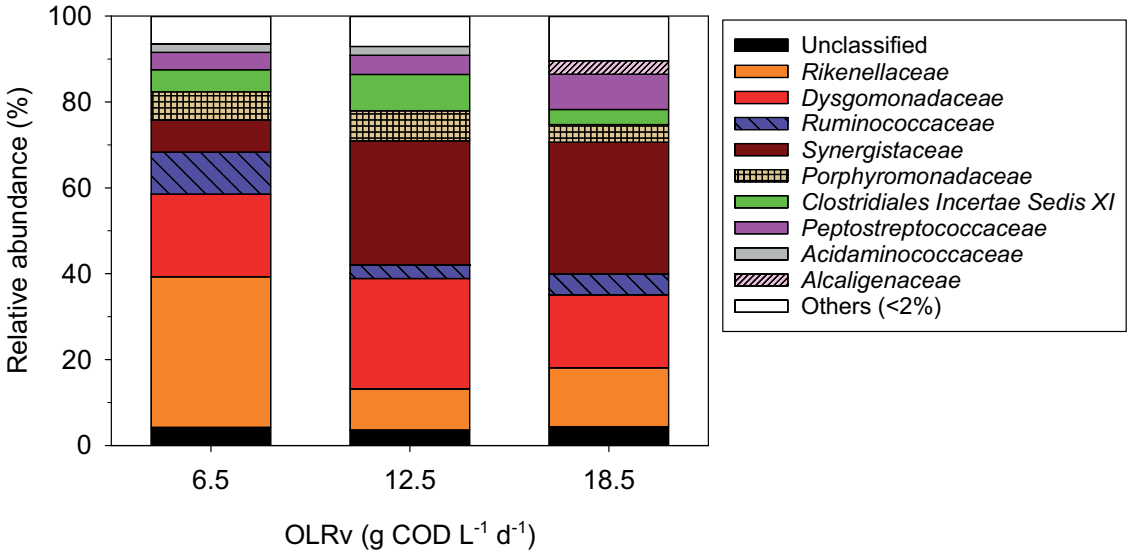


Figure 5.5. Bacterial communities at family level at different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) and at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein isolate as sole carbon source.

Figure 5.6 depicts the relative abundance (in %) of the bacterial communities at genus level at the different tested OLRv. Figure S5.2 (Appendix II) exhibits the difference in the proportions including only the genera that show significant differences in the relative abundance at the three tested values of OLRv. It can be observed that the genera detected changes notably when increasing the OLRv up to 12.5 g COD L^{-1} d^{-1} or above with respect to 6.5 g COD L^{-1} d^{-1} (Figure 5.6). In the tested OLRv, five main genera were found: *Petrimonas, Pyramidobacter, Aminobacterium, Alistipes and Peptostreptococcus*.

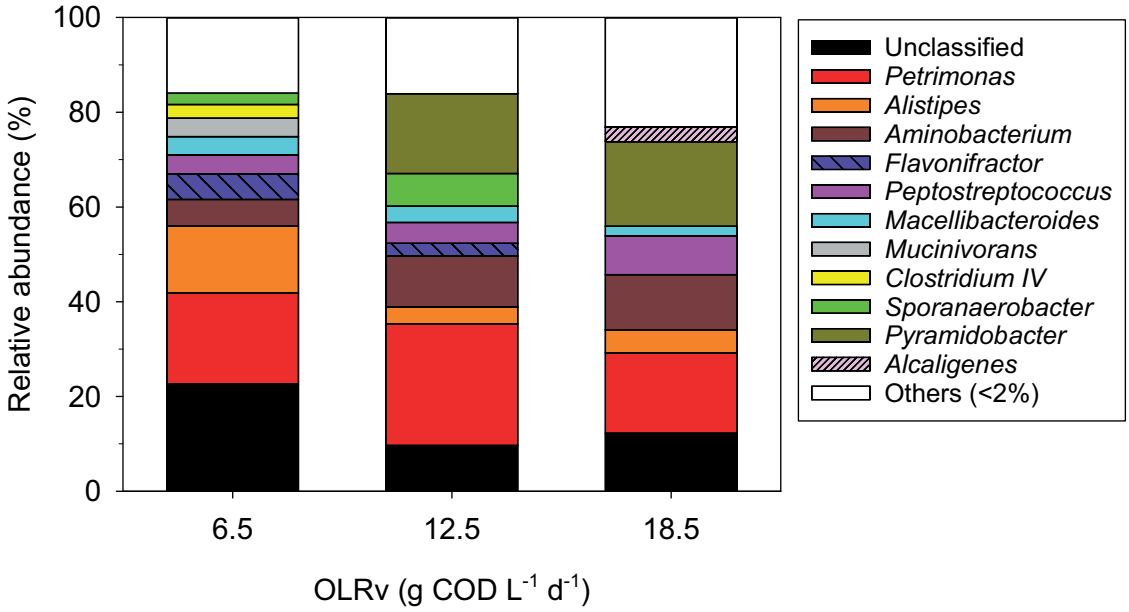


Figure 5.6. Bacterial communities at genus level at different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) and at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein isolate as sole carbon source.

Petrimonas was the genus with largest relative abundance, being 19%, 26%, and 17% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, exhibiting no significant differences at the three levels of tested OLRv (Figure S5.2 in Appendix II). Following *Petrimonas*, the unclassified microorganisms at genus level were the second with highest relative abundance, accounting for the 23%, 10%, and 12% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively. Thus, the unclassified microorganisms significantly increased when decreasing the OLRv to 6.5 g COD L^{-1} d^{-1} with respect to 12.5 and 18.5 g COD L^{-1} d^{-1}, which showed no significant differences between them (Figure S5.2 in Appendix II). The relative abundance of *Pyramidobacter* was 0%, 17%, and 18% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively. Like this, increasing the OLRv up to 12.5 g COD L^{-1} d^{-1} or above significantly increased *Pyramidobacter* relative abundance with respect to the low level of OLRv, while there was no significant difference between 12.5 and 18.5 g COD L^{-1} d^{-1} (Figure S5.2 in Appendix II). With respect to *Aminobacterium*, the relative abundance was 6%, 11%, and 12% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, showing only significant differences between 6.5 and 18.5 g COD L^{-1} d^{-1} (Figure S5.2 in Appendix II). The relative abundance of *Alistipes* was 14%, 4%, and 5% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively. According to this, increasing the OLRv up to 12.5 g

COD L^{-1} d^{-1} or above significantly decreased *Alistipes* relative abundance with respect to the low level of OLRv, while there was no significant difference between 12.5 and 18.5 g COD L^{-1} d^{-1} (Figure S5.2 in Appendix II). The relative abundance of *Peptostreptococcus* was 4%, 4%, and 8% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, showing no significant differences among the different OLRv tested. Finally, under the three tested values of OLRv there was a relatively high fraction of genera with a relative abundance below the 2% (denoted as others (<2%) in Figure 5.6). In particular, the genera with less than the 2% of relative abundance account for the 16%, 16% and 23% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively, showing no significant differences among them (Figure S5.2 in Appendix II).

No significant differences were found among the Shannon Index calculated for quantifying the biodiversity obtained at the different tested OLRv, for both family and genus levels (Table S5.6 in Appendix II).

5.2.4.2 Effect of the OLRs on the bacterial communities

The relative abundance (%) of the bacterial communities at family level at the two OLRs tested is shown in Figure 5.7. Figure S5.3 (Appendix II) depicts the difference in the proportions including only the families which show significant differences in the relative abundance at the two levels of OLRs tested.

Four main families were detected at the two levels of OLRs tested: *Synergistaceae*, *Rikenellaceae*, *Dysgomonadaceae* and *Clostridiales Incertae Sedis XI*. Among these families, only *Clostridiales Incertae Sedis XI* exhibited significant differences at 1.9 and 3.3 g COD g^{-1} VSS d^{-1} (Figure S5.3 in Appendix II). The relative abundance of *Synergistaceae* was 31% and 28% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively. The relative abundance of *Rikenellaceae* was 14% and 21% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively. The relative abundance of *Dysgomonadaceae* was 17% and 10% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively. Finally, the relative abundance of *Clostridiales Incertae Sedis XI* was 3% and 13% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively.

Figure 5.8 depicts the relative abundance (in %) of the bacterial communities at genus level at the two OLRs tested. Figure S5.4 (Appendix II) exhibits the difference in the

proportions including only the genera that show significant differences in the relative abundance at the two OLRs tested.

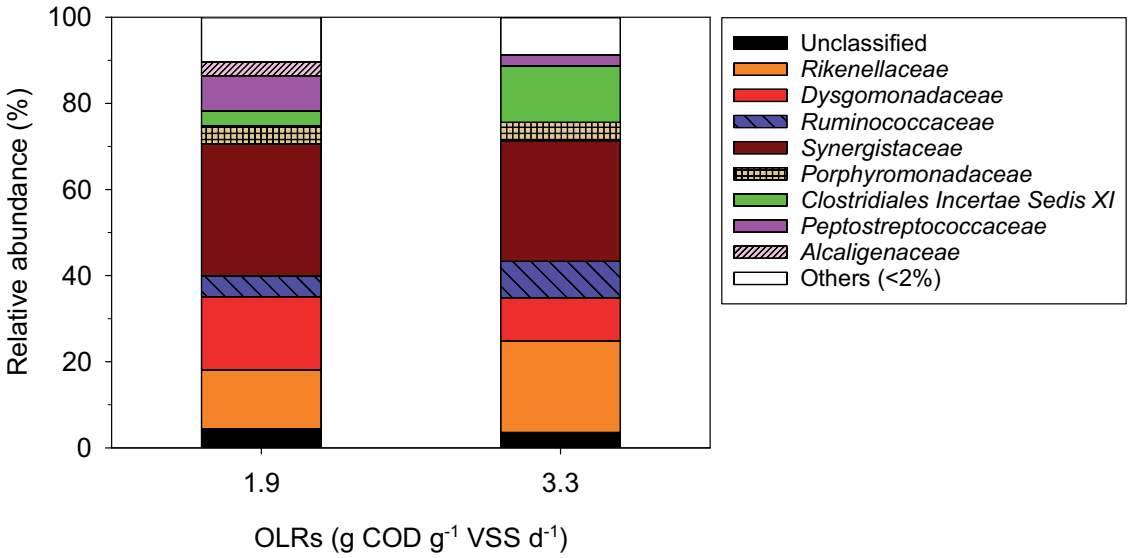


Figure 5.7. Bacterial communities at family level at different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) and at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein isolate as sole carbon source.

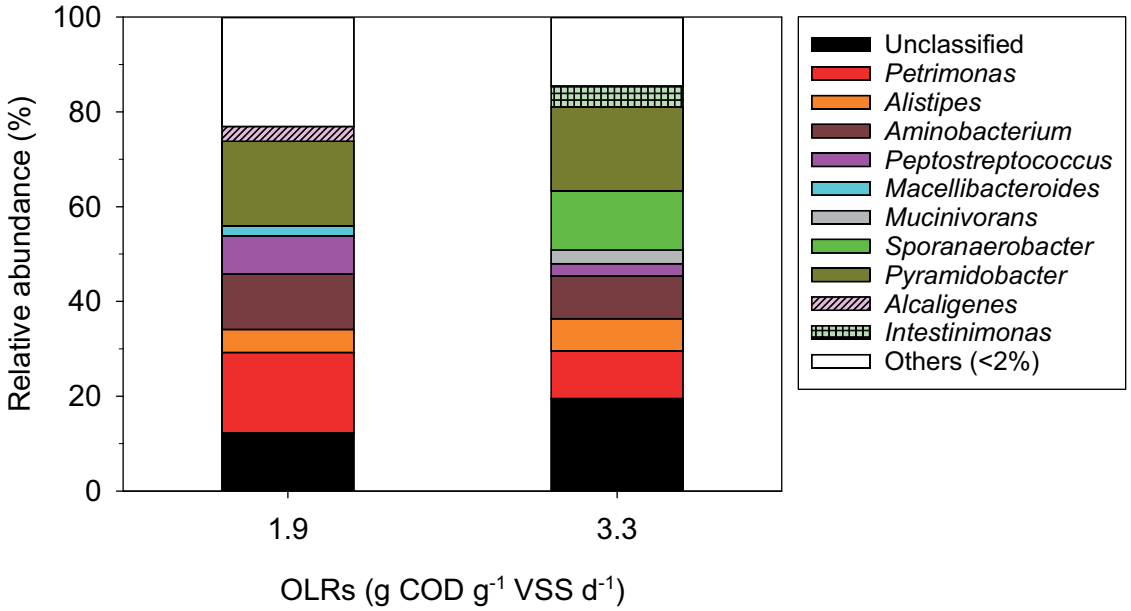


Figure 5.8. Bacterial communities at genus level at different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) and at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein isolate as sole carbon source.

In the examined OLRs, four main genera were found: *Pyramidobacter, Petrimonas, Aminobacterium* and *Sporanaerobacter*. Nevertheless, the fraction corresponding to genera with a relative abundance lower than the 2% (denoted as others (<2%) in Figure 5.8) accounts for higher relative abundance than any of the genera identified. Thus, this fraction corresponded to 23% and 15% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively, showing no significant differences between them (Figure S5.4 in Appendix II). Plus, the unclassified microorganisms at genus level are also a fraction with a higher relative abundance than the relative abundance of any of the identified genera. In this way, the relative abundance of the unclassified microorganisms accounted for the 12% and 20% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively, revealing no significant differences between them (Figure S5.4 in Appendix II). *Pyramidobacter* was the identified genus with the highest relative abundance, being 18% and 18% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively, exhibiting no significant differences at both tested OLRs (Figure S5.4 in Appendix II). Following *Pyramidobacter*, *Petrimonas* relative abundance was 17% and 10% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively, showing no significant differences between both tested OLRs (Figure S5.4 in Appendix II). Regarding to *Aminobacterium*, the relative abundance was 12% and 9% at 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, respectively, exhibiting no significant differences at both tested OLRs tested (Figure S5.4 in Appendix II). Finally, when increasing the OLRs from 1.9 and 3.3 g COD g^{-1} VSS d^{-1}, the relative abundance of *Sporanaerobacter* increased significantly from 0% to 13% (Figure S5.4 in Appendix II).

No significant differences were found among the Shannon Index calculated for quantifying the biodiversity obtained at the two OLRs tested, for both family and genus levels (Table S5.7 in Appendix II).

5.3 Discussion

In the following section, the results presented in the previous section are discussed.

5.3.1 Effect of the OLRv

When the substrate was made up exclusively of proteins, the OLRv did not show a great impact on the acidogenic fermentation process. Firstly, the total COD in the effluent

did not vary significantly when increasing the OLRv from 6.5 up to 18.5 g COD L^{-1} d^{-1} (Figure 5.1) and the difference between the total COD in the influent and the effluent was similar in all the tested OLRv. As stated in Chapter 4, the difference between the total COD in the influent and in the effluent could only correspond to COD consumed for biomass growth or for biogas production. The CA concentration detected in the effluent was in the range of 8000-10000 mg O_2 L^{-1}, showing no significant differences regardless the applied OLRv (Figure 5.1). Moreover, the observed biomass growth yields (Yobs) were calculated for the different tested OLRv and all the values (0.24-0.26 g VSS g^{-1} COD) were very close to the theoretical values for acidogenic biomass growth yield [2]. Hence, from CA accumulation and Yobs, it can be assumed that methane production was totally or strongly inhibited after from day 40 onwards. Besides, the new biomass formed depends on the SRT applied in each of the conditions. The lower the applied SRT, the higher the biomass formation. In this experiment, the SRT was quite similar for all the OLRv applied, with an average value of 10, 9 and 9 days at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively. Therefore, this explains that the COD used for biomass growth was constant and, consequently, the total COD in the effluent did not show significant differences at the different applied OLRv. These results were completely opposite to the ones obtained when using simulated wastewater containing only food-grade sugar as model carbohydrate. As shown in Chapter 4, the changes in the applied OLRv caused modifications of the total COD in the effluent and the resultant SRT. When using a carbohydrate model substrate, decreasing the OLRv to 6.0 g COD L^{-1} d^{-1}, lead to a higher COD concentration in the effluent than the obtained one at 11.0 and 15.8 g COD L^{-1} d^{-1}. Moreover, the resultant SRT at the OLRv of 6.0, to 11.0 and 15.8 g COD L^{-1} d^{-1} were 14, 11 and 5 days, respectively.

The bioconversion, the degree of acidification, the acidified COD and the CA production yield did not vary significantly at the different applied OLRv, since the total COD and the total CAs did not differ significantly (Table S5.2 in Appendix II). When protein isolate was used as substrate, no substrate consumption limitation was recorded since bioconversion was over 98.9% at the three tested OLRv (Table 5.2). Even the whey protein isolate was totally consumed at the three tested OLRv, the degree of acidification (63-63.5%) and the acidified COD (82-83.3%) were not close to 100% due to the large proportion of non-identified compounds in the effluent (corresponding to the 'Rest of COD in the effluent' in Figure 5.3). Moreover, the achieved degree of acidification and

acidified COD were higher than the achieved ones with food-grade sugar at the same OLRv. The notably lower degree of acidification (37.8-59%) and acidified COD (44.9-73%) achieved when the carbon source was food-grade sugar, especially at the medium and high levels of OLRv, was caused by the transformation of part of the substrate to ethanol instead of CA (Figure 4.3 in Chapter 4). However, it is important to consider, unlike the food-grade sugar experiments, that increasing the OLRv does not impact the achieved degree of acidification or the acidified COD value. The CA production yield obtained in the experiments using whey protein isolate (0.6 at any applied OLRv) were equal or higher than the ones obtained in the experiments using food-grade sugar (0.6, 0.5 and 0.4 at the low, medium and high levels of OLRv, respectively). The lower CA production yield at the medium and high levels of OLRv when using food-grade sugar are again explained by the high proportion of ethanol obtained under these conditions (Figure 4.3 in Chapter 4).

The odd-to-even ratio showed significant differences between 6.5 and 12.5 g COD L^{-1} d^{-1} and between 12.5 and 18.5 g COD L^{-1} d^{-1}, but there were no significant differences between 6.5 and 18.5 g COD L^{-1} d^{-1}, so a clear trend could not be described (Table S5.2 in Appendix II). The components with highest proportions in the effluent under the three OLRv were (in decreasing order): acetic, isovaleric, propionic, butyric, isocaproic and isobutyric acids (Figure 5.3). Among these acids, only the isocaproic acid shows a significant increase when increasing the OLRv from 6.5 to 12.5-18.5 g COD L^{-1} d^{-1}, but this acid is not considered in the calculation of the odd-to-even ratio since it is not usually considered one of the possible PHA precursors [29]. The odd-to-even ratios obtained with whey protein isolate (from 0.25 to 0.32) were higher than the achieved ones with food-grade sugar (from 0.0 to 0.2). The odd-to-even ratio was higher with proteins due to the higher proportion of propionic acid and the lower concentration of acetic acid in the effluent (Figures 4.3 and 5.3).

Respect the formation of isocaproic, this acid might be produced by chain elongation reactions from isobutyric acid since branched-SCCA are normally needed to obtain branched-MCCA [211]. Oppositely to the food-grade sugar experiments, where the formation of caproic acid was observed only when the OLRs was increased at the highest level of OLRv, when the substrate was only formed by whey protein isolate, isocaproic acid was detected at all the OLRv tested. When increasing the OLRv from 6.5 to 12.5-18.5 g COD L^{-1} d^{-1}, isocaproic acid was significantly increased. This increase in

isocaproic acid proportion could be expected since the increase of the OLRv was previously pointed out as a strategy to boost MCCA production when the substrates are simple [177]. It must be outlined that isocaproic acid was produced adding any external supply of an electron donor (ED) for the chain elongation reactions. Actually, ethanol was not detected in the effluent at any of the OLRv tested and lactic acid was present in almost negligible concentrations (Table S5.1 in Appendix I). Bevilacqua et al (2022) reported recently that chain elongation might happen in protein mixed culture fermentation without any ED supply, since some amino acids can act as ED [241] Consequently, amino acids might be the ones acting as ED in isocaproic acid formation. Bevilacqua et al (2021) produced isocaproic acid in a chemostat reactor fed by casein at pH 5, but the concentrations were lower than 100 mg L^{-1} [242]. In the present study, isocaproic acid was detected in the effluent with an average concentration of 547 mg L^{-1} when the OLRv was 18.5 g COD L^{-1} d^{-1}. To the best of our knowledge, this is the maximum reported isocaproic acid concentration produced with mixed cultures from proteinic substrates without adding any external ED.

Despite there were no significant changes in the effluent composition of the protein fermentation at the different tested OLRv, the microorganisms present at family and genus level were quite different. There is scarce information of the metabolic products of most of the microorganisms detected when starting fermentation from proteins. Plus, there is a quite high relative abundance of unclassified microorganisms at genus level: 23%, 10% and 12% at 6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}, respectively.

Five main genera were found at the different tested OLRv: *Petrimonas*, *Pyramidobacter*, *Aminobacterium*, *Alistipes* and *Peptostreptococcus*. Firstly, the genus with the highest relative abundance is *Petrimonas* (Figure 5.6), which showed no significant differences at the different OLRv. *Petrimonas* is part of the family *Dysgomonadaceae* that is frequently associated with mesophilic anaerobic digesters fed with food waste or protein-rich substrates and with high ammonia levels [243]. In fact, the presence of *Petrimonas* can be used as an early warning of the anaerobic digestion failure due to the accumulation of ammonia [243]. Therefore, it is reasonable that they were detected in the biomass since the simulated wastewater was only composed by proteins and the ammonium concentration was around 1000 mg $N\text{-}NH_4^+$ L^{-1} during most of the operation time. Members of the genus *Petrimonas* were reported to be able to ferment amino acids (protein monomers), yielding mainly acetic and propionic acids as

products [243]. Therefore, *Petrimonas* might be main responsible of producing acetic and propionic acids under the three OLRv tested.

The second genus with the highest relative abundance was *Pyramidobacter* (Figure 5.6), which showed a significant increase when increasing the OLRv (Figure S5.2 in Appendix II). The genus *Pyramidobacter*, which belongs to family *Synergistaceae*, was described to produce acetic and isovaleric acids and to a lesser extent propionic, isobutyric, succinic and phenylacetic acids [244]. *Pyramidobacter* might be the main responsible of the production of isovaleric acid at the two highest OLRv.

Belonging also to family *Synergistaceae*, the genus *Aminobacterium* was also detected at all the tested OLRv (Figure 5.6). Members of the genus *Aminobacterium* were reported to be able to ferment serine, glycine, threonine and pyruvate obtaining acetate and hydrogen as final products [245–247]. In the whey protein isolate used as substrate, serine, glycine and threonine accounted for a 5.7%, 3.2% and 5.8% (on molar basis), respectively (Figure S5.5 in Appendix II). Moreover, in mixed cultures, members of *Aminobacterium* were reported to synthetize acetic acid from aspartic acid and alanine, isobutyrate from valine, isovalerate from leucine and propionate from glutamate [245–247]. In the whey protein isolate used as substrate, aspartic acid, alanine, valine, leucine and glutamate accounted for a 11%, 6%, 7%, 11%, 16%, respectively (on molar basis, Figure S5.5 in Appendix II). Consequently, *Aminobacterium* might be responsible for the formation of acetic, propionic, isobutyric and isovaleric acids in all the tested OLRv.

Genus *Alistipes*, which belongs to the family *Rikenellaceae* was also present in the reactor at all the OLRv tested (Figure 5.6), and it decreased significantly when increasing the OLRv (Figure S5.2 in Appendix II). Members of genus Alistipes have been described to produce succinic, acetic and propionic acids from peptone-yeast extract-glucose (PYG) broth, which is a culture medium with a high protein content [248]. So, *Alistipes* might be in charge of the production of acetic and propionic acids at all the tested OLRv.

Finally, genus *Peptostreptococcus*, which is part of the family *Peptostreptococcaceae*, was detected at all the OLRv tested (Figure 5.6), showing no significant differences at the different OLRv. Members of the genus *Peptostreptococcus* were reported to produce mainly butyric acid from PYG medium [249]. So, *Peptostreptococcus* might be responsible for butyric acid production at all the tested OLRv.

Among the identified genus, none of them might be responsible of the isocaproic acid. However, to the extent of our knowledge, very few bacteria have been described as isocaproic acid producers, namely genus *Clostridium* and specifically two species of this genus: *Clostridium difficile* and *Clostridium botulinum* [250,251].

5.3.2 Effect of the OLRs.

The OLRs also did not have a significant effect on the acidogenic fermentation process when the substrate was only composed by proteins. First, increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1} did not significantly affect the total COD, the total CAs and the ammonium concentration in the effluent (Figure 5.1). As stated previously, methanogenic activity was inhibited from the day 40 of operation. Since the total CAs did not vary significantly when increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1}, the methanogenic activity remained inhibited at this high CA concentrations. Therefore, the removed COD was mainly used for acidogenic biomass growth. As the total COD was not affected by increasing the OLRs from 1.9 up to 3.3 g COD g^{-1} VSS d^{-1}, the COD used for the acidogenic biomass growth was also constant.

As the total COD and the total CAs did not vary significantly when increasing the OLRs, the bioconversion, the degree of acidification, the acidified COD and the CA production yield did not change significantly (Table S5.3 in Appendix II). At both tested OLRs, the bioconversion was over the 99.8%, so there was no substrate consumption limitation (Table 5.2). Oppositely, at the highest level OLRv when the substrate was food-grade sugar, increasing the OLRs from 2.3 to 3.8 g COD g^{-1} VSS d^{-1} (low and high levels) lead to a decrease of the bioconversion from 99.2% to 96.5% (Table 4.2 in Chapter 4).

Other authors have performed previously acidogenic fermentation experiments starting from model protein compounds in continuous reactors without biomass retention. However, in most of the cases, the conversion of the substrate was lower than in the present study, probably due to protein hydrolysis limitations. Moreover, as we stated in Chapter 1, the ratio between the substrate and the biomass is rarely considered and the protein hydrolysis limitation might be caused by operating the reactor at a very high OLRs. Duong et al. (2021) and Bevilacqua et al. (2020) performed acidogenic fermentation of model proteins (gelatin and casein, respectively) in continuous reactors without biomass retention [102,252]. Duong et al. (2021) reported a bioconversion

(named 'Degree of hydrolysis' in their study) of 35% when feeding the reactor with a gelatin solution at an OLRv of 4.58 g COD L^{-1} d^{-1} at pH 7 and the average biomass concentration was 0.5 g VSS L^{-1} [102]. This value of bioconversion is clearly lower to the 99-100% obtained in this study under higher OLRv (6.5-18.5 g COD L^{-1} d^{-1}) and similar pH conditions. Bevilacqua et al. (2020) reported a maximum degree of acidification of the 48.8% for casein and 40% for gelatin 35% when operating the reactor at an OLRv of 8 g COD L^{-1} d^{-1} at pH 7.2-7.4 [252]. These values of degree of acidification are considerably lower to the 63% obtained in the present study under similar pH conditions. None of these previous studies consider the OLRs, but if calculated from their data their values were 9.2 g COD g^{-1} VSS d^{-1} and 21.3 g COD g^{-1} VSS d^{-1}, for Duong et al. (2021) and Bevilacqua et al (2020) studies, respectively. These values of OLRs were clearly higher than the applied in the present study (1.9-3.3 g COD g^{-1} VSS d^{-1}). Therefore, the low bioconversion and degree of acidification that was obtained in that studies might be caused by hydrolysis limitations due to the high OLRs applied. In fact, Duong et al. (2021) reached a bioconversion of 96% in the reactor fed with gelatin at pH 7 when decreasing the OLRv to 2.31 g COD L^{-1} d^{-1} and the average biomass concentration was 2.3 g VSS L^{-1} [102]. In these conditions, the bioconversion might have increased due to the lower OLRs (1 g COD g^{-1} VSS d^{-1}).

Consequently, operating the reactor in SBR mode has a clear advantage since biomass is retained and OLRs can be kept in lower values. Increasing the OLRv in a system without biomass retention, would inevitably lead to a higher OLRs since the biomass concentration in the reactor is limited by the biomass losses through the effluent. At large OLRs, hydrolysis limitation can occur, leading to a decrease in bioconversion and the degree of acidification. Moreover, at high OLRv, if the reactor is not purged, the effluent would contain high biomass concentration, making its direct use in a subsequent application impossible. On the contrary, performing the acidogenic fermentation process in a SBR reactor would lead to an effluent with a lower concentration of solids, as long as there are no settleability issues, and allows to work at higher OLRv.

Regarding the odd-to-even ratio, it increased when increasing the OLRs from 1.9 up to 3.3 g COD g^{-1} VSS d^{-1} (Table S5.3 in Appendix II). This increase in the odd-to-even ratio was caused by the significant rise of propionic and valeric acids, which were the most significant changes (Table S5.5 in Appendix II). In fact, by increasing the OLRs from 1.9 up to 3.3 g COD g^{-1} VSS d^{-1}, propionic, isobutyric, butyric, valeric and caproic

acids were significantly increased (Table S5.5 in Appendix II), leading to a notably lower proportion of non-identified compounds in the effluent (corresponding to the 'Rest of COD in the effluent' in Figure 5.4). In comparison to the experiments with food-grade sugar as carbon source, the odd-to-even ratio obtained with whey protein isolate as carbon source was higher for both tested OLRs.

The significative differences in the bacterial communities at both tested OLRs are mainly found in families and genera with a low relative abundance (lower than the 10%). The only genus with a relative abundance over the 10% that suffered significative changes between both OLRs was *Sporanaerobacter*. This genus pertains to the family *Clostridiales Incertae Sedis XI* and it increased significantly from 0 to 13% when increasing the OLRs from 1.9 up to 3.3 g COD g^{-1} VSS d^{-1}. The genus *Sporanaerobacter* has been reported to perform the Stickland reaction using isoleucine as electron donor and glycine or serine as electron acceptors [253]. Stickland reaction is a coupled deamination reaction in which one amino acid acting as electron donor is oxidated and other amino acid acting as electron acceptor is reduced [254]. In this reaction, the electron donor is converted to an organic acid one carbon atom shorter than the original amino acid and the electron acceptor is transformed to an organic acid with the same number of carbon atoms [255]. In the whey protein isolate used as carbon source, isoleucine accounted for a 7%, glycine represented the 3% and serine comprised the 6% (all on molar basis) (Figure S5.5 in Appendix II). Therefore, if the Stickland reaction takes part with isoleucine as electron donor and glycine as electron acceptor, the produced acids would be valeric and acetic acids, respectively. On the contrary, if the isoleucine acts as electron donor and serine as electron acceptor, the produced acids would be valeric and propionic. The occurrence of this second reaction would support the significative increase of valeric and propionic acids in the effluent when enlarging the OLRs from 1.9 up to 3.3 g COD g^{-1} VSS d^{-1}.

5.4 Potential application of these effluents

Next, the potential use of the effluents obtained from the acidogenic fermentation of proteins (using whey protein isolate as model) is explored. As opposed to the experiments with carbohydrates (with food-grade sugar as model) in Chapter 4, in this case, the product spectra obtained from whey protein isolate at the different OLRv and OLRs are

very similar. Under all the conditions tested, the effluent is essentially formed by six CA with similar concentrations: acetic, isovaleric, propionic, butyric, isocaproic and isobutyric acids (in decreasing proportions). The resultant odd-to-even ratio at all the conditions is around 0.3.

The relatively high proportions of butyric and propionic acids make convenient the use of these effluents in PHA synthesis. Butyric acid has been identified as one of the preferred substrates because it is very energetically favorable [24,236,237]. Propionic acid is one of the precursors that can be utilized to synthetize 3HV [256]. Polymers containing 3HV have greater mechanical properties than polymers that only contain 3HB [1]. From all the effluents produced, the one more convenient for PHA synthesis would be the one obtained at the highest OLRv (18.6 g COD L^{-1} d^{-1}) and OLRs (3.3 g COD g^{-1} VSS d^{-1}) since the proportion of propionic and butyric acids is higher than the achieved in the rest of conditions (Figure 5.3 and Figure 5.4).

Additionally, the effluents obtained from whey protein isolate contain compounds that could be used as electron acceptor (EA) in the MCCA synthesis reactions. On the one side, acetic and butyric acids could be used as EA for producing caproic and caprylic acids and isobutyric acid could be used as EA to obtain isocaproic and isocaprylic acids [178,257]. On the other side, propionic acid could be used as EA to synthesize valeric and enanthic acids while isovaleric acid could be act as EA in iso-enanthic acid synthesis [178,257]. Therefore, if these effluents are used as feedstock in MCCA synthesis, a mixture of different MCCAs might be produced. Nevertheless, these effluents do not contain any compound that could act as ED in elongation reactions, so it might be provided externally. As aforesaid in the Introduction section, lactate and ethanol were stablished as the more convenient ED since they produce enough NADH and energy in form of ATP to fulfill the next reactions [178,187]. Instead of using pure ethanol or lactic acid currents, one option could be to use effluent rich in these components. As an example, the effluent obtained from the acidogenic fermentation of food-grade sugar at an OLRv of 15.8 g COD L^{-1} d^{-1} and a OLRs of 2.3 g COD g^{-1} VSS d^{-1} could be used as ED provider since the ethanol comprises the 30% of the effluent COD (Figure 4.3 in Chapter 4). However, these effluents already contain isocaproic acid that was produced without providing any external ED. Optimization of the current process operational conditions to increase the isocaproic acid concentration would be an interesting point to consider in future research.

5.5 Conclusions

In this study, a SBR was used to perform acidogenic fermentation experiments starting from a substrate only composed by whey protein isolate as model protein. From the experimental work, it can be observed that both OLRv and OLRs have little impact on the acidogenic fermentation process performance and the effluent composition when the substrate is only formed by a structurally simple protein.

Under all the conditions tested, the bioconversion, the degree of acidification and the CA production yield did not change significantly and their values were around 99%, 63%, 83% and 0.6, respectively. The effluent has a very similar composition under all the conditions tested and it was formed by six main carboxylic acids with a balanced proportion: acetic acid (15.4-18.1%), isovaleric acid (15.0-17.5%), propionic acid (11.5-15.5%), butyric acid (11.9-15.8%), isocaproic acid (7.2-11.2%) and isobutyric acid (7.2-9.1%).

When increasing the OLRv from 6.5-12.5 to 18.5 g COD L^{-1} d^{-1}, the only relevant change was the increase of isocaproic acid from 7.5% to 11.0%. As far as we know, at the highest OLRv tested, isocaproic acid achieved the maximum reported concentration (547 mg L^{-1}) obtained in a mixed culture reactor without the addition of any external electron donor (ED). Future work might consider the optimization of the operational parameters to maximize isocaproic acid production without adding ED, as it would be beneficial to apply these conditions in a large-scale application when starting from protein-rich real wastes or wastewaters since operational costs would be reduced.

The odd-to-even ratio slightly increased from 0.25 to 0.35 when increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1} due to the significant increase of propionic and valeric acids. Among the main acids, butyric and isobutyric were also increased when increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1}, leading to a lower fraction of non-identified compounds in the effluent.

Regarding the bacterial communities present in the reactor, under all the OLRv tested five main genera were detected: *Petrimonas*, *Pyramidobacter*, *Aminobacterium*, *Alistipes* and *Peptostreptococcus*. In the examined OLRs, four main genera were found: *Pyramidobacter, Petrimonas, Aminobacterium* and *Sporanaerobacter*. For all the conditions tested, it was possible to link all the effluent components except isocaproic acid to the different genera detected in the reactor.

The effluents obtained from whey protein isolate had a very similar composition under all the OLRv and OLRs tested, and they would be suitable to be used in PHB-co-PHV synthesis due to its high content of butyric and propionic acids. Plus, the effluent contains several acids to be used as EA in MCCA synthesis reactions, but an external stream of ethanol or lactic acid would be needed as ED provider.

Chapter 6:

Acidogenic fermentation of mixtures of carbohydrates and proteins: effect of the substrate organic composition on the process performance and the biomass composition

6.1 Overview

In this chapter, simulated wastewater composed by food-grade sugar, as model carbohydrate, and whey protein isolate, as model protein, was used as substrate in the acidogenic fermentation carried out in a sequencing batch reactor (SBR). The objective of this study was to assess the influence of the organic composition of the substrate, with different proportions of carbohydrate and protein, on the process effectiveness parameters and the effluent and biomass compositions. Statistical methods were used to compare if the results obtained with the different tested substrate organic compositions were significantly different and therefore, to assess if the substrate composition has a significant effect on the acidogenic fermentation performance. Likewise, microbial communities favoured with the different substrate composition and indirectly, the product spectrum formed, were evaluated. Experiments were performed using the same operational conditions in all the cases.

In this sense, this chapter starts with the description of the results regarding the reactor operation, process effectiveness parameters, effluent composition and biomass composition. Next, the effect of the substrate composition on the above-mentioned variables is jointly assessed. Finally, the potential applications of the obtained effluents is evaluated. Materials and methods corresponding to the experiments of this chapter can be found in Chapter 3.

6.2 Results

6.2.1 Reactor operation

The reactor was operated for a total of 85 days using different substrate compositions (Table 6.1). Three different proportions of food-grade sugar (C, model carbohydrate) and whey protein isolate (P, model protein) were used (% in terms of COD-chemical oxygen demand): 50%C/50%P, 25%C/75%P and 75%C/25%P, for a total COD concentration of 14000 mg O_2 L^{-1}. The inoculum was a mixture in equal proportions (in mass basis) of biomass gathered from the reactor where the experiments with an influent containing only carbohydrate were performed (Chapter 4) and of biomass collected from the reactor after the operation with an influent containing only protein (Chapter 5). Table 6.1 contains the different operational periods and the conditions applied in each one of them. Figure 6.1

shows the profiles against time of the total COD concentrations of the influent and of the effluent; the COD concentrations corresponding to food-grade sugar, whey protein isolate and total Carboxylic Acids (CAs) in the effluent; and the ammonium concentration in the effluent.

The reactor was started up with an OLRv of 12.0 g COD L^{-1} d^{-1} and an OLRs of 1.8 g COD g^{-1} VSS d^{-1} using a simulated wastewater composed by 50%C/50%P (Table 6.1). The influent pH was 7.4 and there was not ammonium in the influent. The total COD and the total CAs of the effluent reached values over 10000 and 6000 mg O_2 L^{-1}, respectively, from the very beginning of the operation (Figure 6.1), since the inoculum proceeded from acidogenic reactors. Food-grade sugar was totally removed during the whole reactor operation. However, whey protein isolate was not fully consumed and its concentration in the effluent was accumulated ca. 5000 mg O_2 L^{-1} (Figure 6.1), even though half of the biomass came from an acidogenic fermentation reactor consuming only whey protein. Two possible reasons could be responsible of the protein accumulation: the low pH in the bulk liquid and the ammonium concentration in the reactor. On the one side, protein hydrolysis has been reported to be inhibited at pH comprised between 4.6-5.5 [102]. On the other side, low concentrations of ammonium in the effluent might indicate possible limitation due to the low nitrogen content while very high ammonium concentration (over 1700-1800 mg N-NH_4^+ L^{-1}) was reported to inhibit acidogenic fermentation with an unacclimated inoculum [84]. For this reason, several pH adjustments (PA in Figure 6.1), ammonium concentration adjustments (AA in Figure 6.1) and pH and ammonium concentration adjustments (PAA in Figure 6.1) were performed in the influent during the start-up period attempting to increase the whey protein consumption (Figure 6.1). After all these adjustments, the pH and ammonium concentrations in the influent were 9.2 and 130 mg N-NH_4^+ L^{-1}, respectively. However, whey protein isolate was still not fully consumed (Figure 6.1). For this reason, the OLRv was reduced by half (from 12 to 6 g COD L^{-1} d^{-1}) on day 43rd and it was determined as the one to be used to assess the different substrate organic composition. Consequently, the period before day 43rd was considered the start-up period.

Table 6.1. Experimental conditions tested throughout the study of the acidogenic fermentation using mixtures of food-grade sugar and whey protein isolate as carbon source.

Period	**Duration (d)**	**Substrate composition**	**OLRv ($g\ COD\ L^{-1}\ d^{-1}$)**	**OLRs ($g\ COD\ g^{-1}\ VSS\ d^{-1}$)**	**TRH (d)**	**pH of the influent**	**Ammonium concentration in the influent ($mg\ N\text{-}NH_4^+\ L^{-1}$)**	**Average pH in the bulk liquid**
Start-up	43	50%C/50%P	12.0±0.5	1.8±0.3	1.1±0.1	7.7-9.3	0-1000	5.5-6.8
I	14	50%C/50%P	6.1±0.1	1.7±0.5	2.2±0.1	9.4±0.1	130	7.0±0.1
II	14	25%C/75%P	6.2±0.2	1.8±0.4	2.2±0.0	9.0±0.1	0	7.3±0.1
III	14	75%C/25%P	6.0±0.2	1.9±0.5	2.2±0.1	8.9±0.3	130	6.4±0.2

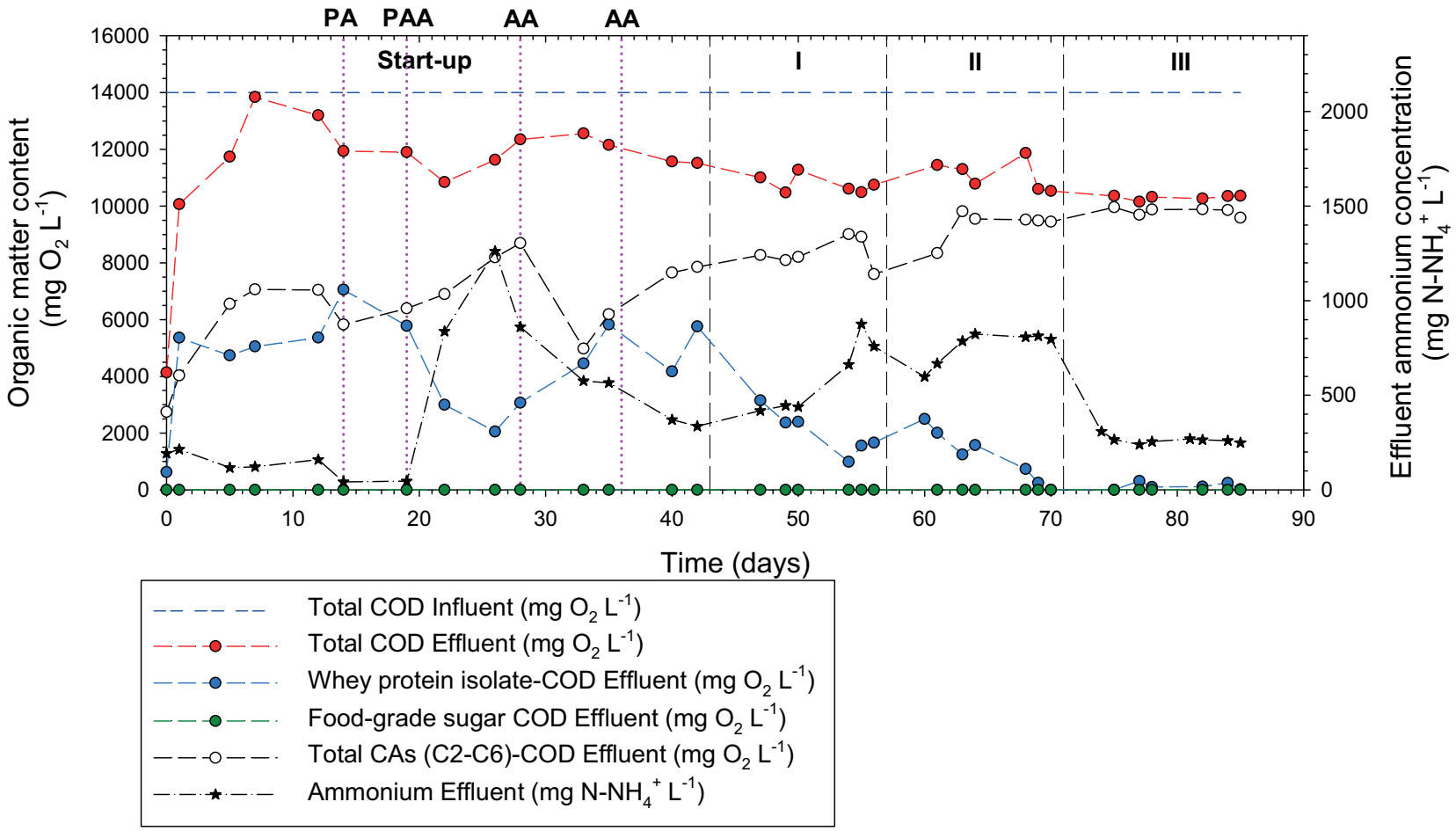


Figure 6.1. Reactor operation during the study of the acidogenic fermentation using mixtures of food-grade sugar and whey protein isolate as carbon source. Vertical pink dotted lines indicate changes in the influent preparation (PA: pH adjustment, AA: ammonium concentration adjustment, PAA: pH and ammonium concentration adjustment). Vertical black discontinuous lines indicate the beginning of a new period.

Once the OLRv was decreased, the whey protein isolate concentration in the effluent started to sharply decrease. Each of the substrate compositions was tested until reaching a pseudo-steady state in the following order: 50%C/50%P, 25%C/75%P and 75%C/25%P. Despite not having a pH automatic control loop, the pH value in each of the achieved steady states remained around a value of 7 (Table 6.1). Consequently, the effect of the substrate composition on the process performance, the effluent composition and the biomass composition was not affected by the variation of the pH value. The ammonium concentration in the influent was kept at 130 mg $N\text{-}NH_4^+$ L^{-1} when the composition was 50%C/50%P and 75%C/25%P and zero when the composition was 25%C/75%P.

The whey protein isolate concentrations in the effluent in the pseudo-steady states of the different periods were the following: period I (1400±361 mg O_2 L^{-1}), period II (327±373 mg O_2 L^{-1}) and period III (117±92 mg O_2 L^{-1}) (Figure 6.1). On the one side, period I showed whey protein concentrations significantly higher than period II ($p=0.0059$) and period III ($p=0.0015$). On the other side, there were no significant differences between periods II and III ($p=0.6192$). This fact implies that the whey protein isolate concentration was significantly higher when the influent was composed by 50%C/50%P in comparison to the remaining whey protein isolate when the substrate was comprised by 25%C/75%P and 75%C/25%P.

The total COD in the effluent varied between 10000 and 12000 mg O_2 L^{-1} with the following mean values in the pseudo-steady states of the different periods: period I (10617±130 mg O_2 L^{-1}), period II (11001±753 mg O_2 L^{-1}) and period III (10325±43 mg O_2 L^{-1}) (Figure 6.1). No significant differences were found among the total COD obtained for the different periods, indicating that the organic composition of the substrate did not affect the total COD of the effluent.

Regarding the total CAs in the effluent, the concentrations ranged between 7500 and 10000 mg O_2 L^{-1} with the following mean values for the pseudo-steady states in the different periods: period I (8511±787 mg O_2 L^{-1}), period II (9488±36 mg O_2 L^{-1}) and period III (9804±144 mg O_2 L^{-1}). Significant differences were only found between periods I and III ($p=0.0138$), revealing that the total CAs yielded with an influent composed by 75%C/25%P was significantly higher than the obtained using an influent composed by 50%C/50%P.

With respect to the reactor operation, proper biomass settleability of the biomass, i.e. low biomass concentration in the effluent is chased in order to obtain effluents that could be directly used in a subsequent application. Despite of using a flocculent biomass, the reactor performance was successful since the effluent had an average solid concentration of 0.3±0.1 g VSS L^{-1} after the start-up period, while the average biomass concentration in the reactor was 3.6±1.0 g VSS L^{-1}. It is important to highlight that at the same OLRv and OLRs, the solid concentration in the effluent did not vary notably with the influent organic composition. In consequence, the SBR appears to be a satisfactory system to obtain effluents from the acidogenic fermentation of wastewaters composed by mixtures of carbohydrates and proteins that could be use in posterior applications without any post-treatment.

6.2.2 Process effectiveness parameters: bioconversion, degree of acidification, acidified COD, CA production yield and odd-to-even ratio

In this section, the effect of the substrate organic composition on the process effectiveness parameters is presented. Table 6.2 collects the values of these effectiveness parameters at the different proportions of carbohydrates and proteins tested during the acidogenic fermentation study using mixtures of food-grade sugar and whey protein isolate as carbon sources (25%C/75%P, 50%C/50%P and 75%C/25%P). Additionally, Table 6.2 also contains the effectiveness parameters resultant from the acidogenic fermentation study when the reactor was fed by only food-grade sugar as carbon source (100%C) and from the acidogenic fermentation study when the reactor was fed by only whey protein isolate as carbon source (100%P) at similar OLRv and OLRs conditions (6 g COD L^{-1} d^{-1} and 2 g COD g^{-1} VSS d^{-1}, respectively). The results of the acidogenic fermentation using only food-grade sugar and only whey protein isolate as carbon sources were previously described in Chapter 4 and Chapter 5, respectively. The statistical results of the ANOVA test and Tukey's multiple comparisons test to evaluate the effect of the substrate organic composition on the effectiveness parameters can be found in Table S6.2 in Appendix III.

Firstly, the bioconversion values were over 98% except for 50%C/50%P (Table 6.2). The bioconversion obtained when the substrate was composed by 50%C/50%P (90%) was significantly lower than the resultant of using any of the other proportions, which showed no significant differences among them (Table S6.2 in Appendix III).

In relation to the degree of acidification, the values ranged between 59 and 73% for the different substrate compositions tested (Table 6.2). The degree of acidification obtained with an influent composition of 75%C/25%P was significantly higher than the obtained with the rest of the influent composition tested, except for 25%C/75%P (Table S6.2 in Appendix III). There were no significant differences among the resultant degree of acidification when using a substrate composed by any of the remaining compositions (Table S6.2 in Appendix III).

With respect to the acidified COD, it was comprised between 73 and 95% for the different substrate compositions tested (Table 6.2). The maximum acidified COD corresponded to a substrate composition of 75%C/25%P, which was significantly higher to the rest of the composition tested unless when the substrate was composed by 25%C/75%P (Table S6.2 in Appendix III). The lowest acidified COD were obtained when the substrate was formed by only carbohydrates, only proteins or carbohydrates and proteins in equal proportions (100%C, 100%P and 50%C/50%P), which showed no significant differences among them (Table S6.2 in Appendix III).

Regarding the CA production yield, its value was around 0.6 when the substrate was formed only by proteins or only by carbohydrates (100%P and 100%C, respectively) and around 0.7 when the substrate was composed by both carbohydrates and proteins (25%C/75%P, 50%C/50%P and 75%C/25%P) (Table 6.2). The CA production yield resultant of using a mixture of carbohydrates and proteins was significantly higher than the obtained when the substrate was only formed by carbohydrates. However, the CA production yield for a substrate containing only protein (100%P) was not significantly different from the obtained with a substrate containing 25%C/75%P, 50%C/50%P (Table S6.2 in Appendix III).

Finally, the odd-to-even ratio was comprised between 0.03 and 0.60 for the different substrate composition tested (Table 6.2). The odd-to-even ratio showed significant differences among all the substrate organic compositions tested, except for 25%C/75%P and 75%C/25%P which led to the highest values: 0.60 and 0.56, respectively (Table S6.2 in Appendix III). Below these values, the resultant odd-to-even ratio in descending order were the corresponding to the following substrate organic compositions: 50%C/50%P, 100%P and 100%C (Table 6.2).

Table 6.2. Bioconversion, Degree of Acidification, Acidified COD, Carboxylic Acid yield and odd-to-even ratio for the different substrates organic composition tested in the study of the acidogenic fermentation using mixtures of food-grade sugar and whey protein isolate as carbon source.

Substrate composition	**OLRv ($g\ COD\ L^{-1}\ d^{-1}$)**	**OLRs ($g\ COD\ g^{-1}\ VSS\ d^{-1}$)**	**Bioconversion (%)**	**Degree of Acidification (%)**	**Acidified COD (%)**	**CA production yield ($g\ COD\ g^{-1}\ COD$)**	**Odd-to-even ratio**
100%P	6.5±0.6	1.9±0.4	98.9±1.9	63±3	82±3	0.64±0.03	0.26±0.03
25%C/75%P	6.2±0.2	1.8±0.4	98±3	68.0±0.2	86±6	0.70±0.02	0.60±0.10
50%C/50%P	6.1±0.1	1.7±0.5	90±3	63±6	80±9	0.70±0.05	0.38±0.01
75%C/25%P	6.0±0.2	1.9±0.5	99.1±0.7	73±2	95.0±1.7	0.74±0.02	0.56±0.09
100%C	6.0±0.3	2.2±0.2	99.4±0.1	59±7	73±8	0.60±0.06	0.03±0.01

6.2.3 Effluent composition

In this section, the effect of the substrate organic composition on the effluent composition is presented. Figure 6.2 shows the effluent composition in terms of % of COD equivalents for the different substrate organic composition tested, including the data corresponding to the experiments using mixtures of food-grade sugar and whey protein isolate as carbon source (this Chapter) and to the experiments using only food-grade sugar or whey protein isolate as single carbon sources (Chapter 4 and Chapter 5, respectively) at similar OLRv and OLRs conditions. The statistical difference between the effluent components can be deduced from the ANOVA test and Tukey's multiple comparisons test in Table S6.3a and Table S6.3b in Appendix III.

The substrate organic composition strongly impacts the effluent composition (Figure 6.2). The higher the proportion of protein in the substrate, the lower the selectivity for a particular product, since the number of compounds in the effluent is clearly increased. When carbohydrates were used as the only carbon source (100%C), the effluent was mainly composed by two acids: butyric and acetic. If the substrate was composed by 75%C/25%P or 50%C/50%P, the effluent had three main acids: butyric, propionic and acetic acids. When the composition of the substrate was 25%C/75%P, the effluent contained four main acids: acetic, isovaleric, butyric and propionic. Finally, when protein was the only carbon source (100%P), the effluent was composed by six acids: acetic, isovaleric, butyric, propionic, isobutyric and isocaproic. Moreover, the majoritarian acid produced depended on the substrate composition: (i) butyric acid for 100%C, (ii) butyric and propionic acids for 75%C/25%P, (iii) acetic acid for 50%C/50%P, (iv) propionic acid for 25%C/75%P and (v) acetic and isovaleric acids for 100%P.

Acetic acid was one of the main acids for all the substrate organic composition tested (Figure 6.2). The acetic acid proportion ranged from 18.1 to 28.0% between the different substrate compositions. However, it is not possible to describe a clear trend of the acetic acid proportion regarding the different proportions of carbohydrates and proteins in the substrate.

Propionic acid proportion in the effluent ranged from 2.4 to 31.0% between the different substrate compositions (Figure 6.2). The highest propionic acid proportion was achieved for substrates compositions of 25%C/75%P (29.1%) and 75%C/25%P (31.0%), which showed no significant differences among them (Table S6.3 in Appendix III), and

they were followed by 50%C/50%P (20.3%), 100%P (13.3%) and finally, 100%C (2.4%). However, it is not possible to describe a clear trend of the propionic acid proportion regarding the different proportions of carbohydrates and proteins in the substrate.

Butyric acid was also present in the effluent when feeding the reactor with all the substrate organic composition tested (Figure 6.2). The butyric acid proportion in the effluent was 14.8%, 10.5%, 19.2%, 33.6% and 47%, for 100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C, respectively. So, butyric acid proportion increased significantly when the proportion of carbohydrates was increased over the 25% (Figure 6.2, Table S6.3 in Appendix III).

Isobutyric acid proportion ranged from 0.9 to 5.9% between the different substrate compositions (Figure 6.2). The proportion of isobutyric acid was significantly increased when the proportion of protein in the substrate was increased over the 50% (Figure 6.2, Table S6.3 in Appendix III).

Isovaleric acid proportion in the effluent ranged from 0.0 to 17.0% and it showed significant differences among all the organic compositions tested (Table S6.3 in Appendix III). In this case, there was also a clear trend: the higher the protein proportion, the higher the isovaleric acid proportion (Figure 6.2).

Isocaproic acid was only found when the substrate contained 100%P and 25%C/75%P, yielding a 7.2% and a 4.8% of the effluent, respectively, which were significantly different between them (Figure 6.2, Table S6.3 in Appendix III).

Ethanol was only detected when the composition of the substrate was 100%C resulting in an 8% of the effluent (Figure 6.2).

Whey protein isolate proportion in the effluent was 0.5%, 3.0%, 13.0% and 1.1% for 100%P, 25%C/75%P, 50%C/50%P and 75%C/25%P in the substrate, respectively. However, the whey protein isolate composition was only significantly higher than obtained with the rest of the conditions tested when the substrate composition was 50%C/50%P (Table S6.2 in Appendix III).

The rest of the compounds (valeric acid, caproic acid, lactic acid and glucose) comprised less than the 5% of the effluent with all the tested influent composition.

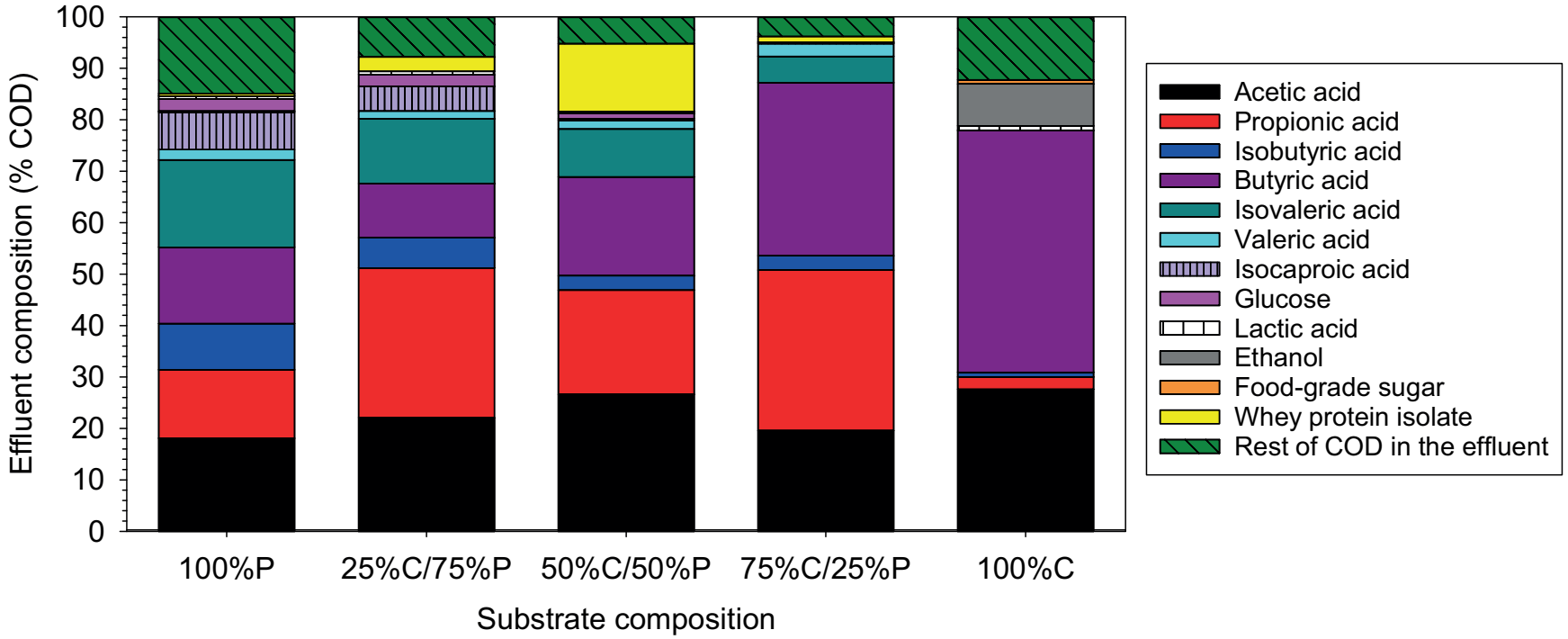


Figure 6.2. Effect of the substrate composition on the effluent composition yielded at an OLRv of 6 g COD L^{-1} d^{-1} and an OLRs of 2 g COD g^{-1} VSS d^{-1}.

6.2.4 Bacterial communities

In this section, the bacterial communities present in the reactor when using the different substrate organic composition tested are presented at family and genus level.

6.2.4.1 Bacterial communities at family level

The relative abundances (in %) of the bacterial communities at family level for the different substrate organic composition used are shown in Figure 6.3. Figures S6.1a-S6.1g in Appendix III depict the difference in the proportions including only the families that showed significant differences in the relative abundance for the different substrate organic composition. The substrate composition strongly affected the spectrum of families present in the reactor (Figure 6.3). At the conditions tested, nineteen families were detected with a relative abundance over 2% (Figure 6.3). For simplicity, in this section, only the seven families with a relative abundance over the 15% are analyzed: *Rikenellaceae*, *Dysgomonadaceae*, *Ruminococcaceae*, *Synergistaceae*, *Porphyromonadaceae*, *Spirochaetaceae* and *Bifidobacteriaceae*.

The relative abundance of *Rikenellaceae* ranged from 0 to 35% for the different substrate compositions (Figure 6.3). *Rikenellaceae* was the majoritarian family when the substrate was only composed by proteins (100%P). In this case, all the relative abundances which were different from zero were significantly different among them (Figures S6.1a-S6.1g in Appendix III).

The relative abundance of *Dysgomonadaceae* ranged from 0 to 19% for the different substrate compositions (Figure 6.3). Thus, this family was only present when the protein proportion was over the 50% of substrate. The relative abundance of *Dysgomonadaceae* was significantly higher with a substrate composed only by protein (100%P) with respect to 25%C/75%P and 50%C/50%P, which were not significantly different between them (Figures S6.1a-S6.1g in Appendix III).

The relative abundance of *Ruminococcaceae* ranged from 0 to 29% for the different substrate compositions (Figure 6.3). It can be observed that the proportion of this family increased when the substrate was only formed by proteins (100%P) or especially when it is only composed by carbohydrates (100%C).

The relative abundance of *Synergistaceae* ranged from 0 to 19% for the different substrate compositions (Figure 6.3). *Synergistaceae* was the majoritarian family when the substrate was composed by 50%C/50%P (Figure 6.3). There is not a clear trend between the composition of the substrate and the proportion of *Synergistaceae* beyond this family was absent when the substrate is completely composed by carbohydrates (100%C).

The relative abundance of *Porphyromonadaceae* ranged from 0 to 23% for the different substrate compositions (Figure 6.3). *Porphyromonadaceae* was the majoritarian family when the substrate was composed by 25%C/75%P and 75%C/25%P (Figure 6.3). There is not a clear trend between the composition of the substrate and the proportion of *Porphyromonadaceae* beyond this family was absent when the substrate is completely composed by carbohydrates (100%C).

Spirochaetaceae was only detected when the substrate was a mixture of carbohydrates and proteins, and its relative abundance was 4%, 15% and 5%, for 25%C/75%P, 50%C/50%P and 75%C/25%P in the substrate, respectively (Figure 6.3). The relative abundance of *Spirochaetaceae* was significantly higher for 50%C/50%P than the achieved for 25%C/75%P and 75%C/25%P, which showed no significant differences between them. Therefore, *Spirochaetaceae* is a family specially promoted when the substrate is a mixture of carbohydrates and proteins in similar proportions.

Finally, *Bifidobacteriaceae* was the majoritarian family with a relative abundance of 38% when the substrate was only formed by carbohydrates (100%C), and it was not detected with any of the other substrate compositions.

Table 6.3 displays the Shannon Index for the bacterial communities present in the reactor at family and genus level for the different substrate organic composition tested. The highest Shannon Index at family level corresponds to the influent composed by 75%C/25%P. The only compositions that did not lead to significantly different Shannon Indexes at family level were 25%C/75%P and 50%C/50%P. The presence of both carbohydrates and proteins in the substrate leads to more diverse bacterial communities (at family level), especially when the influent composition was 75%C/25%P, in comparison to using only carbohydrates or only proteins as carbon source.

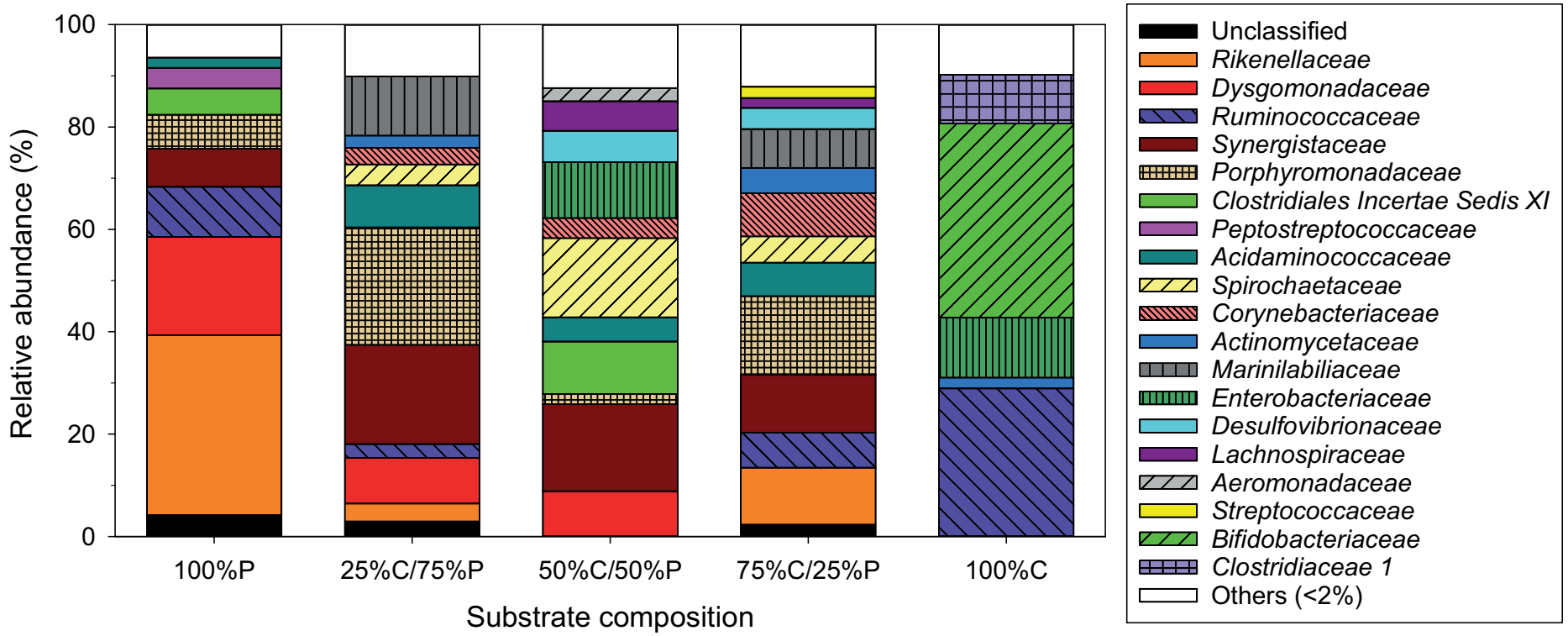


Figure 6.3. Bacterial communities at family level present in the reactor for the different substrate composition used at an OLRv of 6 g COD L^{-1} d^{-1} and an OLRs of 2 g COD g^{-1} VSS d^{-1}.

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Table 6.3. Shannon index and p values for t-test statistical comparison of the bacterial diversity for the different influent organic compositions tested (100%P, 25%C /75%P, 50%C /50%P, 75%C /25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Shannon index					**p values (Diversity t test)**									
	100%P	25%C/ 75%P	50%C/ 50%P	75%C/ 25%P	100%C	100%P vs. 25%C/ 75%P	100%P vs. 50%C/ 50%P	100%P vs. 75%C/ 25%P	100%P vs. 100%C/	25%C/ 75%P vs. 50%C/ 50%P	25%C/ 75%P vs. 75%C/ 25%P	25%C/ 75%P vs. 100%C	50%C/ 50%P vs. 75%C/ 25%P	50%C /50%P vs. 100%C	75%C/ 25%P vs. 100%C
Family level	1.9555	2.2075	2.3188	2.4813	1.5108	0.0269	0.0005	<0.0001	<0.0001	0.2367	0.0039	<0.0001	0.0472	<0.0001	<0.0001
Genus level	2.1151	2.2028	2.3331	2.3400	1.6822	0.3520	0.0198	0.0275	<0.0001	0.1143	0.1361	<0.0001	0.9391	<0.0001	<0.0001

6.2.4.2 Bacterial communities at genus level

The relative abundances (in %) of the bacterial communities at genus level for the different substrate organic composition used are shown in Figure 6.4. Figures S6.2a-S6.2h in Appendix III depict the difference in the proportions including only the genera that showed significant differences in the relative abundance for the different substrate organic composition. The spectrum of genera detected in the reactor was strongly affected by the substrate organic composition (Figure 6.4). At the conditions tested, twenty-five genera were detected with a relative abundance over 2% (Figure 6.4). For simplicity, in this section, only the nine genera with a relative abundance over the 10% are analyzed: *Petrimonas*, *Alistipes*, *Sporanaerobacter*, *Pyramidobacter*, *Odoribacter*, *Saccharicrinis*, *Sphaerochaeta*, *Bifidobacterium* and *Ethanoligenens*.

The relative abundance of *Petrimonas* ranged from 0 to 19% for the different substrate compositions (Figure 6.4). *Petrimonas* was the majoritarian genus when the substrate was only composed by proteins (100%P). The relative abundance of *Petrimonas* clearly decreased when the proportion of proteins of the substrate also decreased, disappearing when the proportion of proteins was 25% or lower.

Alistipes was only detected when the substrate was composed by 100%P and 75%C/25%P, with relative abundances of 14% and 5%, respectively (Figure 6.4). So, the relative abundance of *Alistipes* resultant of using a substrate composed by 100%P was significantly higher than one obtained with a substrate formed by 75%C/25%P (Figures S6.2a-S6.2h in Appendix III).

Sporanaerobacter was only detected when the substrate was composed by 100%P and 50%C/50%P, with relative abundances of 2% and 10%, respectively (Figure 6.4). Thus, the relative abundance of *Sporanaerobacter* resultant of using a substrate composed by 50%C/50%P was significantly higher than one obtained with a substrate formed by 100%P (Figures S6.2a-S6.2h in Appendix III).

Pyramidobacter was only detected when the substrate was a mixture of carbohydrates and proteins, and its relative abundance was 19%, 16% and 11%, for 25%C/75%P, 50%C/50%P and 75%C/25%P in the substrate, respectively (Figure 6.4). Moreover, it was the majoritarian genus for these three substrate compositions and the resultant relative abundances showed no significant differences among them (Figures S6.2a-S6.2h in Appendix III).

Odoribacter was only detected when the substrate was a mixture of carbohydrates and proteins in different proportions, and its relative abundance was 12% and 8%, for 25%C/75%P and 75%C/25%P in the substrate, respectively, which were not significantly different between them (Figure 6.4, Figures S6.2a-S6.2h in Appendix III).

Similarly to *Odoribacter, Saccharicrinis* was only detected when the substrate was a mixture of carbohydrates and proteins in different proportions, and its relative abundance was 11% and 7%, for 25%C/75%P and 75%C/25%P in the substrate, respectively, which were not significantly different between them (Figure 6.4, Figures S6.2a-S6.2h in Appendix III).

Sphaerochaeta was only detected when the substrate was a mixture of carbohydrates and proteins, and its relative abundance was 3%, 13% and 4%, for 25%C/75%P, 50%C/50%P and 75%C/25%P in the substrate, respectively (Figure 6.4). In this case, the relative abundance obtained with a substrate composition of 50%C/50%P was significantly higher than the obtained when the substrate was composed by 25%C/75%P and 75%C/25%P, which were not significantly different between them (Figures S6.2a-S6.2h in Appendix III).

Finally, *Bifidobacterium, Ethanoligenens* and *Clostridium sensu stricto* were only present when the substrate was only formed by carbohydrates (100%C), with a relative abundance of 38%, 22% and 10%, respectively (Figure 6.4). Moreover, *Bifidobacterium* was the majoritarian genus when the composition of the substrate was 100%C.

The highest Shannon Indexes at genus level correspond to the influents composed by mixtures of carbohydrates and proteins (25%C/75%P, 50%C/50%P and 75%C/25%P) which showed no significant differences among them, and they were followed by 100%P, and finally 100%C (Table 6.3). The presence of both carbohydrates and proteins in the substrate leads to more diverse bacterial communities (at genus level) in comparison to using only carbohydrates or only proteins as carbon source.

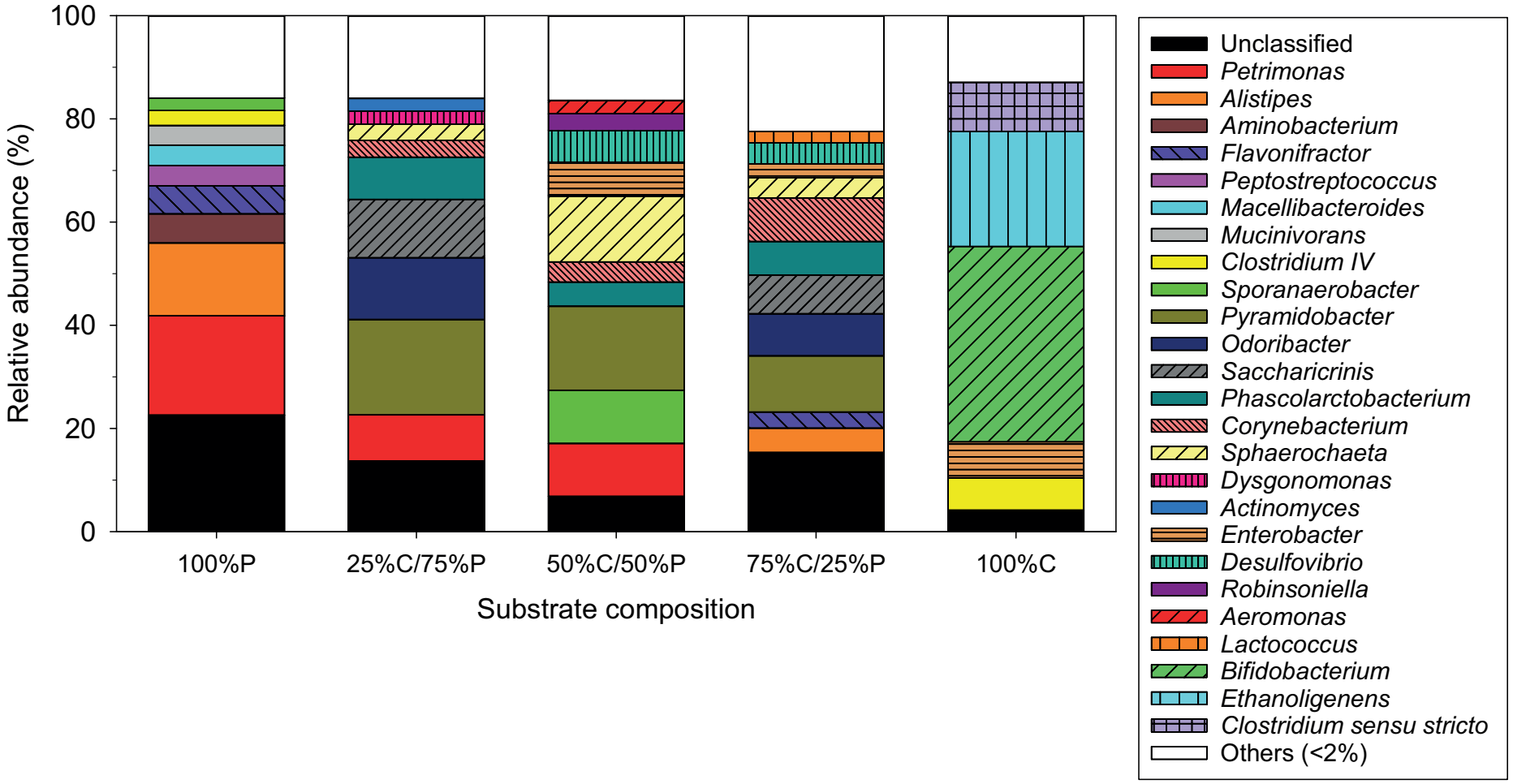

Figure 6.4. Bacterial communities at genus level present in the reactor for the different substrate composition used at an OLRv of 6 g COD L^{-1} d^{-1} and an OLRs of 2 g COD g^{-1} VSS d^{-1}.

6.3 Discussion

In this section, the results presented in the previous section are discussed. It is aimed to analyze jointly the effect of the organic composition of the substrate on the acidogenic fermentation performance, effluent composition and bacterial communities. As exposed previously, the substrate organic composition impacted on the acidogenic fermentation performance, mainly in the resultant effluent and biomass composition because of the different products favoured with the different influent compositions and the degree of consumption of the protein in the substrate.

First, the bioconversion was significantly lower when the substrate was composed by 50%C/50%P in comparison to the rest of substrate composition tested (Table S6.2 in Appendix III). The main cause of this is the limited substrate consumption during the period I (Figure 6.1) since whey protein isolate was not totally consumed as in the rest of the periods. Probably, if period I had been briefly extended, whey protein isolate might have been totally consumed because whey protein isolate was fully consumed when the proportion of protein in the influent was increased from 50% (period I) to 75% (period II) (Figure 6.1). In that hypothetical case, the bioconversion would be closer to the values obtained with the rest of the substrate composition tested. Ammonium inhibition could not be responsible of the limited consumption of whey protein isolate when the influent was composed by 50%C/50%P, as the ammonium concentration under these conditions were below 900 mg $N\text{-}NH_4^+$ L^{-1}, which is a value lower than the ammonium concentration reported to inhibit acidogenesis reactions with unacclimated inoculum (1700-1800 mg $N\text{-}NH_4^+$ L^{-1}) [84].

Regarding the degree of acidification and the acidified COD, their values when the influent was composed by 75%C/25%P were significantly higher to the values obtained with the rest of the substrate composition tested except 25%C/75%P (Table S6.2 in Appendix III). Just like the bioconversion, the low values of the degree of acidification and the acidified COD when the substrate was composed by 50%C/50%P might be conditioned by the limited whey protein isolate consumption (Figure 6.2). The values of the degree of acidification and the acidified COD when the substrate was composed by 100%P and 100%C were lower to the obtained with 75%C/25%P due to the effluent composition obtained, which comprised a larger proportion of non-identified compounds

(Figure 6.2). Moreover, the effluent produced when the substrate composition was 100%C contained an 8% of ethanol (Figure 6.2).

The maximum CA production yields were obtained with a substrate comprised by mixtures of carbohydrates and proteins (25%C/75%P, 50%C/50%P and 75%C/25%P) (Table 6.2), which were a 9-16% higher than the CA production yield obtained with a substrate only containing carbohydrates (100%C). However, there was no significant difference between the CA production yields obtained with only proteins (100%P) and with mixtures of carbohydrates and proteins (25%C/75%P, 50%C/50%P and 75%C/25%P).

In general, it could be concluded that substrates containing both carbohydrates and proteins are preferable if it is aimed to maximize CA production. Influents comprised by 25%C/75%P and 75%C/25%P led to the highest values of degree of acidification, acidified COD and CA production yield and did not show significant differences between them. Regarding to the influent containing 50%C/50%P, it conducted to significantly lower degree of acidification and acidified COD than the achieved ones with influent with 25%C/75%P and 75%C/25%P, but these values might be conditioned by the limited protein consumption.

Previously, other authors have already concluded that the mixture of different organic matter components (carbohydrates, proteins and lipids) lead to higher CA production yields than when using as substrates any of these organic matter components individually. Yin et al. (2016) performed batch experiments using glucose, peptone and glycerol (carbohydrate, protein and lipid hydrolysates, respectively) and a mixture of these three components at a COD ratio of 1:1:1 in different bottles with a starting substrate concentration of 50 g O_2 L^{-1} of COD. They reported that the CA production yields obtained using the mixture of the three components were higher than the SCCAs obtained when each of the components was used individually [64]. Other authors arrived at similar conclusions working with real wastes. As an example, Ma et al. (2017) carried out experiments in 500 mL serum bottles mixing pretreated secondary sludge from a WWTP (protein-rich waste) with potato peel waste (carbohydrate-rich waste). They found that when using a ratio pretreated sludge/potato peel waste of 1:3 (in VS terms) the CA production yield was a 160% higher than the CA production yield resultant of fermenting the pretreated sludge individually [89]. Similarly, Feng et al. (2009) performed

experiments in 5 L-reactors mixing waste activated sludge (protein-rich waste) with rice (carbohydrate-rich waste). In this case, when mixing the two substrates in a 1:1 ratio (in VSS terms) at pH=8, the CA production yield was a 413% higher than the CA production yield resultant of fermenting the waste activated sludge individually under the same pH conditions [86]. The co-fermentation of substrates rich in carbohydrates and substrates rich in proteins conducts to the increase of the CA production yield mainly due to the balancing of the carbon-to-nitrogen ratio [1].

As stated in the results section, the organic composition of the substrate impacts strongly on the resultant effluent composition. Besides the different proportions of the effluent components yielded, the organic composition of the substrate affects the selectivity for a specific product. In general, the higher the proportion of carbohydrates. the lower the number of components in the effluent (Figure 6.2).

Among the main carboxylic acids, only butyric, isovaleric, isobutyric showed a clear trend with the proportion of carbohydrates and proteins. On the one hand, butyric acid proportion significantly increased when increasing the proportion of carbohydrates in the substrate (Figure 6.2). On the other hand, isobutyric and isovaleric acid proportions decreased when decreasing the proportion of carbohydrates in the substrate (Figure 6.2).

In addition to the statistical analysis presented in the results section, we were able to find a linear correlation of the resultant compositions of butyric, isobutyric and isovaleric acids in the effluent and the organic composition of the substrate (Figure 6.5). On the contrary, the rest of the SCCA produced (acetic, propionic and valeric acids) did not show a linear dependence on the organic composition of the substrate (Figure 6.5). Table 6.4 presents the coefficients of the linear regression calculated for the butyric, isobutyric and isovaleric acids concentrations (% COD) as a function of the protein proportion (% COD) in the substrate. The resultant R^2 were quite high: 0.84, 0.91 and 0.99 for butyric, isobutyric and isovaleric acids, respectively (Table 6.4). On the one hand, the correlation for butyric acid predicts a linear increase of its proportion when the proportion of carbohydrates in the substrate increases. This result agrees with the fact that butyric acid is commonly the majoritarian component in the effluent when starting from carbohydrates-rich substrates (see Tables 1.3a and 1.3b in Chapter 1). On the other hand, the correlations for isobutyric and isobutyric acids predict a linear increase of their proportions when the proportion of proteins in the substrate increases. This result agrees

with the fact that branched SCCAs, such as are isobutyric and isobutyric acids, are product of the catabolism of amino acids [258].

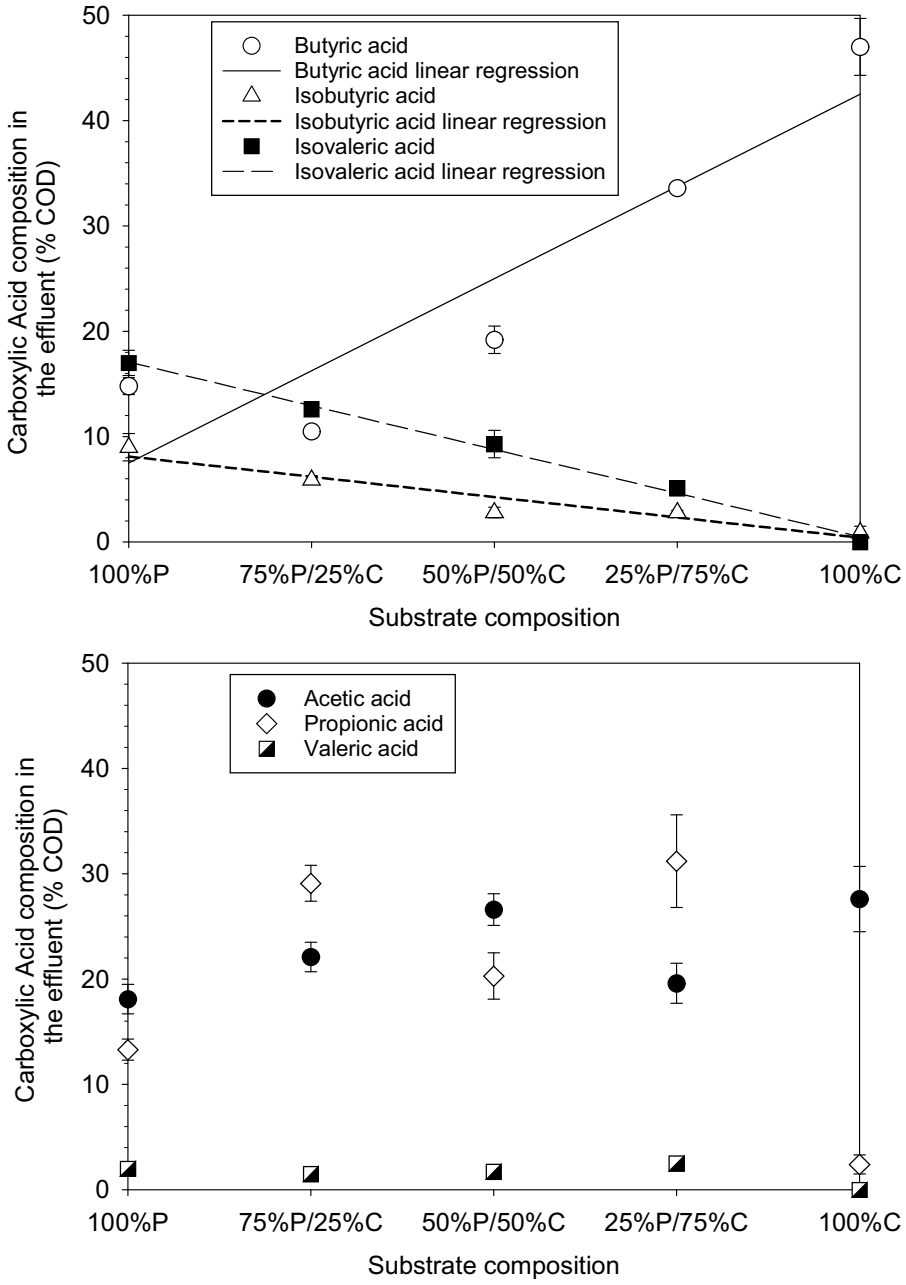

Figure 6.5. a) Butyric, isobutyric and isovaleric composition in the effluent and linear correlations for the different substrate compositions tested at an OLRv of 6 g COD L^{-1} d^{-1} and an OLRs of 2 g COD g^{-1} VSS d^{-1}. b) Acetic, propionic and valeric acid composition in the effluent for the different substrate compositions tested at an OLRv of 6 g COD L^{-1} d^{-1} and an OLRs of 2 g COD g^{-1} VSS d^{-1}.

Table 6.4. Results on the linear regression analysis on butyric, isobutyric and isovaleric acids concentration.

Carboxylic Acid concentration (%COD)	Intercept		%Protein		R^2
	Coefficient	Standard error	Coefficient	Standard error	
Butyric acid	7.52	5.29	8.75	2.16	0.84
Isobutyric acid	17.10	0.41	-4.15	0.17	0.91
Isovaleric acid	8.14	0.83	-1.93	0.34	0.99

Regarding the other SCCAs that were not correlated with the substrate composition, acetic and valeric acids, despite not presenting a correlation, were always in a constant proportion ranged between 18-28% and 0-2.5%, respectively (Figure 6.5). Finally, propionic was the most unpredictable acid. The highest propionic acid proportion was achieved with substrates composed by 25%C/75%P and 75%C/25%P, followed by 50%C/50%P, 100%P and finally, 100%C (Figure 6.2). The trend of the odd-to-even ratio with the different influent compositions is in accordance with the proportion of propionic acid in the effluent, since valeric acid accounted for less than a 3%. The higher the propionic acid proportion, the higher odd-to-even ratio obtained.

To the best of our knowledge, this is the first study that is able to find a correlation from experimental data between the SCCAs concentrations and the substrate composition in a long-term reactor. The most closely related results found in literature were some correlations found in experiments carried out in batch. For example, Alibardi & Cossu (2016) performed batch experiments with mixtures of different organic wastes varying the carbohydrate, protein and lipid proportions. In their work, they were only able to correlate the butyric acid concentration with the carbohydrate, protein and lipid proportions in a multiple regression analysis with an R^2 value of 0.809. As in our study, carbohydrate content was the main factor influencing butyric acid concentration [54]. Ma et al. (2017) performed batch fermentations experiments starting from mixtures in different ratios of waste activated sludge and food waste or potato peel. They tried to correlate propionic, butyric and valeric acid concentrations with lipid, starch, and protein consumption, but the resultant regressions were quite weak and the correlations were not linear in most of the cases. The only linear correlation linked the butyric acid concentration with the starch consumption, but the R^2 was only 0.71. The best correlation found was a logarithmic regression line to link valeric acid concentration with starch

consumption (R^2=0.82), which showed that the lower the carbohydrate consumption, the higher the valeric acid production [89].

The reason why the linear correlations found in our study are successful in correlating butyric, isobutyric and isovaleric acids concentrations with the substrate composition might be the experimental design. Since the OLRv, OLRs and temperature were controlled to be fixed, the resultant concentrations are only affected by the substrate composition. Habitually, experimental studies have analyzed the effect of the substrate composition on the effluent composition, but by modifying simultaneously the influent composition and the OLRv and without controlling the biomass concentration [259,260]. Therefore, effluent composition might be concurrently affected by the substrate composition, the OLRv and the OLRs, making more difficult to find a correlation.

Besides the SCCAs, ethanol was only present when the influent was only formed by carbohydrates (100%C) and isocaproic acid when the influent was only composed by proteins (100%P) and 25%C/75%P (Figure 6.2). On the one side, ethanol is usually found as a fermentation product of carbohydrate-rich substrates. As an example, Breure et al. (1986) carried out fermentation experiments of a gelatin (protein-model compound) solution in a 350-mL chemostat reactor and found that ethanol was detected in the effluent only when glucose was added to the medium [260]. On the other side, isocaproic acid was found when the isobutyric acid proportions reached their highest values. This fact could be related to the formation of isocaproic acid from isobutyric acid through carbon chain elongation reactions [211]. It is important to emphasize that isocaproic acid was produced without adding any external electron donor (ED) for the chain elongation reaction. It was recently reported that chain elongation might happen in protein mixed culture fermentation without any ED supply, since some amino acids can act as ED [241].

Concerning the bacterial communities present in the reactor at family and genus level, they varied considerably depending on the substrate composition (Figure 6.3 and Figure 6.4). Table 6.5 gathers the most relevant information about the metabolism of the genera found in the reactor. Once known the metabolic products of each of the main genera present, the production of the different acids can be linked to the different genera.

Table 6.5. Metabolism of the main found in the reactor when using mixtures of food-grade sugar and whey protein isolate as carbon source.

Genus	Family	Grown frequently in	Metabolism products	References
Petrimonas	*Dysgomonadaceae*	Mesophilic anaerobic reactors fed with substrates rich in proteins and with high ammonia concentrations.	Acetic and propionic acids from sugars or amino acids.	[243]
Alistipes	*Rikenellaceae*	Gut bacteria in individuals with a protein-rich diet	Succinic, acetic and propionic acids from peptone yeast glucose (PYG) medium.	[248,261,262]
Aminobacterium	*Synergistaceae*	Isolated from protein-rich anaerobic dairy wastewater lagoon.	Acetic acid from serine, glycine and threonine (in pure cultures). Acetic acid from alanine and aspartate, propionic acid from glutamate, isobutyric acid from valine, isovaleric acid from leucine and 2-methylbutyrate from isoleucine (in mixed cultures)	[245–247]
Flavonifractor	*Ruminococcaceae*	Human gut microbiota in individuals with a rich-fiber diet.	Propionic and butyric acids	[263]
Peptostreptococcus	*Peptostreptococcaceae*	Isolated from a swine-manure storage pit	Butyric acid from PYG medium	[249,264]
Sporanaerobacter	*Clostridiales Incertae Sedis XI*		Acetate, H_2 and CO_2 from glucose. Valeric and acetic acids from isoleucine and glycine (Stickland reaction). Valeric and propionic acids from isoleucine and serine (Stickland reaction).	[253]
Pyramidobacter	*Synergistaceae*	Found in the distal colon where the protein degradation takes place.	Acetic and isovaleric acids and in a lesser extent propionic, isobutyric, succinic and phenylacetic acids from PYG medium.	[244,265,266]
Odoribacter	*Porphyromonadaceae*		Succinic, acetic and isovaleric acids from peptone yeast extract (PY) or PYG medium.	[267]
Saccharicrinis	Marinilabiliaceae		Acetic, succinic and propionic acids.	[268]
Sphaerochaeta	*Spirochaetaceae*		Acetic acid, formic acid and ethanol.	[269]
Bifidobacterium	*Bifidobacteriaceae*	Gastrointestinal tract of mammals and some insects (carbohydrate degraders)	Acetic and lactic acids from monosaccharides (bifid shunt pathway).	[220,221]
Ethanoligenens	*Ruminococcaceae*	Isolated from molasses wastewater	Ethanol, acetic acid and hydrogen from carbohydrates.	[223,224,270]
Clostridium sensu stricto	*Clostridiaceae 1*		Butyric and acetic acids from monosaccharides and butyric acid from lactic acid (in presence of acetic acid) (*Clostridium tyrobutyricum*) Isocarboxylic acids (*Clostridium luticellarii*)	[227,228]
Clostridium IV	*Ruminococcaceae*	Human gut	Acetic and butyric acids	[229]

Firstly, acetic acid was a majoritarian acid produced with all the tested substrate composition. When the influent was composed only by carbohydrates (100%C), acetic acid might be produced by three of the main detected genera (Figure 6.4): *Bifidobacterium*, *Ethanoligenens* and *Clostridium sensu stricto*, all of them previously described as acetic acid producers (Table 6.5). In the rest of the tested substrate compositions, there were several genera that might be responsible of acetic acid production: *Pyramidobacter*, *Odoribacter*, *Saccharicrinis*, *Sphaerochaeta*, *Alistipes*, *Sporanaerobacter, Aminobacterium* and *Petrimonas* (Table 6.5). For each of the substrate compositions tested, with a carbohydrate content between 0 and 75%, at least three of the aforementioned genera were present in the reactor (Figure 6.4).

Butyric acid was the other SCCA that was produced with all the tested substrate compositions. Its production can be linked to the bacterial communities for each substrate composition as follows: (i) For a substrate composed only by carbohydrates (100%C), genera *Clostridium sensu stricto* and *Clostridium IV* were detected (Figure 6.4) and members of these genera were described as butyric acid producers (Table 6.5). (ii) For a substrate composed by 75%C/25%P, genus *Flavonifractor* was detected in a significant amount (Figure 6.4) and members of this genus were described as a butyric acid producer (Table 6.5). (iii) For a substrate composed by 50%C/50%P, none of the genus detected in the reactor with a relative abundance over the 2% (Figure 6.4), were reported to produce butyric acid. Therefore, butyric acid might be produced from several genera with a relative abundance lower than the 2% (which comprise in total the 16% of the relative abundance) or by the bacterial communities unclassified at genus level (6% of the relative abundance). (iv) For a substrate composed by 25%C/75%P, the family *Porphyromonadaceae* was found (Figure 6.3) although it was not identified at genus level in a large extent. However, genus *Butyricimonas*, comprised in the family *Porphyromonadaceae*, was described as butyric acid producer [271] and it might be responsible of the production of butyric acid at this composition. Finally, (v) for a substrate composed only by proteins (100%P), three of the detected genera, *Peptostreptococcus*, *Flavonifractor* or *Clostridium IV* (Figure 6.4), include species that were previously described as butyric acid producers (Table 6.5).

Propionic acid was another of the majoritarian acids present in the effluent for all the tested substrate compositions except of a substrate comprised only by carbohydrates (100%C). Among the genera identified in this study, *Pyramidobacter*, *Saccharicrinis*,

Alistipes, *Sporanaerobacter, Aminobacterium* and *Petrimonas* include species that were reported to produce propionic acid (Table 6.5). For each of the tested substrate compositions with a carbohydrate content between 0 and 75%, at least three of the aforementioned genera were present in the reactor (Figure 6.4).

Isovaleric acid was one of the majoritarian acids present in the effluent when the substrate was composed by 25%C/75%P or 100%P. Its production can be linked to the bacterial communities for both substrate composition as follows: (i) For a substrate composed by 25%C/75%P, genera *Pyramidobacter* and *Odoribacter* were detected (Figure 6.4) and some species of these genera were reported to produce isovaleric acid (Table 6.5). (ii) For a substrate composed by 100%P, genus *Aminobacterium* was identified (Figure 6.4) and some species of this genus were reported to produce isovaleric acid from leucine (Table 6.5). Leucine accounts for the 11% (in molar basis) of the whey protein isolate used in the influent as model protein (Figure S5.5 in Appendix II), so *Aminobacterium* might be responsible of isovaleric production.

Isobutyric and isocaproic acids were part of the majoritarian components of the effluent only when the substrate was exclusively composed by proteins (100%P). Regarding the isobutyric acid, among the identified genera, some species of the genus *Aminobacterium* were reported to produce isobutyric acid from valine (Table 6.5). Valine accounts for the 7% (in molar basis) of the whey protein isolated used as carbon source Figure S5.5 in Appendix II), so *Aminobacterium* might be responsible of isobutyric acid production. With respect to the isocaproic acid, none of the genera identified in proportions higher than the 2% was reported to produce isocaproic acid. In fact, to the best of our knowledge, there are very few species that have been identified as isocaproic acid producers, such as *Clostridium difficile* and *Clostridium botulinum* [250,251]. More research to identify isocaproic acid producing bacteria is needed.

Finally, ethanol was only found when the substrate was only made up by carbohydrates (100%C) and, as explained in Chapter 4 (Section 4.3.1), *Ethanoligenens* might be the responsible genus of its production (Table 6.5).

6.4 Potential applications of the acidogenic fermentation effluents

In this section, the potential use of the effluents obtained from the different substrate composition tested is analyzed. The effluent composition changed notably with the influent organic composition (Figure 6.2). As stated before, the higher the proportion of protein, the lower selectivity of the reactions taking place for a specific compound. Then, when increasing the protein proportion in the influent, the number of components of the effluent increases and their proportions tend to be more balanced. Consequently, the potential applications for each effluent can be different.

Regarding the use of any of the effluents for polyhydroxyalkanoates (PHA) synthesis, the effluent yielded when the influent was only composed by carbohydrates would be appropriate for polyhydroxybutyrate (PHB) production. As explained in Chapter 4 (Section 4.4), this effluent has a large proportion of butyric acid, which has been reported as one of the more energetically favourable substrates for PHA production [24,236,237]. However, the resultant odd-to-even ratio was very low (almost 0) due to the absence of propionic and valeric acids, so this effluent would lead mainly to PHB production. PHB has worse mechanical properties than other polymers such as poly(hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV), that have incorporated a 3-hydroxyvalerate monomer [1,29]. Propionic acid is one of the precursors that can be used to synthetize 3-hydroxyvalerate (3HV) [256]. The effluents obtained with carbohydrate proportions between 0% and 75%, despite having lower proportions of butyric acid, would be more convenient for PHB-co-PHV synthesis due to their larger propionic acid proportions (Figure 6.2). Among these effluents, the more convenient would be the ones obtained with substrates composed by 25%C/75%P and 75%C/25%P since they led to the highest odd-to-even ratio (around 0.6) (Table 6.2). Nevertheless, between both, the effluent obtained with the substrate composed by 75%C/25%P would be more appropriate due to its larger proportion of butyric acid (Figure 6.2).

As explained in detail in Chapter 1 (Section 1.5.1.), for medium chain carboxylic acids (MCCA) synthesis, an electron donor (ED) and an electron acceptor (EA) are needed. Ethanol and lactic acid have been pointed out as the most convenient EDs, since they provide enough energy (in form of ATP) and reducing equivalents to support MCCA synthesis [187]. In this case, the production of lactic acid was negligible for all the substrate compositions tested and ethanol was only detected when the substrate was only

composed by carbohydrates (100%C) (Figure 6.2). The effluent obtained with an influent only containing food-grade sugar as carbon source was mainly formed by three components: butyric acid, acetic acid and ethanol (Figure 6.2). Therefore, this effluent would be appropriate for producing caproic acid, since both acetic and butyric acids can act as EA and ethanol as ED in the reverse β-oxidation (RBO) cycle [187]. As mentioned in Chapter 4 (Section 4.4.), the ED:EA ratio when using acetic acid as EA and ethanol as ED is still being discussed. Some authors reported a ratio ED:EA of 1:1 in terms of maximizing the MCCA production yield [194], while other authors found the ratio 4:1 as the optimal in terms of MCCA production and selectivity [239,240]. In any case, the ED:EA carbon molar ratio of the effluent obtained when the reactor was fed only with carbohydrates (considering only the acetic acid in the calculation) was 0.38, so additional ethanol might be provided externally to produce caproic acid.. The effluents obtained with a substrate containing a carbohydrate proportion between 0 and 75% were mixtures of several carboxylic acids that could act as EA. Therefore, they can also be used as feedstock in a chain elongation process if the ED is provided externally. However, the effluent obtained might be a mixture of MCCAs with less selectivity for the caproic acid due to the varied EAs present in the feedstock.

6.5 Conclusions

In this study, a SBR was used to perform acidogenic fermentation experiments starting from a substrate composed by mixtures with different proportions of food-grade sugar and whey protein isolate as carbohydrate and protein model compounds, respectively. From the experimental work, we discussed the effect of the organic composition of the influent on the process performance.

When comparing the acidogenic fermentation effectiveness parameters and the effluent and the biomass composition for substrates composed by 100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C, it was observed the following:

- The bioconversion was over the 90% for all the influent compositions tested.
- The highest degree of acidification and acidified COD were obtained when the substrate was composed by 75%C/25%P and 25%C/75%P. The maximum CA production yield (0.7) was obtained with substrates composed by mixtures of carbohydrates and proteins (25%C/75%P, 50%C/50%P and 75%C/25%P). In

general, using mixtures of carbohydrates and proteins as substrates benefits CA formation over the use of substrates formed predominantly by carbohydrates or by proteins.

- The higher the proportion of proteins in the influent, the higher the number of organic components in the effluent (and, consequently the lower selectivity).
- Isocaproic acid was produced when the protein proportion in the feed was higher than the 75% without the addition of an electron donor.
- Branched acids (isobutyric, isovaleric and isocaproic acids) proportion was increased when increasing the protein proportion over the 50%.
- It was possible to find a linear correlation between the concentration of three of the six SCCAs (isobutyric, butyric and isovaleric acids) with the substrate composition. Under fixed OLRv and OLRs, isobutyric and isovaleric acids were directly proportional to the protein concentration in the substrate and butyric acid was directly proportional to the carbohydrate concentration in the substrate.
- The odd-to-even ratio was maximum (around 0.6) for the substrates composed by 25%C/75%P and 75%C/25%P, which led to effluents with a higher propionic proportion in comparison to the effluents obtained with other substrate compositions. Between these two effluents, the one obtained with a substrate composition of 75%C/25%P would be the more suitable for PHB-co-PHV production due to its higher proportion of butyric acid.
- The effluent obtained with a substrate composed only by carbohydrates was appropriate for caproic acid bioproduction since it was mainly comprised by acetic acid, butyric acid and ethanol.
- The influent organic composition had a great impact on the resultant biomass composition. The highest microbial diversity at family and genus level was found when the influent was composed by a mixture of carbohydrates and proteins.
- In general, it was possible to link the different genera present in the reactor with the effluent components obtained with the different substrate organic compositions tested.

Chapter 7:

General conclusions

Biological synthesis of carboxylic acids (CAs), including short chain carboxylic acids (SCCAs) and medium chain carboxylic acids (MCCAs), by acidogenic fermentation using microbial mixed cultures is gaining attention in the last years. CAs are value-added products and their obtention from waste or wastewater by acidogenic fermentation would avoid the use of the traditional fossil fuels-based production processes. Nevertheless, acidogenic fermentation is a complex process and there is still scarce information about how to control the effluent composition and the resultant CA production yield. Therefore, deeper understanding of the acidogenic fermentation is necessary to implement the process at large scale. This thesis aimed to contribute to go beyond current knowledge of the acidogenic fermentation process. For that reason, it started from a literature review of the state-of-the-art (Chapter 1) to identify the research gaps, which were covered at least partially in the remaining chapters, as explained in the following paragraphs:

1) *Establishment of standard parameters to quantify both SCCA production yield and SCCA composition.*

When doing the literature review, it was noticed that different authors used different ways to quantify the yields of SCCA production, making very difficult to compare results of the different studies. So, in this thesis, five effectiveness parameters were proposed for evaluating the process performance: bioconversion, degree of acidification, acidified COD, CA production yield and odd-to-even ratio. In Chapter 3 (Section 3.5.3), these parameters were defined and the utility of each of them was explained in detail. After using them in the present study, we can assess the convenience of each of them: (i) Bioconversion is a parameter that can inform of substrate hydrolysis limitations. In our study, carbohydrate and protein model compounds were used as substrate and hydrolysis limitations did not occur but it was useful when comparing with other studies where substrate consumption was limited. (ii) The degree of acidification and acidified COD measure the produced CAs in relation to the available organic matter in the substrate and the total organic matter content of the effluent, respectively. (iii) The CA production yield informs of the CAs produced per amount of substrate consumed, so it is useful to quantify how much substrate is being used for CA formation. The degree of acidification, the acidified COD and the CA production yield allow us to compare the CA production efficiency and the CA content in the effluents when using different substrates and different operational conditions. (iv) Finally, the odd-to-even ratio is a widely used

parameter to measure the quality of the effluents to be used in PHA production. This parameter was used to choose which of the effluents obtained were more appropriate for being utilized as feedstock in PHA synthesis processes. The use of all these effectiveness parameters as standard parameters in further studies would make easier to compare results, and consequently, to generate more knowledge of the process.

2) *Study of the specific organic loading rate (OLRs) as another parameter that can affect both SCCA production yield and SCCA composition.*

One limitation we also observed from the literature review was that authors rarely consider the effect of the ratio between substrate and biomass concentration in the acidogenic fermentation performance, specially in those studies that do not involve solid wastes. OLRs was proposed as a parameter to measure the impact of the ratio between the substrate and biomass concentration in long term fermentation reactors. It was hypothesized that OLRs could affect the acidogenic fermentation performance. In fact, we detected that increasing the OLRs when the reactor was fed by food-grade sugar as sole carbon source led to an increase of caproic acid concentration since the organic overloading favoured chain elongation reactions. When increasing the OLRs in the reactor fed by whey protein isolate as sole carbon source led to a decrease in the non-identified fraction of the effluent due to the increase of the concentration of CAs in the effluent. Moreover, we found previous studies starting from protein model compounds that achieved values of bioconversion and degree of acidification considerably lower to the ones attained in this study under similar pH conditions and with a similar substrate. In those works, the applied OLRs was 3 to 6.5 times higher than the maximum OLRs tested in our study and that might have caused substrate consumption limitations that lead to lower bioconversion and degree of acidification. In conclusion, we consider that OLRs is a parameter that must be taken into consideration in acidogenic fermentation processes.

3) *Further study of the SCCA composition obtained from different substrates regarding their organic components rather than considering it only as a concrete type of substrate.*

There were very few previous works analyzing the effect of the organic composition of the substrate (in terms of carbohydrates, proteins and lipids) on the resultant effluent composition. In the present study, we analyzed in depth the influence of the operational parameters, namely volumetric organic rate (OLRv) and OLRs, on the acidogenic fermentation performance, effluent and biomass composition when the reactor was fed

only by carbohydrate (C) or protein (P) model compounds. It was observed that the effect of these two parameters on the process performance was different depending on the carbon source used. When the reactor was fed by an influent containing only food-grade sugar, the effluent was mainly formed by two components that changed from butyric and acetic acid to acetic acid and ethanol by increasing the OLRv. However, the effluent obtained from the reactor fed by an influent only formed by whey protein isolate was comprised by six CAs, whose composition was almost unaltered by the OLRv. Moreover, under fixed OLRv and OLRs conditions, five substrate organic compositions were tested, showing that the influent organic composition strongly impacted the effluent composition. The higher the protein proportion, the higher the branched acids content in the effluent and the higher the number of compounds in the effluent. These results emphasize the need for considering the organic composition of the substrate instead of regarding it only as a specific type of substrate even when using real wastes or wastewaters, as the effluent composition obtained depends, among other parameters, on the organic composition of the substrate.

4) *Further analysis of the microbial communities to link the different genera to the different SCCAs obtained.*

In the present study, biomass samples from the reactor were taken for the different steady states achieved. In all the experiments, the bacterial communities present at family and genus level under the different conditions were analyzed and it was possible to link each of the effluent components to the reported metabolism of one or more of the genera present in the reactor. The only compounds that it was not possible to link to the metabolism of any genera were caproic and isocaproic acids, since there are very few bacteria reported that can produce them. Further research is needed to identify caproic and isocaproic producing bacteria. It was also difficult to find the metabolic products of some genera when the substrate was proteinaceous. The metabolism of the different genera starting from proteins or amino acids requires to be more deeply explored.

Besides the research gaps addressed in this thesis, the most relevant conclusions obtained from the experimental work presented in Chapters 4, 5 and 6 are detailed below.

In **Chapter 4**, fermentation experiments in a sequencing batch reactor (SBR) fed by simulated wastewater composed only by food-grade sugar were performed to study the individual impact of the OLRv, OLRs and influent carbon-to-nitrogen ratio on the process

performance, effluent composition and biomass composition. Among the three parameters tested, the OLRv was the one that impacted more the process performance.

The effect of increasing the OLRv from 6.0 to 11.0-15.8 g COD L^{-1} d^{-1} (at an around 2 g COD g^{-1} VSS d^{-1} and the influent carbon-to-nitrogen ratio at 12) was the following:

- Bioconversion did not change significantly and remained over 99%.
- The degree of acidification, acidified COD and the CA production yield significantly decreased since the main effluent components changed from a mixture of butyric and acetic acid to a mixture of acetic acid and ethanol.
- Odd-to-even ratio significantly increased since butyric acid decreased and propionic acid increased.

The most relevant effect of increasing the OLRs from 2.3 up to 3.8 g COD g^{-1} VSS d^{-1} (at an OLRv of 16 g COD L^{-1} d^{-1} and the influent carbon-to-nitrogen ratio of 12) was that chain elongation reactions were favoured and caproic acid composition was increased.

Finally, the increase of the influent carbon-to-nitrogen ratio from 12 to 25 (at an OLRv of 16 g COD L^{-1} d^{-1} and a OLRs of 3.8 g COD g^{-1} VSS d^{-1}) had almost no impact on acidogenic process performance.

Regarding the bacterial communities present, under all the conditions tested there were four main genera: *Bifidobacterium*, *Ethanoligenens*, *Clostridium IV* and *Clostridium sensu stricto.* It was possible to link the main effluent components to the metabolism of the different genera present.

In **Chapter 5**, fermentation experiments in SBR fed by simulated wastewater composed only by whey protein isolate were performed to study the individual impact of the OLRv and OLRs on the process performance, effluent composition and biomass composition. The OLRv and OLRs had low impact on the process performance when the carbon source was whey protein isolate.

Under all the conditions tested, the bioconversion, the degree of acidification and the CA production yield did not change significantly, and their values were around 99%, 63%, 83% and 0.6, respectively. The effluent has a very similar composition under all the conditions tested and it was formed by a balanced proportion of acetic, isovaleric, propionic, butyric, isocaproic and isobutyric acids.

When increasing the OLRv from 6.5-12.5 to 18.5 g COD L^{-1} d^{-1} (at an OLRs of 1.9 g COD g^{-1} VSS d^{-1}) the only relevant change was the increase of isocaproic acid composition. At the highest OLRv, it was reached the maximum reported isocaproic acid concentration obtained in a mixed culture reactor without the addition of any external electron donor (ED). Future research might consider to optimize the operational conditions that maximize the isocaproic acid production without the addition of any external ED to reduce the operational costs in a large scale application when starting from real protein-rich wastes or wastewater.

By increasing the OLRs from 1.9 to 3.3 g COD g^{-1} VSS d^{-1} (at an OLRv of 18.5 g COD L^{-1} d^{-1}), propionic, isobutyric, butyric and valeric acids were significantly increased leading to a effluent with lower proportion of non-identified compounds and a higher odd-to-even ratio.

The main genera detected in the reactor when the substrate was whey protein isolate were *Petrimonas*, *Pyramidobacter*, *Aminobacterium*, *Alistipes* and *Peptostreptococcus.* It was possible to link the main effluent components (except for isocaproic acid) to the metabolism of the different genera present.

In **Chapter 6**, fermentation experiments in SBR fed by simulated wastewater composed by mixtures of food-grade sugar and whey protein isolate in different ratios were performed under fixed conditions of OLRv and OLRs and it was observed that the resultant process effectiveness parameters, effluent composition and biomass composition were strongly impacted by the substrate organic composition.

It can be concluded that substrates containing both carbohydrates and proteins benefit CA formation in comparison to using substrates only formed by carbohydrates and proteins since higher CA yields were obtained. Since the substrates were structurally simple, the bioconversion remained over the 90% for all the influent composition tested.

Regarding the effluent composition, increasing the protein proportion in the substrate, increased the number of compounds and the branched CA proportion in the effluent. It was found a linear correlation for the composition of three CAs with the substrate composition: isobutyric and isovaleric acids were directly proportional and butyric acid was directly proportional to the carbohydrate proportion (%) in the substrate.

Isocaproic acid was produced when the protein proportion was higher than the 75% in the influent without the addition of an ED.

The influent organic composition had a great impact on the resultant biomass composition. It was possible to link the different genera present in the reactor with the effluent components obtained with the different substrate organic compositions tested except for isocaproic acid.

Finally, general conclusions extracted from the data generated in all the experimental part of this thesis are stated:

- The degree of acidification, acidified COD, CA yield obtained with a substrate only composed by whey protein isolate were higher than the obtained with a substrate only formed by food-grade sugar under all the conditions of OLRv and OLRs tested.
- Modifying the OLRv when the substrate was only formed by carbohydrates or changing the carbohydrate and protein proportions seem the best strategies to alter the effluent composition. Thus, the aforementioned parameters can be tuned to obtain the desired effluent composition depending on the subsequent application of these effluents.
- Ethanol was only obtained when the influent was only formed by carbohydrates and it was produced at all the conditions tested.
- It was demonstrated that SBR is an ideal reactor configuration to perform acidogenic fermentation of influents composed by simple carbohydrates and/or simple proteins since even using a flocculent biomass, good settleability was attained.
- The SBR is also a convenient configuration to carry acidogenic fermentation processes since the biomass is retained and the OLRs, which is a key parameter as demonstrated in this study, can be more easily tuned in comparison to a chemostat reactor.
- Further research is needed to identify MCCA-producing genera and to understand the acidogenic metabolism of the genera involved in protein degradation.

Chapter 8:

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Appendix I:

Supporting Information for Chapter 4

The methodology used to perform statistical tests to compare the process effectiveness parameters and the effluent composition obtained at the different OLRv, OLRs and influent carbon-to-nitrogen ratio is presented in detail in Chapter 3. Section 3.5.4 can be consulted for better understanding of Tables S4.2 to S4.7.

Table S4.1. Effluent composition (mg O_2 L^{-1}) in the different conditions tested in the reactor when using food-grade sugar as sole carbon source. ND: Non-Detectable.

OLRv (g COD L^{-1} d^{-1})	**6.0±0.3**	**11.0±0.6**	**15.8±0.7**	**17.6±1.1**	**17.3±0.8**
OLRs (g COD g^{-1} VSS d^{-1})	**2.2±0.2**	**1.9±0.2**	**2.3±0.3**	**3.8±0.9**	**3.4±0.6**
Influent carbon-to-nitrogen ratio	**12**	**12**	**12**	**12**	**25**
Total COD in the effluent (mg O_2 L^{-1})	10886±502	9644±660	9577±445	9825±695	9868±500
Acetic acid (mg O_2 L^{-1})	2898±356	2163±521	2157±97	2181±579	2189±425
Propionic acid (mg O_2 L^{-1})	255±93	655±69	429±32	442±145	736±135
Isobutyric acid (mg O_2 L^{-1})	93±65	815±240	499±70	433±153	440±14
Butyric acid (mg O_2 L^{-1})	4912±185	1339±478	1063±118	1295±83	1450±266
Isovaleric acid (mg O_2 L^{-1})	ND	ND	ND	ND	23±21
Valeric acid (mg O_2 L^{-1})	ND	267±86	173±6	326±48	422±65
Isocaproic acid (mg O_2 L^{-1})	ND	ND	ND	ND	ND
Caproic acid (mg O_2 L^{-1})	ND	ND	ND	634±254	494±218
Glucose (mg O_2 L^{-1})	6±8	0.9±1.6	ND	1.1±1.9	ND
Lactic acid (mg O_2 L^{-1})	92±134	3±4	11±16	7±3	8±7
Ethanol (mg O_2 L^{-1})	854±232	2319±417	2904±250	1353±249	1748±237
Food-grade sugar (mg O_2 L^{-1})	82±5	119±23	90±8	456±6	465±2

Table S4.2. ANOVA test and Tukey's multiple comparisons test for comparing the resultant process effectiveness parameters at the different OLRv tested (6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}) at an OLRs of approximately 2 g COD g^{-1} VSS d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Brown-Forsythe test (to compare standard deviations)	**ANOVA test (for equal standard deviations)**	**Brown-Forsythe ANOVA test (for different standard deviations)**	**Tukey's multiple comparisons test**		
	p value	**p value**	**p value**	**p value**		
				OLRv 6.0 and 11.0 g COD L^{-1} d^{-1}	**OLRv 6.0 and 15.8 g COD L^{-1} d^{-1}**	**OLRv 11.0 and 15.8 g COD L^{-1} d^{-1}**
Bioconversion (%)	0.2233	0.0066	-	0.0059	0.4293	0.1230
Degree of Acidification (%)	0.3485	0.0001	-	0.0010	0.0001	0.0714
Acidified COD (%)	0.3577	0.0002	-	0.0022	0.0002	0.0424
CA production yield (g COD g^{-1} COD)	0.3013	0.0002	-	0.0013	0.0002	0.0780
Odd-to-even ratio	0.1529	<0.0001	-	<0.0001	0.0002	0.1042

Table S4.3. t-test for comparing the resultant process effectiveness parameters at the different OLRs tested (2.3 and 3.8 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	P value (F test to compare variances)	**P value (t-test considering same standard deviations)**	**P value (t-test using Welch's correction to compare mean values)**
Bioconversion (%)	0.6642	<0.0001	-
Degree of Acidification (%)	0.0273	-	0.6239
Acidified COD (%)	0.0318	-	0.1746
CA production yield (g COD g^{-1} COD)	0.0182	-	0.4798
Odd-to-even ratio	0.0177	-	0.2916

Table S4.4. t-test for comparing the resultant process effectiveness parameters at different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	P value (F test to compare variances)	**P value (t-test considering same standard deviations)**	**P value (t-test using Welch's correction to compare mean values)**
Bioconversion (%)	0.6595	0.1549	-
Degree of Acidification (%)	0.8201	0.2912	-
Acidified COD (%)	0.4540	0.1904	-
CA production yield (g COD g^{-1} COD)	0.7842	0.3181	-
Odd-to-even ratio	0.2500	0.1370	-

Table S4.5. ANOVA test and Tukey's multiple comparisons test for comparing the effluent composition obtained at different OLRv tested (6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}) at an OLRs of approximately 2 g COD g^{-1} VSS d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Brown-Forsythe test (to compare standard deviations)	**ANOVA test (for equal standard deviations)**	**Brown-Forsythe ANOVA test (for different standard deviations)**	**Tukey's multiple comparisons test**		
	p value	**p value**	**p value**	**p value**		
				OLRv 6.0 and 11.0 g COD L^{-1} d^{-1}	**OLRv 6.0 and 15.8 g COD L^{-1} d^{-1}**	**OLRv 11.0 and 15.8 g COD L^{-1} d^{-1}**
% Acetic acid	0.0984	0.2313	-	-	-	-
% Propionic acid	0.2084	<0.0001	-	<0.0001	0.0169	0.0026
% Isobutyric acid	0.0872	0.0003	-	0.0002	0.0422	0.0722
% Butyric acid	0.3071	<0.0001	-	<0.0001	<0.0001	0.4359
% Isovaleric acid	-	-	-	-	-	-
% Valeric acid	0.0042	-	0.0002	0.0002	0.0172	0.1409
% Isocaproic acid	-	-	-	-	-	-
% Caproic acid	-	-	-	-	-	-
% Glucose	<0.0001	-	0.2338	-	-	-
% Lactic acid	0.1971	0.1459	-	-	-	-
% Ethanol	0.6658	<0.0001	-	<0.0001	<0.0001	0.1150
% Food-grade sugar	0.2402	0.0060	-	0.0061	0.5663	0.0805

Table S4.6. t-test for comparing the effluent composition obtained at different OLRs tested (2.3 and 3.8 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	P value (Brown-Forsythe test to compare standard deviations)	**P value (t-test considering same standard deviations)**	**P value (t-test using Welch's correction to compare mean values)**
% Acetic acid	0.0263	-	0.5459
% Propionic acid	0.0482	-	0.7680
% Isobutyric acid	0.2962	0.2375	-
% Butyric acid	0.8004	0.1769	-
% Isovaleric acid	-	-	-
% Valeric acid	0.0371	-	0.0628
% Isocaproic acid	-	-	-
% Caproic acid	<0.0001	-	0.0189
% Glucose	<0.0001	-	0.4226
% Lactic acid	0.0652	0.6578	-
% Ethanol	0.5642	0.0007	-
% Food-grade sugar	0.2645	<0.0001	-

Table S4.7. t-test for comparing the effluent composition obtained at different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	P value (F test to compare variances)	**P value (t-test considering same standard deviations)**	**P value (t-test using Welch's correction to compare mean values)**
% Acetic acid	0.6490	0.6990	-
% Propionic acid	0.8266	0.0234	-
% Isobutyric acid	0.0211	-	0.7167
% Butyric acid	0.2643	0.3477	-
% Isovaleric acid	<0.0001	-	0.1885
% Valeric acid	0.9895	0.1091	-
% Isocaproic acid	-	-	-
% Caproic acid	0.7728	0.5715	-
% Glucose	<0.0001	-	0.4226
% Lactic acid	0.4000	0.9367	-
% Ethanol	0.4974	0.0777	-
% Food-grade sugar	0.9113	0.3096	-

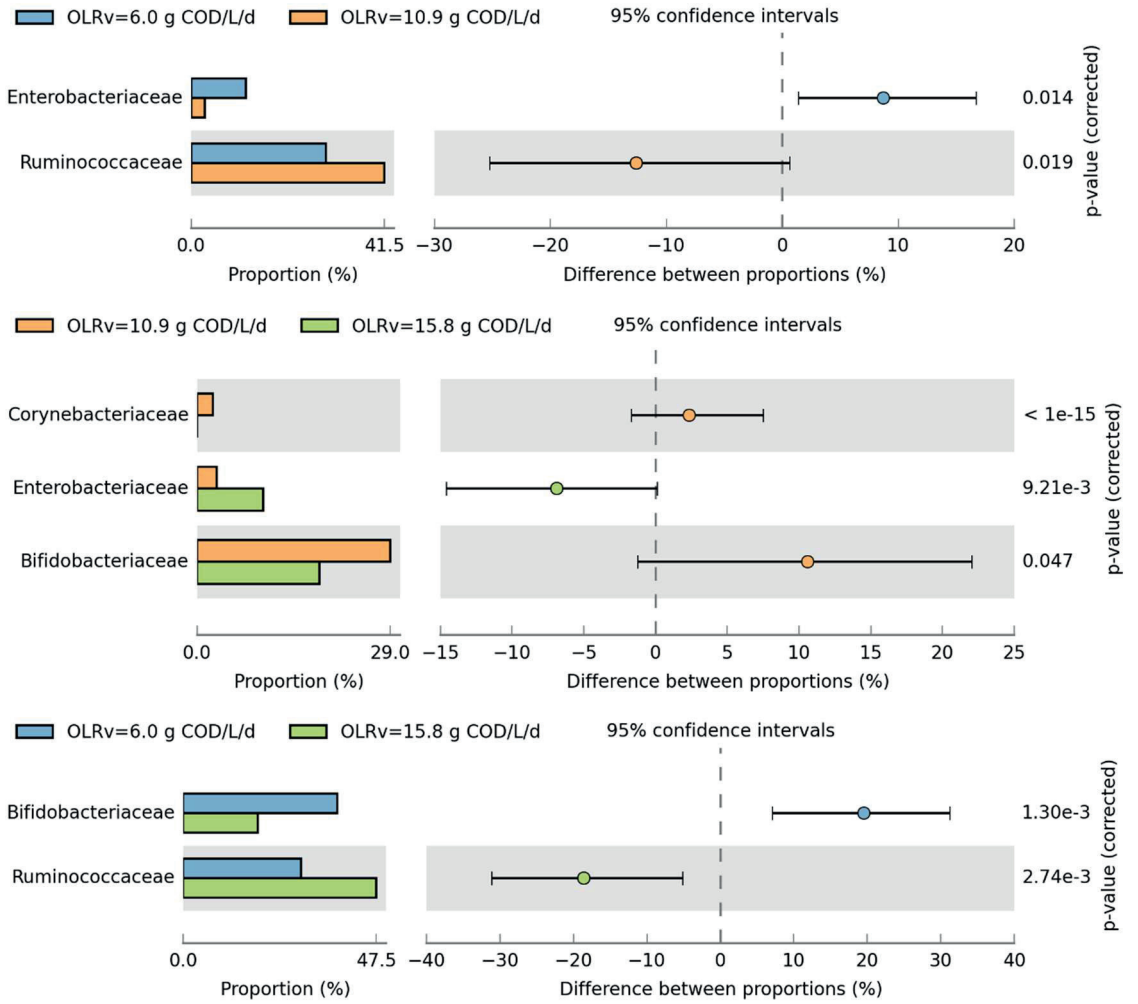

Figure S4.1. Fisher's exact test for comparison of bacterial communities relative abundance at family level at different OLRv tested (6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}) at an OLRs of approximately 2 g COD g^{-1} VSS d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

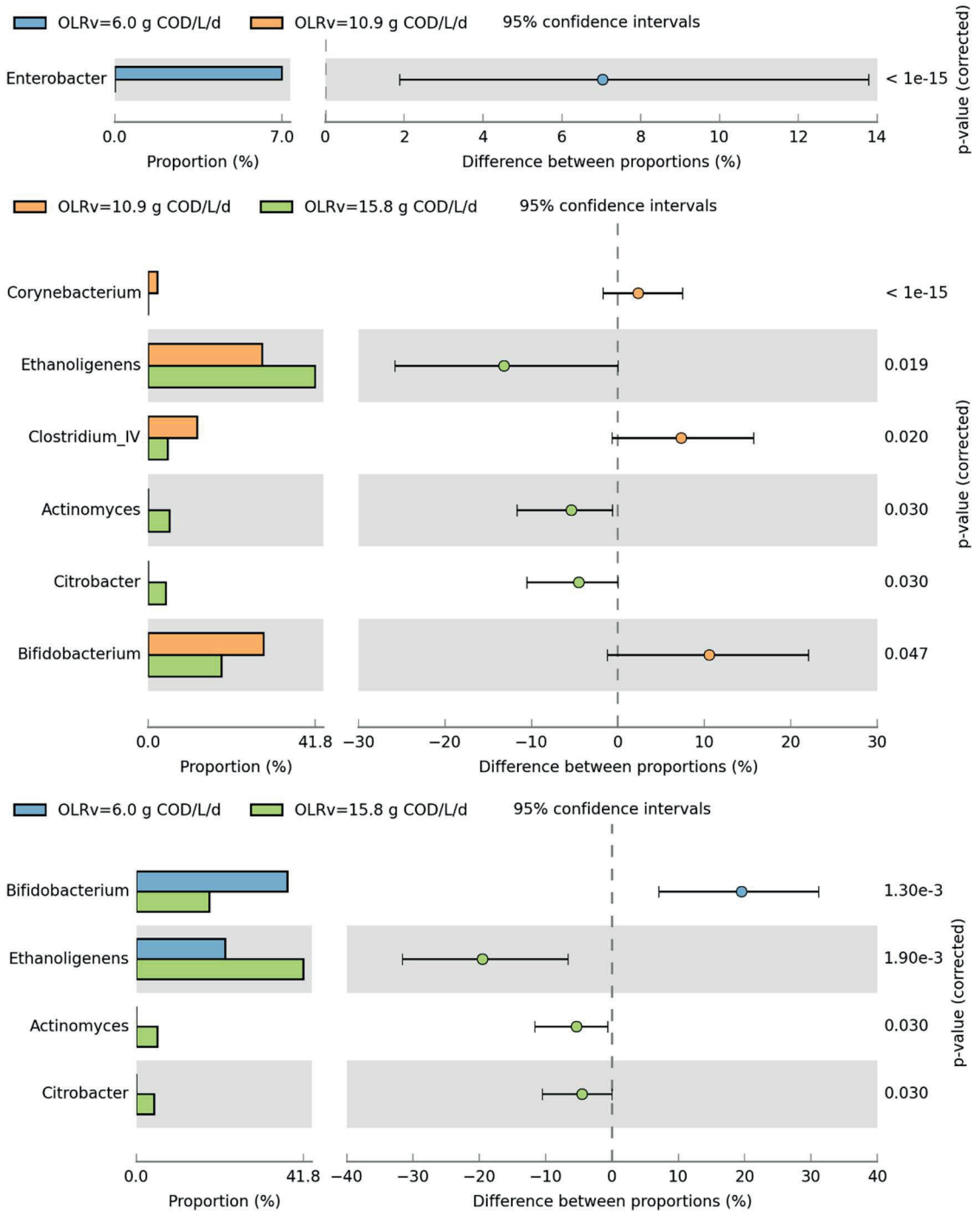

Figure S4.2. Fisher's exact test for comparison of bacterial communities relative abundance at genus level at different OLRv tested (6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}) at an OLRs of approximately 2 g COD g^{-1} VSS d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

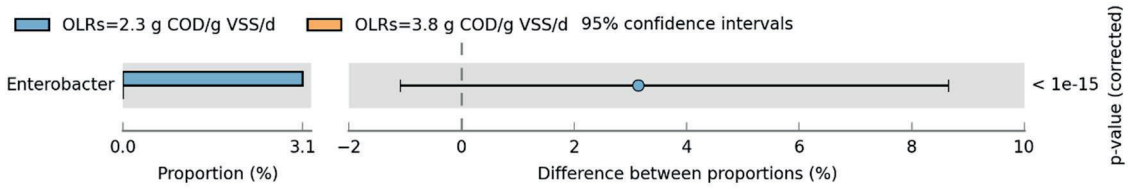

Figure S4.3. Fisher's exact test for comparison of bacterial communities relative abundance at genus level at different OLRs tested (2.3 and 3.8 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source.

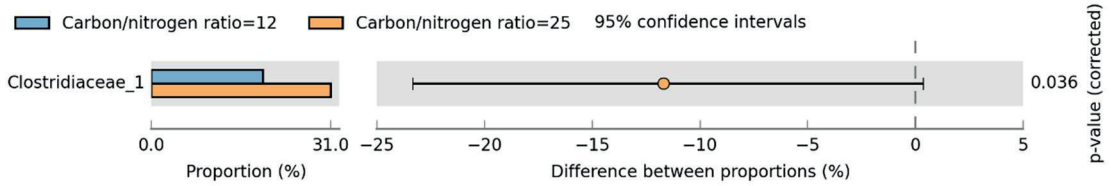

Figure S4.4. Fisher's exact test for comparison of bacterial communities relative abundance at family level at different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source.

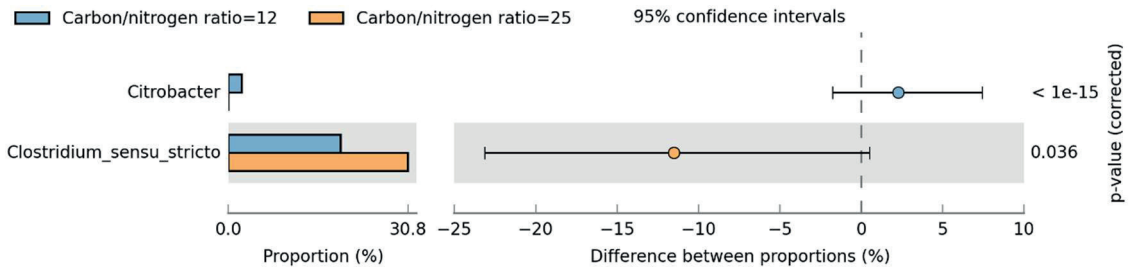

Figure S4.5. Fisher's exact test for comparison of bacterial communities relative abundance at genus level at different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source.

Table S4.8. Shannon index and p values for t-test statistical comparison of the bacterial diversity under the effect of the different OLRv tested (6.0, 11.0 and 15.8 g COD L^{-1} d^{-1}) at an OLRs of approximately 2 g COD g^{-1} VSS d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Shannon index			**p values (Diversity t test)**		
	OLRv (g COD L^{-1} d^{-1})			**OLRv (g COD L^{-1} d^{-1})**		
	6.0	**11.0**	**15.8**	**6.0 and 11.0**	**6.0 and 15.8**	**11.0 and 15.8**
Family level	1.5109	1.5116	1.4809	0.9949	0.7772	0.7894
Genus level	1.6822	1.6858	1.7637	0.9704	0.8305	0.4885

Table S4.9. Shannon index and p values for t-test statistical comparison of the bacterial diversity under the effect of the different OLRs tested (2.3 and 3.8 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and with an influent carbon-to-nitrogen ratio of 12 using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Shannon index		**p values (Diversity t test)**
	OLRs (g COD g^{-1} VSS d^{-1})		
	2.3	**3.8**	
Family level	1.4809	1.4131	0.5382
Genus level	1.7637	1.5536	0.0845

Table S4.10. Shannon index and p values for t-test statistical comparison of the bacterial diversity under the effect of the different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Shannon index		**p values (Diversity t test)**
	Influent carbon-to-nitrogen ratio		
	12	**25**	
Family level	1.4131	1.5216	0.2857
Genus level	1.5536	1.6874	0.2300

Appendix II:

Supporting Information for Chapter 5

The methodology used to perform statistical tests to compare the process effectiveness parameters and the effluent composition obtained at the different OLRv and OLRs is presented in detail in Chapter 3. Section 3.5.4 can be consulted for better understanding of Tables S5.2 to S5.5.

Table S5.1. Effluent composition (mg O_2 L^{-1}) in the different conditions tested in the reactor when using whey protein isolate as sole carbon source. ND: Non-Detectable.

OLRv (g COD L^{-1} d^{-1})	**6.5±0.6**	**12.5±0.6**	**18.5±0.3**	**18.6±0.4**
OLRs (g COD g^{-1} VSS d^{-1})	**1.9±0.4**	**1.8±0.4**	**1.9±0.1**	**3.3±0.3**
Total COD in the effluent (mg O_2 L^{-1})	11376±539	11209±550	10916±526	10093±737
Acetic acid (mg O_2 L^{-1})	2094±233	1681±131	2106±443	1781±420
Propionic acid (mg O_2 L^{-1})	1528±111	1617±418	1243±63	1490±55
Isobutyric acid (mg O_2 L^{-1})	1037±105	787±156	807±30	875±19
Butyric acid (mg O_2 L^{-1})	1704±118	1382±380	1289±252	1517±159
Isovaleric acid (mg O_2 L^{-1})	1956±74	1635±359	1629±64	1671±52
Valeric acid (mg O_2 L^{-1})	225±21	168±23	239±27	317±35
Isocaproic acid (mg O_2 L^{-1})	830±125	813±87	1205±98	1058±51
Caproic acid (mg O_2 L^{-1})	34±3	27±3	48±5	66±14
Glucose (mg O_2 L^{-1})	264±86	126±170	132±66	152±5
Lactic acid (mg O_2 L^{-1})	74±31	60±57	42±32	19±16
Ethanol (mg O_2 L^{-1})	ND	ND	ND	ND
Whey protein isolate (mg O_2 L^{-1})	64±96	137±142	5.5±9.5	27±47

Table S5.2. ANOVA test and Tukey's multiple comparisons test for comparing the resultant process effectiveness parameters at the different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Brown-Forsythe test (to compare standard deviations)	**ANOVA test (for equal standard deviations)**	**Brown-Forsythe ANOVA test (for different standard deviations)**	**Tukey's multiple comparisons test**		
	p value	**p value**	**p value**	**p value**		
				OLRv 6.5 and 12.5 g COD L^{-1} d^{-1}	**OLRv 6.5 and 18.5 g COD L^{-1} d^{-1}**	**OLRv 12.5 and 18.5 g COD L^{-1} d^{-1}**
Bioconversion (%)	0.5501	0.5686	-	-	-	-
Degree of Acidification (%)	0.7441	0.9611	-	-	-	-
Acidified COD (%)	0.2504	0.6426	-	-	-	-
CA production yield (g COD g^{-1} COD)	0.6306	0.4029	-	-	-	-
Odd-to-even ratio	0.6943	0.0083	-	0.0096	0.9951	0.0185

Table S5.3. t-test for comparing the resultant process effectiveness parameters at the different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	P value (F test to compare variances)	**P value (t-test considering same standard deviations)**	**P value (t-test using Welch's correction to compare mean values)**
Bioconversion (%)	0.0821	0.4807	-
Degree of Acidification (%)	0.2411	0.9937	-
Acidified COD (%)	0.8624	0.1993	-
CA production yield (g COD g^{-1} COD)	0.9878	0.3645	-
Odd-to-even ratio	0.4194	0.0266	-

Table S5.4. ANOVA test and Tukey's multiple comparisons test for comparing the effluent composition obtained at the different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Brown-Forsythe test (to compare standard deviations)	**ANOVA test (for equal standard deviations)**	**Brown-Forsythe ANOVA test (for different standard deviations)**	**Tukey's multiple comparisons test**		
	p value	**p value**	**p value**	**p value**		
				OLRv 6.5 and 12.5 g COD L^{-1} d^{-1}	**OLRv 6.5 and 18.5 g COD L^{-1} d^{-1}**	**OLRv 12.5 and 18.5 g COD L^{-1} d^{-1}**
% Acetic acid	0.5436	0.1003	-	-	-	-
% Propionic acid	0.3718	0.1874	-	-	-	-
% Isobutyric acid	0.6649	0.1136	-	-	-	-
% Butyric acid	0.4012	0.1423	-	-	-	-
% Isovaleric acid	0.5342	0.2719	-	-	-	-
% Valeric acid	0.5971	0.0011	-	0.0084	0.0816	0.0009
% Isocaproic acid	0.6724	0.0012	-	0.9213	0.0011	0.0049
% Caproic acid	0.5946	<0.0001	-	0.0123	<0.0001	<0.0001
% Glucose	0.5886	0.2113	-	-	-	-
% Lactic acid	0.8205	0.6081	-	-	-	-
% Ethanol	-	-	-	-	-	-
% Whey protein isolate	0.3417	0.2719	-	-	-	-

Table S5.5. t-test for comparing the effluent composition obtained at the different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	P value (F test to compare variances)	**P value (t-test considering same standard deviations)**	**P value (t-test using Welch's correction to compare mean values)**
% Acetic acid	0.9657	0.3938	-
% Propionic acid	0.1046	0.0128	-
% Isobutyric acid	0.9757	0.0339	-
% Butyric acid	0.0772	0.0494	-
% Isovaleric acid	0.4279	0.1068	-
% Valeric acid	0.8373	0.0002	-
% Isocaproic acid	0.1781	0.8540	-
% Caproic acid	0.0558	0.0418	-
% Glucose	0.1164	0.4053	-
% Lactic acid	0.4125	0.3619	-
% Ethanol	-	-	-
% Whey protein isolate	0.0507	0.4910	-

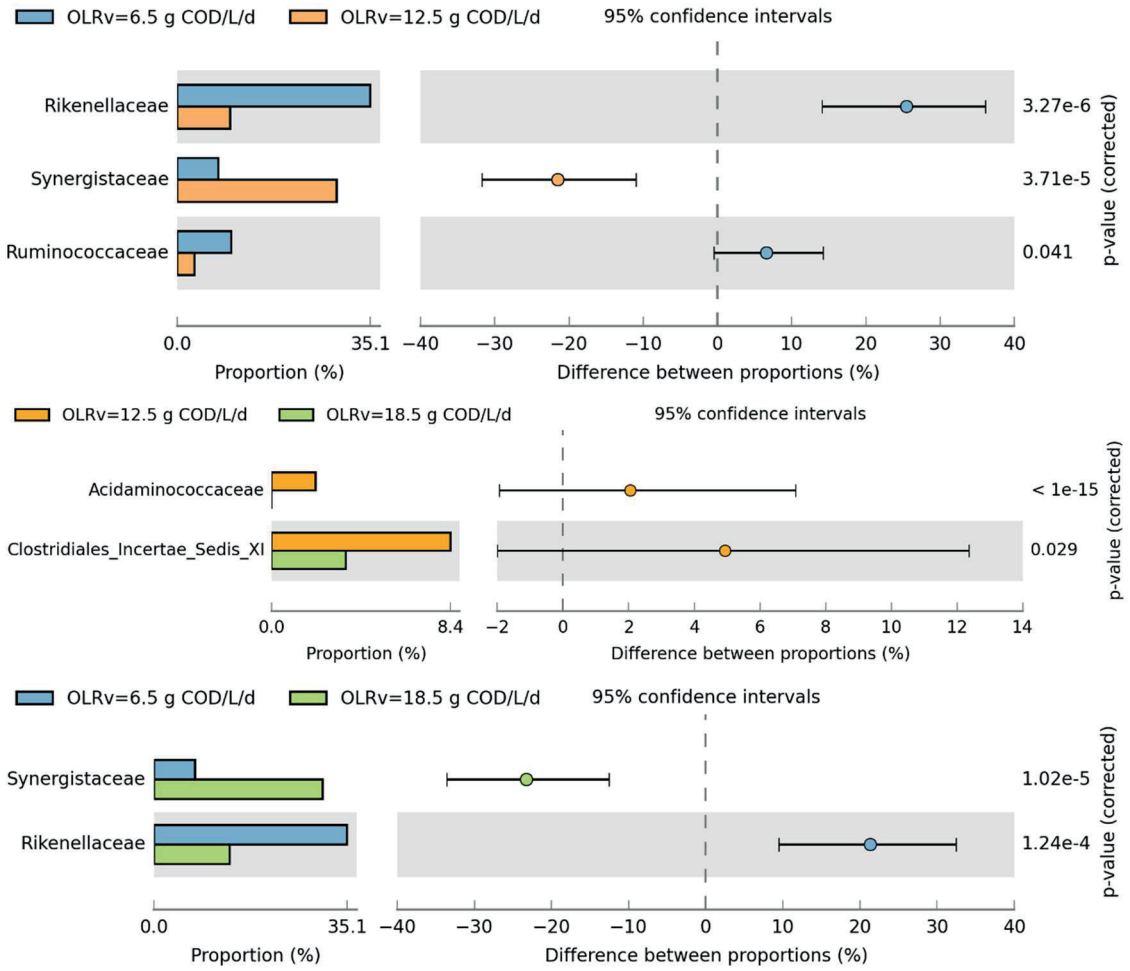

Figure S5.1. Fisher's exact test for comparison of bacterial communities relative abundance at family level at different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein isolate as sole carbon source.

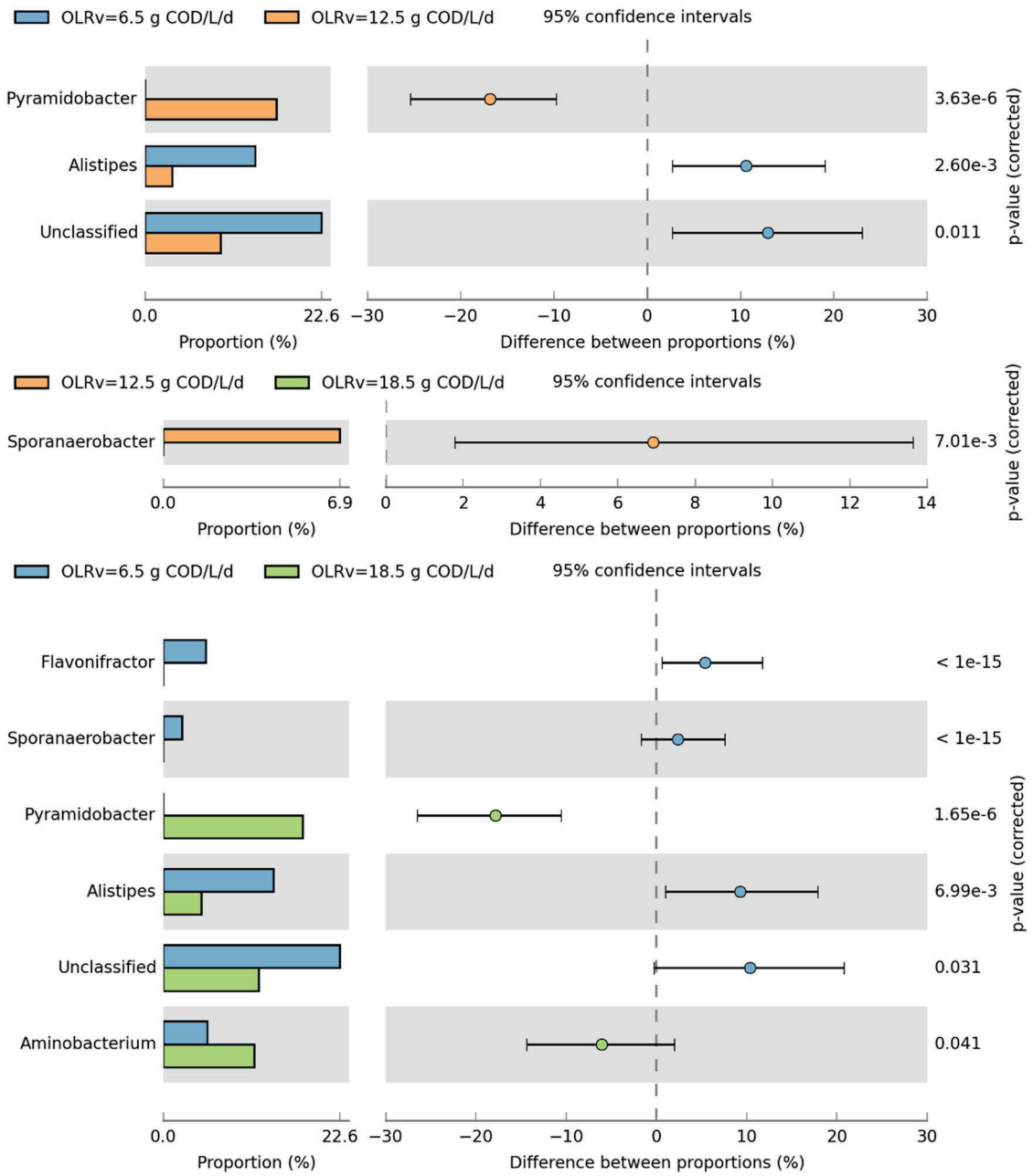

Figure S5.2. Fisher's exact test for comparison of bacterial communities relative abundance at genus level at different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein isolate as sole carbon source.

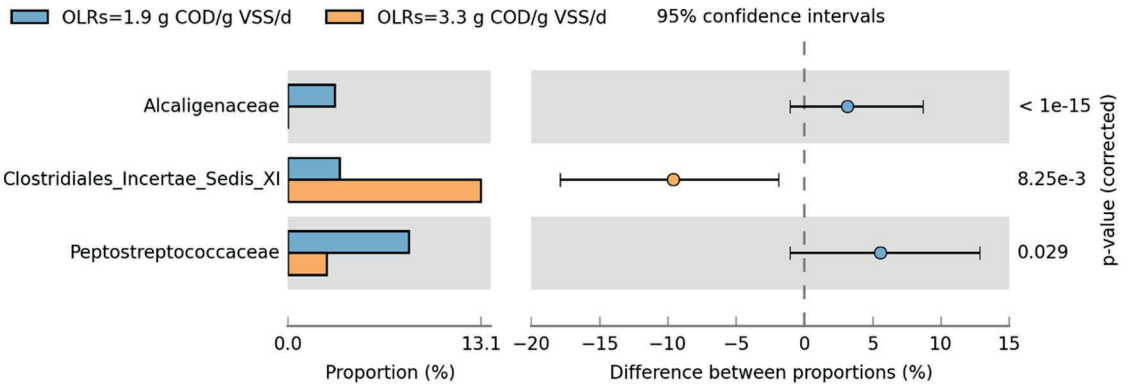

Figure S5.3. Fisher's exact test for comparison of bacterial communities relative abundance at family level at different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein isolate as sole carbon source.

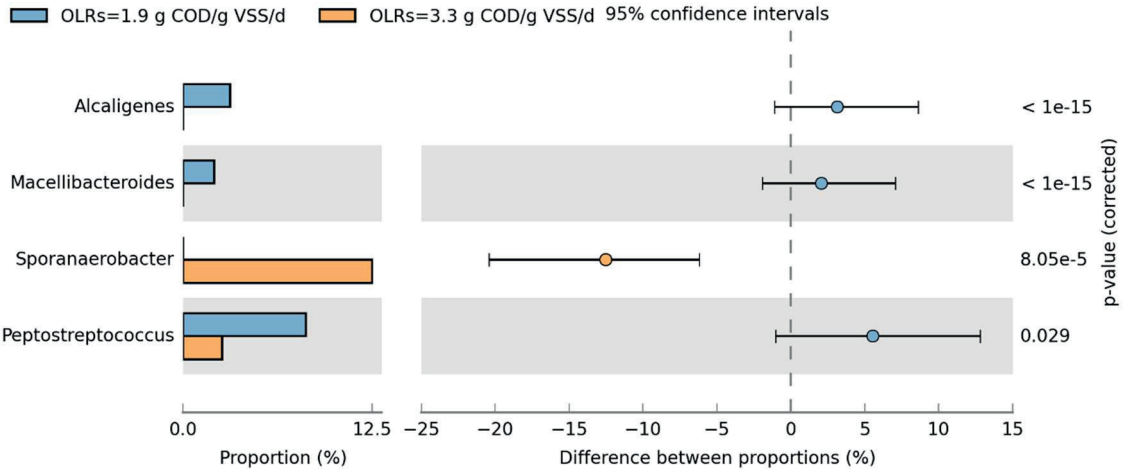

Figure S5.4. Fisher's exact test for comparison of bacterial communities relative abundance at genus level at different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein isolate as sole carbon source.

Table S5.6. Shannon index and p values for t-test statistical comparison of the bacterial diversity under the effect of the different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein isolate as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Shannon index			p values (Diversity t test)		
	OLRv = 6.5 g COD L^{-1} d^{-1}	**OLRv = 12.5 g COD L^{-1} d^{-1}**	**OLRv = 18.5 g COD L^{-1} d^{-1}**	**OLRv 6.5 and 12.5 g COD L^{-1} d^{-1}**	**OLRv 6.5 and 18.5 g COD L^{-1} d^{-1}**	**OLRv 12.5 and 18.5 g COD L^{-1} d^{-1}**
Family level	1.9554	1.9617	2.0181	0.9578	0.4991	0.6141
Genus level	2.1151	2.0617	1.9943	0.5936	0.1991	0.4533

Table S5.7. Shannon index and p values for t-test statistical comparison of the bacterial diversity under the effect of the different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein isolate as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Shannon index		p values (Diversity t test)
	OLRs = 1.9 g COD g^{-1} VSS d^{-1}	**OLRs = 3.3 g COD g^{-1} VSS d^{-1}**	
Family level	2.0181	1.9521	0.5199
Genus level	1.9943	2.1298	0.0980

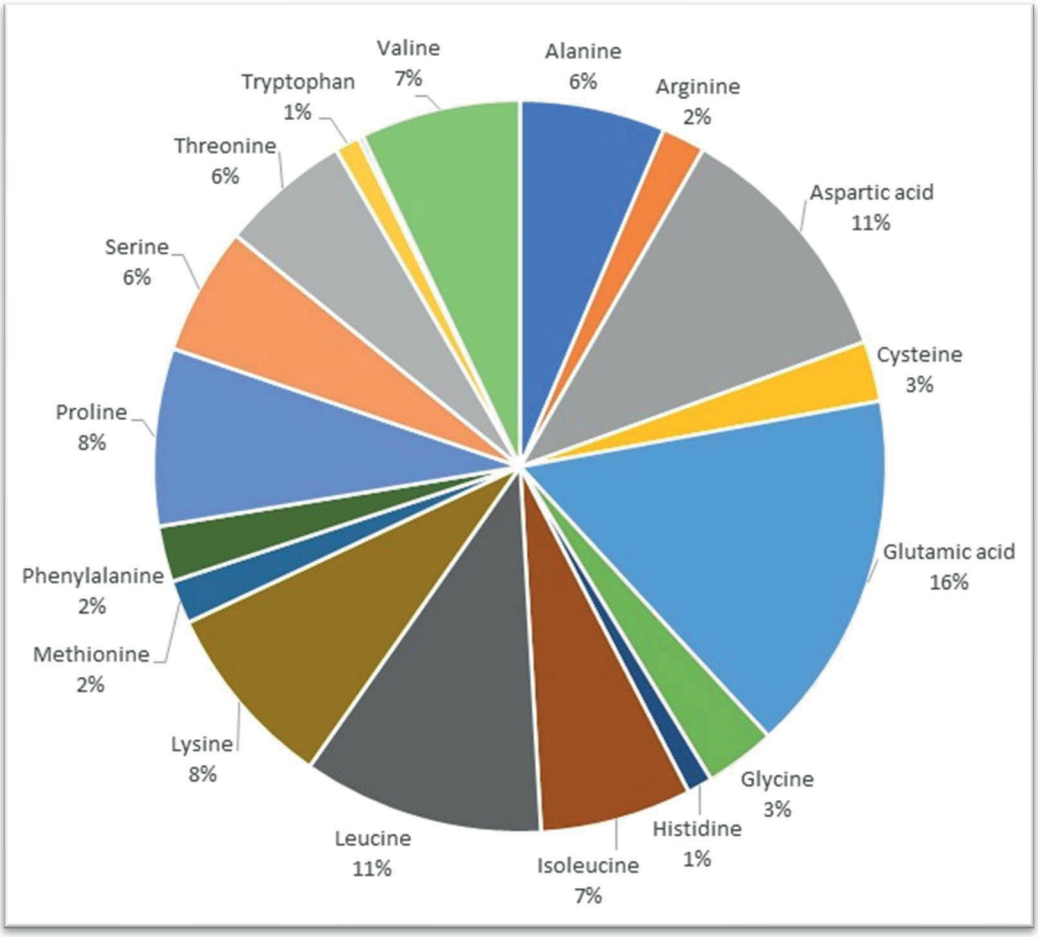

Figure S5.5. Amino acids composition (% mol) of the whey protein isolate used as substrate (information provided by the manufacturer (HSN ®)).

Appendix III:

Supporting Information for Chapter 6

The methodology used to perform statistical tests to compare the fermentation effectiveness parameters and the effluent composition obtained with the different influent organic compositions is presented in detail in Chapter 3. Section 3.5.4 can be consulted for better understanding of Table S6.2, Table S6.3a and Table S6.3b.

Table S6.1. Effluent composition (% COD) yielded for the different substrate organic compositions tested at an OLRv of 6 g COD L^{-1} d^{-1} and an OLRs of 2 g COD g^{-1} VSS d^{-1}.

Substrate composition	**100%P**	**25%C /75%P**	**50%C /50%P**	**75%C /25%P**	**100%C**
OLRv (g COD L^{-1} d^{-1})	**6.5±0.6**	**6.2±0.2**	**6.1±0.1**	**6.0±0.2**	**6.0±0.3**
OLRs (g COD g^{-1} VSS d^{-1})	**1.9±0.4**	**1.8±0.4**	**1.7±0.5**	**1.9±0.5**	**2.2±0.2**
Total COD in the effluent (mg O_2 L^{-1})	11376±539	11001±753	10617±130	10325±43	10886±502
% Acetic acid (%COD)	18.1±1.4	22.1±1.5	26.6±1.6	19.6±1.9	28±4
% Propionic acid (%COD)	13.3±1.1	29.1±1.8	20.3±2.3	31±5	2.4±0.9
% Isobutyric acid (%COD)	9.0±1.3	5.9±0.5	2.8±0.6	2.8±0.3	0.9±0.7
% Butyric acid (%COD)	14.8±0.9	10.5±0.6	19.2±1.4	33.6±0.3	47±3
% Isovaleric acid (%COD)	17.0±1.2	12.6±0.6	9.3±1.4	5.1±0.3	0.0±0.0
% Valeric acid (%COD)	2.0±0.2	1.5±0.1	1.7±0.4	2.5±0.5	0.0±0.0
% Isocaproic acid (%COD)	7.2±0.9	4.8±0.8	0.0±0.0	0.2±0.4	0.0±0.0
% Caproic acid (%COD)	0.3±0.1	0.0±0.0	0.3±0.3	0.0±0.0	0.0±0.0
% Glucose (% COD)	2.3±0.8	2±4	1.0±0.9	0.1±0.1	0.1±0.1
% Lactic acid (%COD)	0.6±0.3	0.6±0.8	0.4±0.6	0.0±0.0	0.9±1.3
% Ethanol (%COD)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	8±3
% Food-grade sugar (%COD)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.8±0.1
% Whey protein isolate (%COD)	0.5±0.8	3±4	13±4	1.1±0.9	0.0±0.0

Table S6.2. ANOVA test and Tukey's multiple comparisons test for comparing the resultant acidogenic fermentation effectiveness parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Brown-Forsythe test (to compare standard deviations)	**ANOVA test (for equal standard deviations)**	**Brown-Forsythe ANOVA test (for different standard deviations)**	**Tukey's multiple comparisons test**									
				p values									
	p value	**p value**	**p value**	100%P vs. 25%C /75%P	100%P vs. 50%C /50%P	100%P vs. 75%C /25%P	100%P vs. 100%C	25%C /75% P vs. 50%C /50%P	25%C /75%P vs. 75%C /25%P	25%C /75%P vs. 100%C	50%C /50%P vs. 75%C /25%P	50%C /50%P vs. 100%C	75%C /25%P vs. 100%C
Bioconversion (%)	0.4495	<0.0001	-	0.8599	<0.0001	0.9994	0.9918	0.0004	0.8072	0.7097	<0.0001	<0.0001	0.9996
Degree of Acidification (%)	0.5072	0.0014	-	0.3865	0.9999	0.0074	0.6142	0.4496	0.4374	0.0678	0.0193	0.8140	0.0012
Acidified COD (%)	0.6434	0.0006	-	0.6014	0.9956	0.0104	0.1703	0.6067	0.2727	0.0381	0.0173	0.4727	0.0003
CA production yield (g COD g^{-1} COD)	0.6886	0.0015	-	0.2987	0.2516	0.0110	0.6002	>0.9999	0.6388	0.0475	0.7014	0.0388	0.0016
Odd-to-even ratio	0.0007	-	0.0005	<0.0001	0.0158	<0.0001	<0.0001	0.0002	0.7492	<0.0001	0.0010	<0.0001	<0.0001

Table S6.3a. ANOVA test and Tukey's multiple comparisons test for the different effluent organic components proportion (%COD) obtained with the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Brown-Forsythe test (to compare standard deviations)	**ANOVA test (for equal standard deviations)**	**Brown-Forsythe ANOVA test (for different standard deviations)**	**Tukey's multiple comparisons test**									
				p values									
	p value	**p value**	**p value**	100%P vs. 25%C /75%P	100%P vs. 50%C /50%P	100%P vs. 75%C /25%P	100%P vs. 100%C	25%C /75% P vs. 50%C /50%P	25%C /75%P vs. 75%C /25%P	25%C /75%P vs. 100%C	50%C /50%P vs. 75%C /25%P	50%C /50%P vs. 100%C	75%C /25%P vs. 100%C
% Acetic acid	0.2515	<0.0001	-	0.0766	0.0002	0.7654	<0.0001	0.0802	0.4900	0.0165	0.0024	0.9651	0.0003
% Propionic acid	0.0201	-	<0.0001	<0.0001	0.0054	<0.0001	<0.0001	0.0026	0.7451	<0.0001	0.0002	<0.0001	<0.0001
% Isobutyric acid	0.2032	<0.0001	-	0.0008	<0.0001	<0.0001	<0.0001	0.0030	0.0015	<0.0001	>0.9999	0.0555	0.0393
% Butyric acid	0.0402	-	<0.0001	0.2324	0.1940	<0.0001	<0.0001	0.0111	<0.0001	<0.0001	<0.0001	<0.0001	0.0001
% Isovaleric acid	0.1544	<0.0001	-	<0.0001	<0.0001	<0.0001	<0.0001	0.0026	<0.0001	<0.0001	0.0001	<0.0001	<0.0001
% Valeric acid	0.0648	<0.0001	-	0.0940	0.5266	0.0342	<0.0001	0.8542	0.0007	<0.0001	0.0053	<0.0001	<0.0001
% Isocaproic acid	0.2002	<0.0001	-	0.0003	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.9954	>0.9999	0.9938

Table S6.3b. ANOVA test and Tukey's multiple comparisons test for the different effluent organic components proportion (%COD) obtained with the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

	Brown-Forsythe test (to compare standard deviations)	**ANOVA test (for equal standard deviations)**	**Brown-Forsythe ANOVA test (for different standard deviations)**	**Tukey's multiple comparisons test**									
				p values									
	p value	**p value**	**p value**	100%P vs. 25%C /75%P	100%P vs. 50%C /50%P	100%P vs. 75%C /25%P	100%P vs. 100%C	25%C /75% P vs. 50%C /50%P	25%C /75%P vs. 75%C /25%P	25%C /75%P vs. 100%C/	50%C /50%P vs. 75%C /25%P	50%C /50%P vs. 100%C	75%C /25%P vs. 100%C
% Caproic acid	0.1714	0.0002	-	0.0062	0.9985	0.0029	0.0029	0.0115	>0.9999	>0.9999	0.0068	0.0068	>0.9999
% Glucose	0.3186	0.0503	-	-	-	-	-	-	-	-	-	-	-
% Lactic acid	0.4591	0.4169	-	-	-	-	-	-	-	-	-	-	-
% Ethanol	0.3637	<0.0001	-	>0.9999	>0.9999	>0.9999	<0.0001	>0.9999	>0.9999	<0.0001	>0.9999	<0.0001	<0.0001
% Food-grade sugar	0.1858	<0.0001	-	>0.9999	>0.9999	>0.9999	<0.0001	>0.9999	>0.9999	<0.0001	>0.9999	<0.0001	<0.0001
% Whey protein isolate	0.2523	<0.0001	-	0.4535	<0.0001	0.9871	0.9916	<0.0001	0.7434	0.3029	<0.0001	<0.0001	0.9002

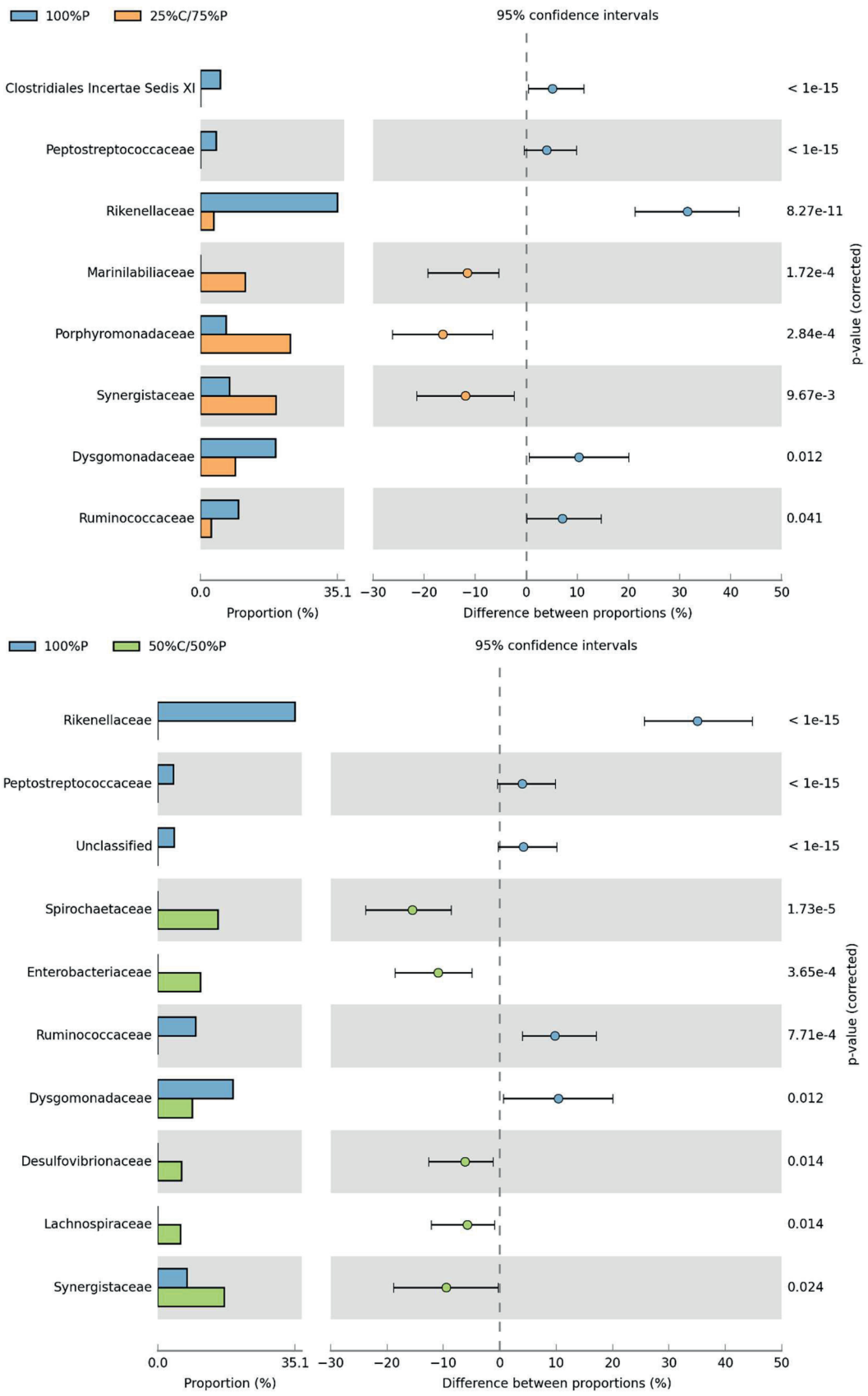

Figure S6.1a. Fisher's exact test for comparison of bacterial communities' relative abundance at family level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

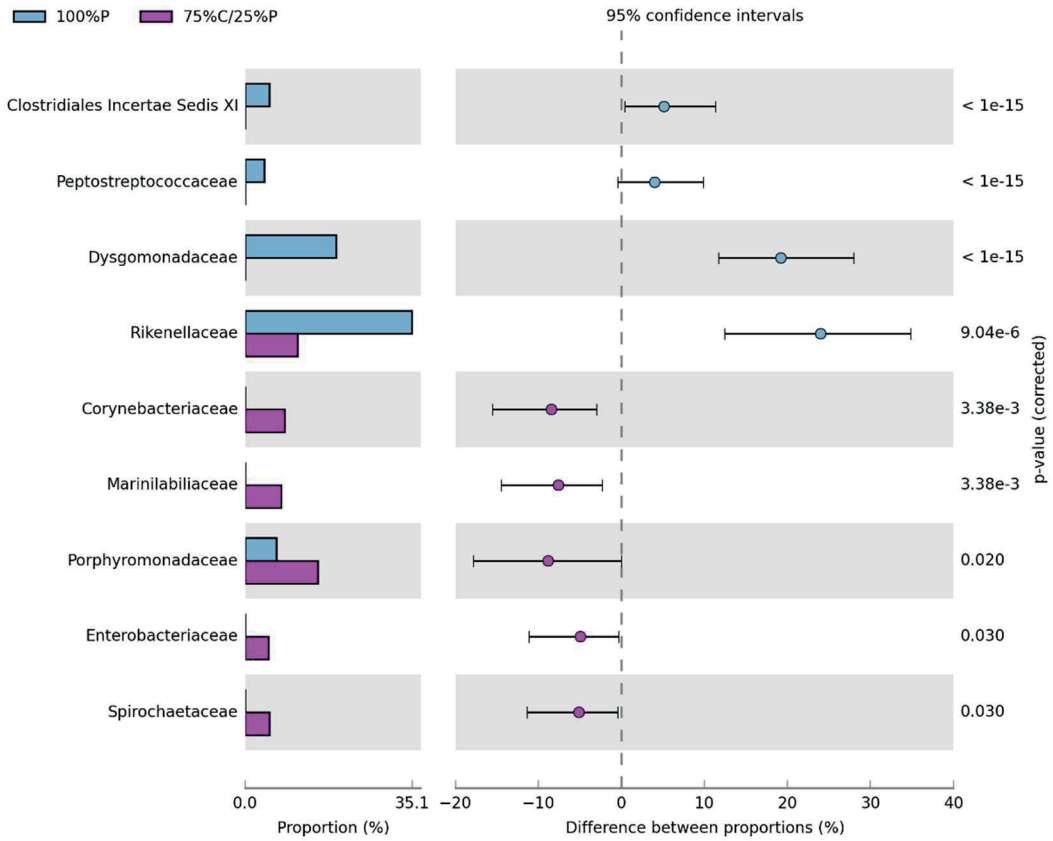

Figure S6.1b. Fisher's exact test for comparison of bacterial communities' relative abundance at family level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

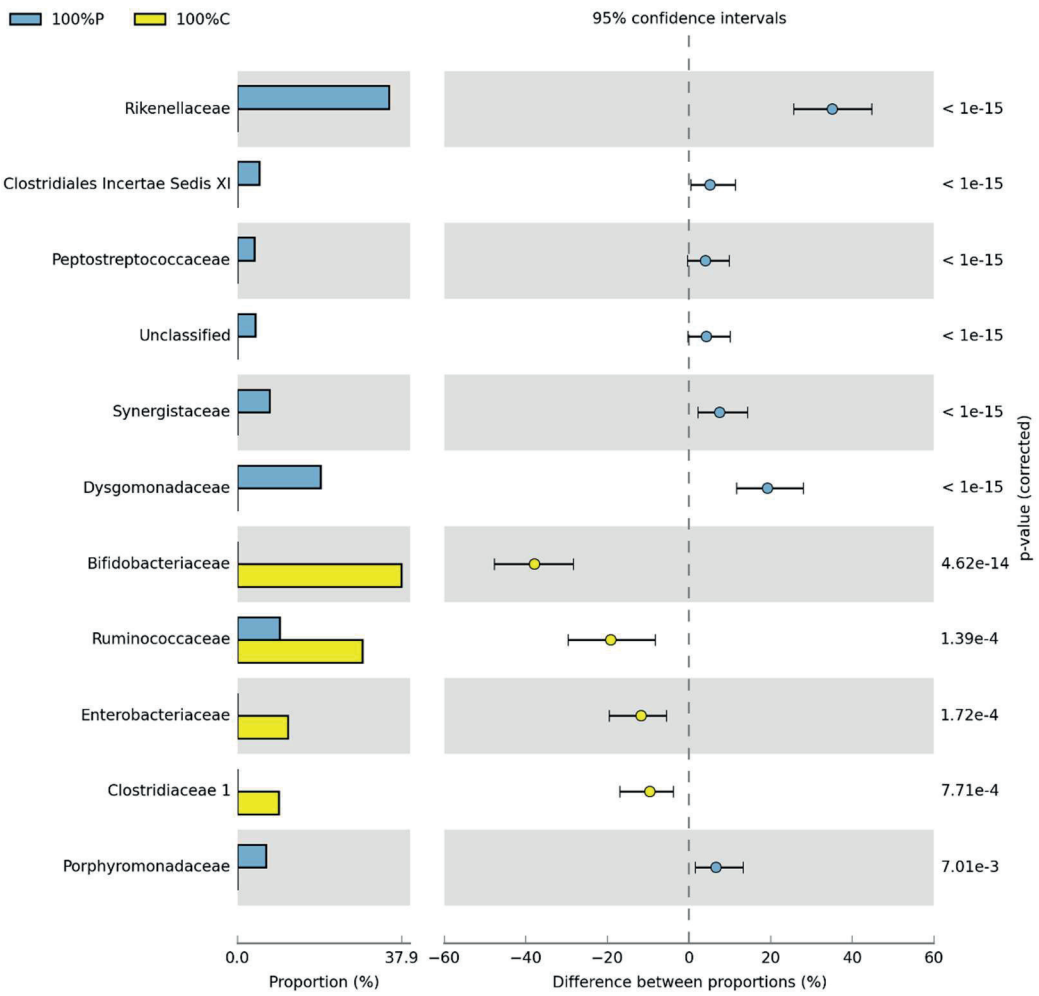

Figure S6.1c. Fisher's exact test for comparison of bacterial communities' relative abundance at family level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

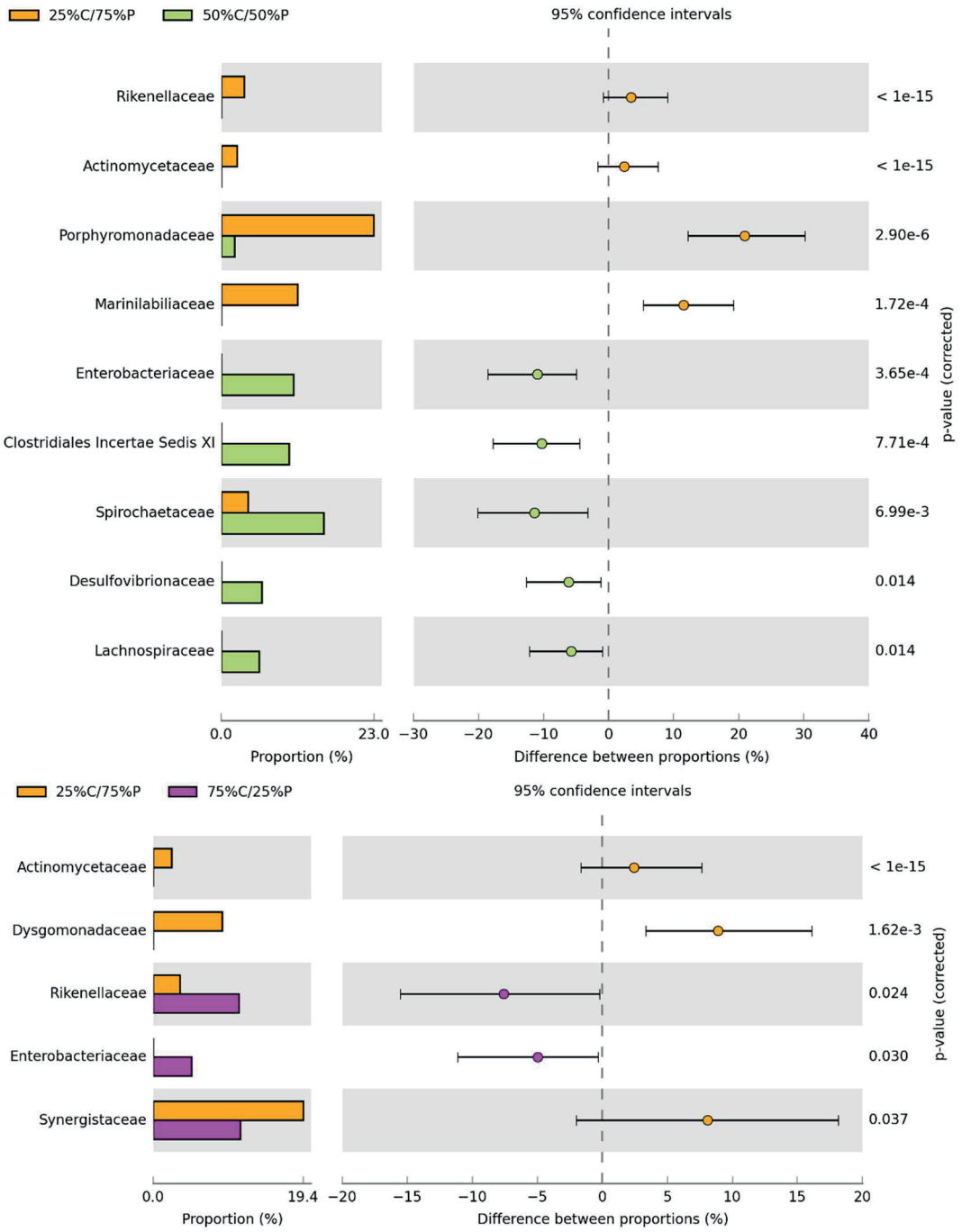

Figure S6.1d. Fisher's exact test for comparison of bacterial communities' relative abundance at family level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

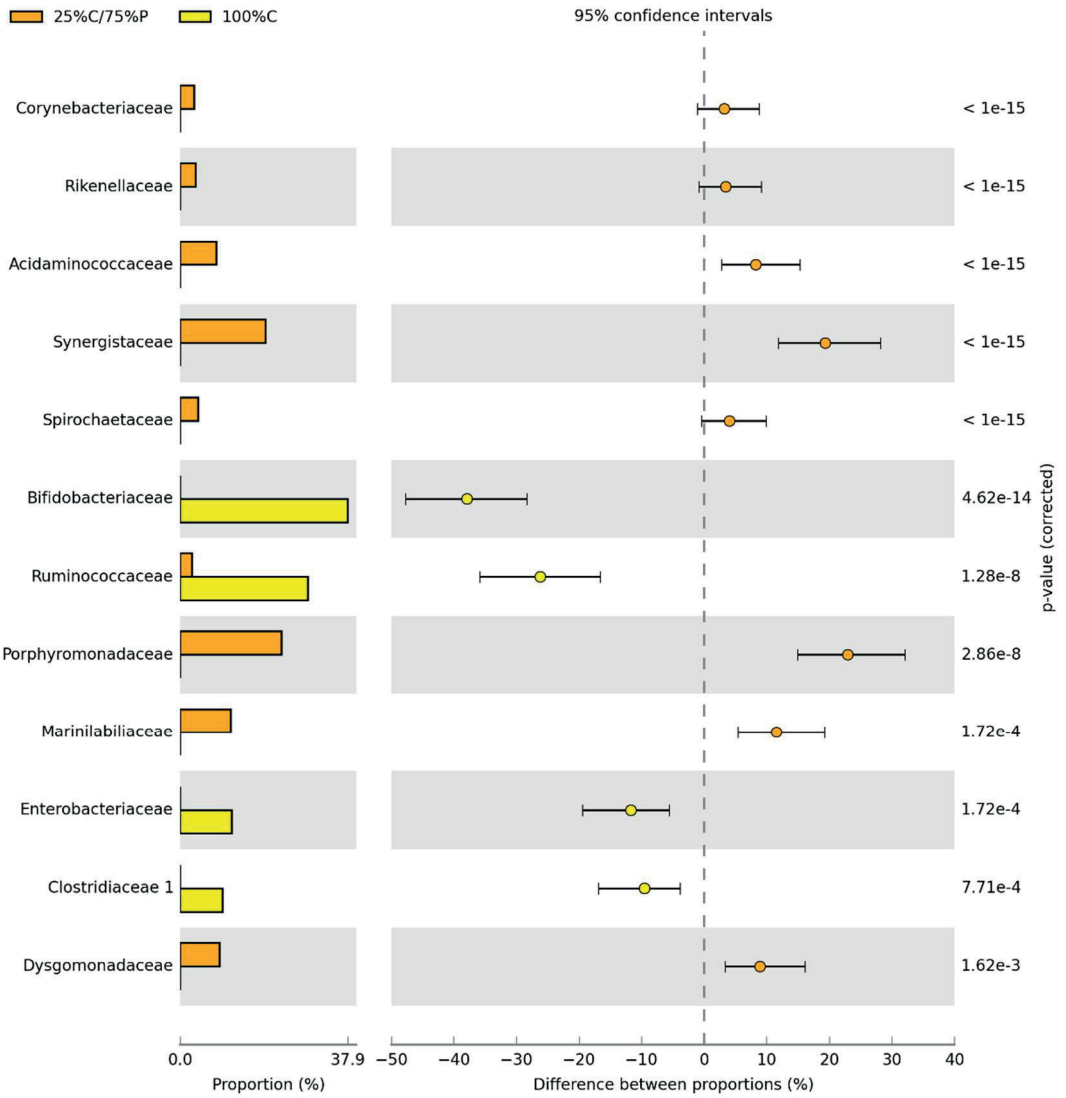

Figure S6.1e. Fisher's exact test for comparison of bacterial communities' relative abundance at family level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

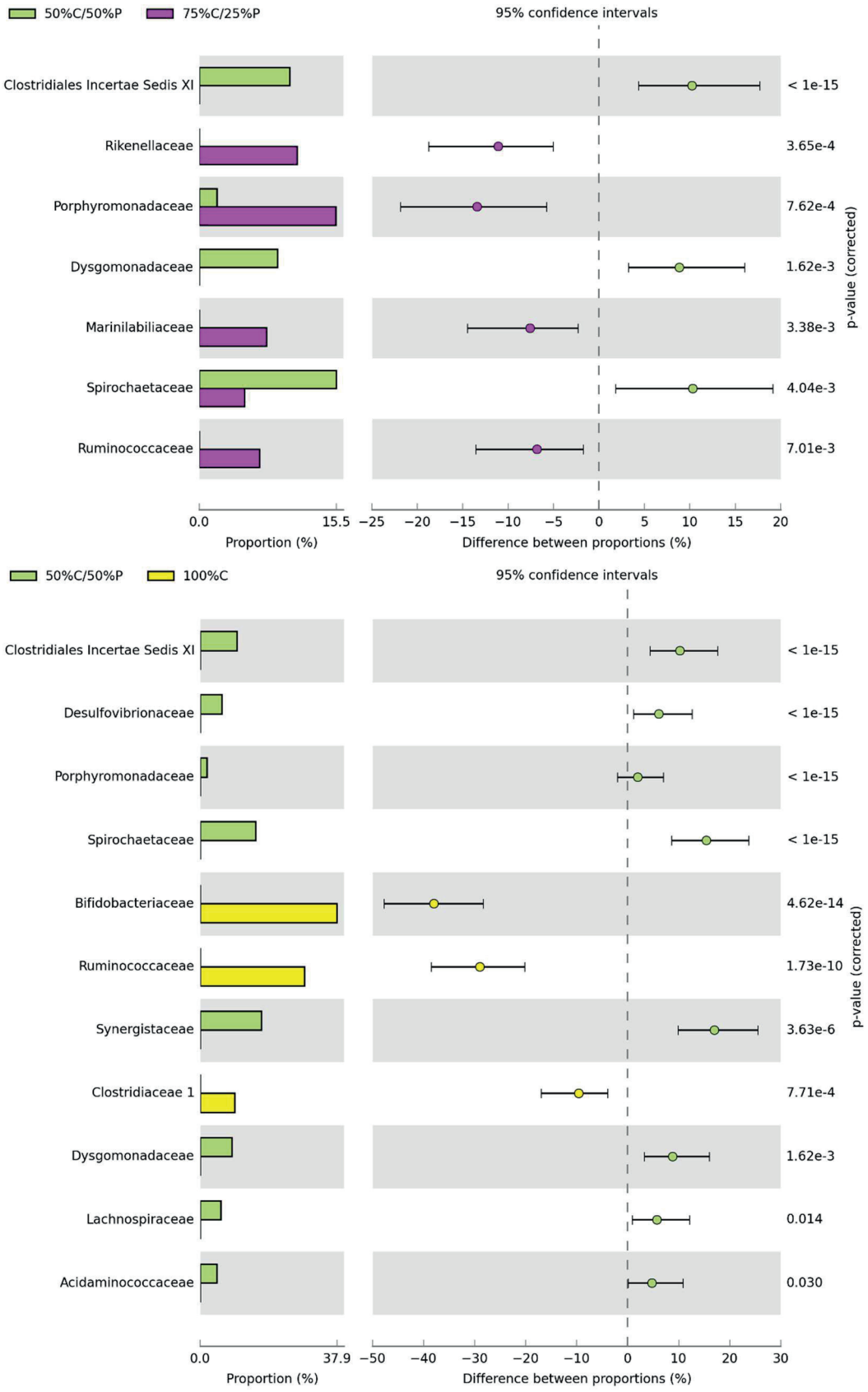

Figure S6.1f. Fisher's exact test for comparison of bacterial communities' relative abundance at family level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

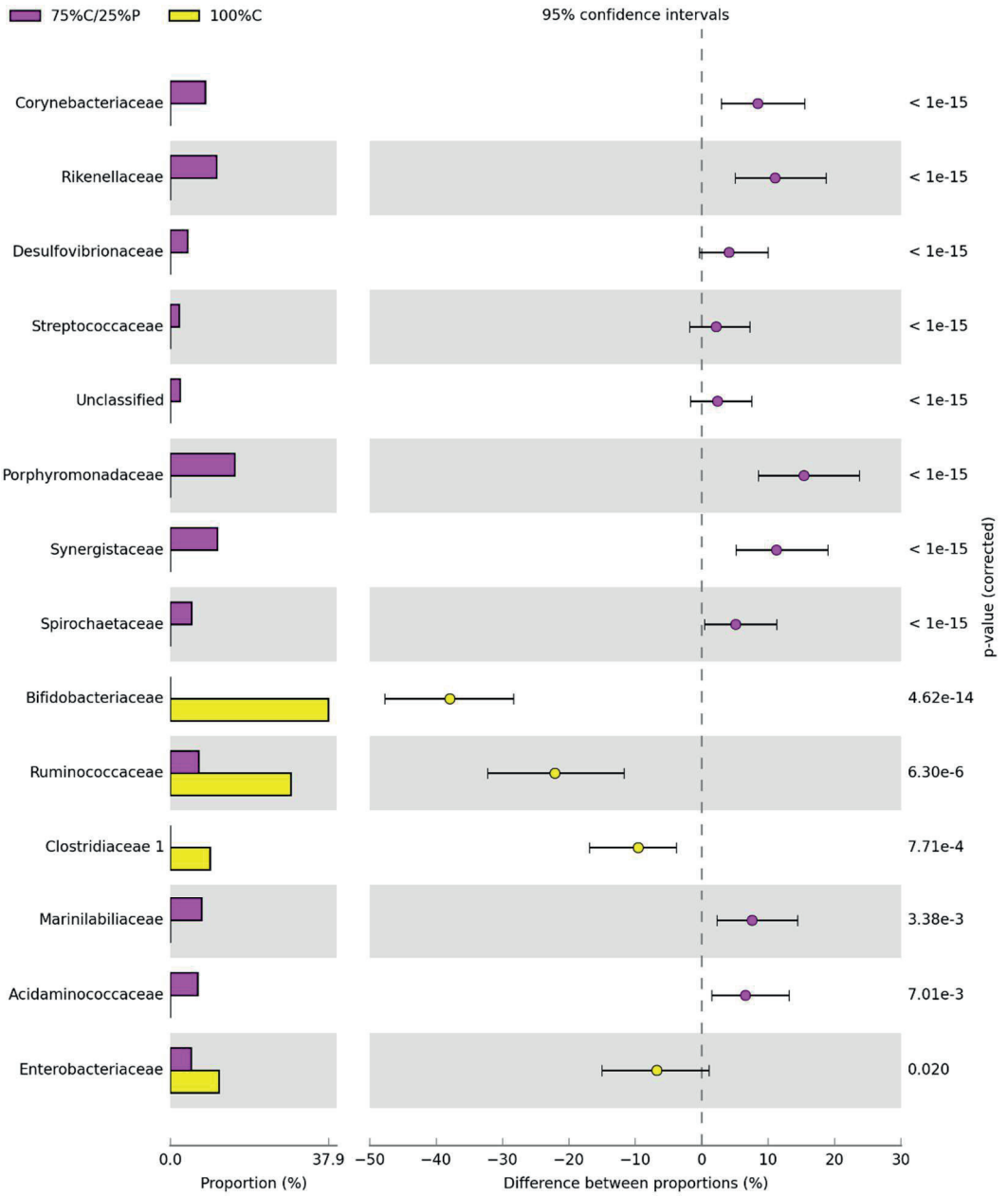

Figure S6.1g. Fisher's exact test for comparison of bacterial communities' relative abundance at family level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

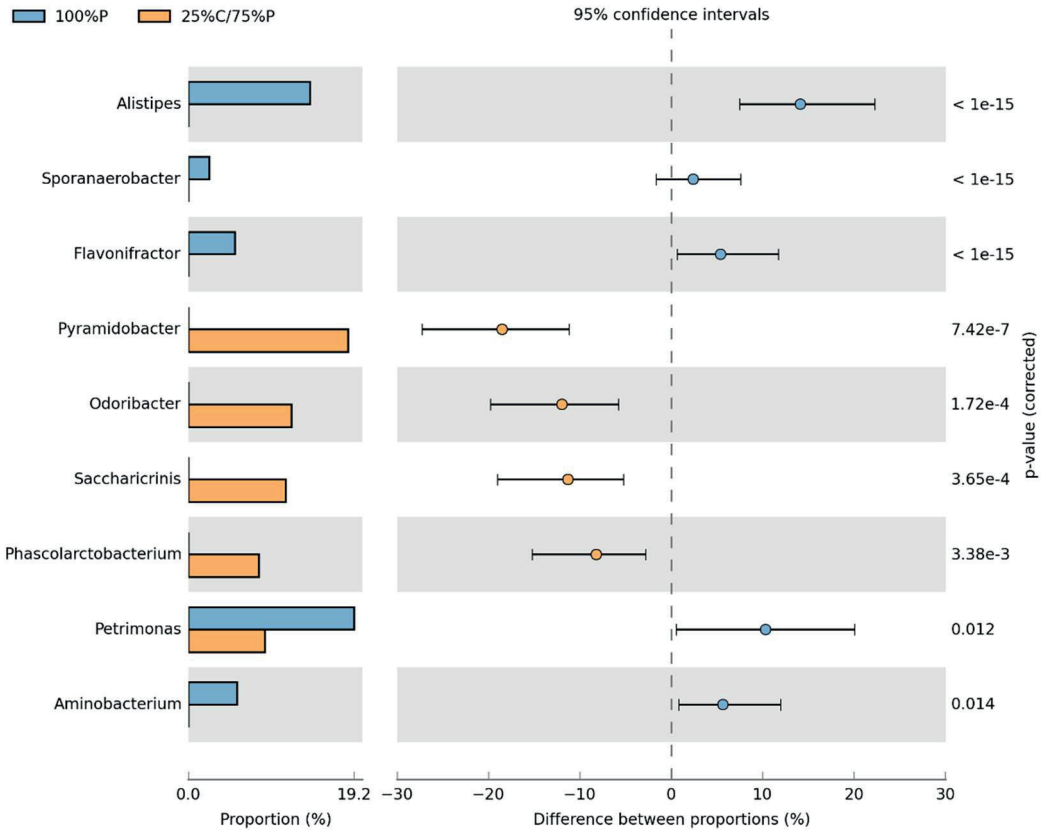

Figure S6.2a. Fisher's exact test for comparison of bacterial communities' relative abundance at genus level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

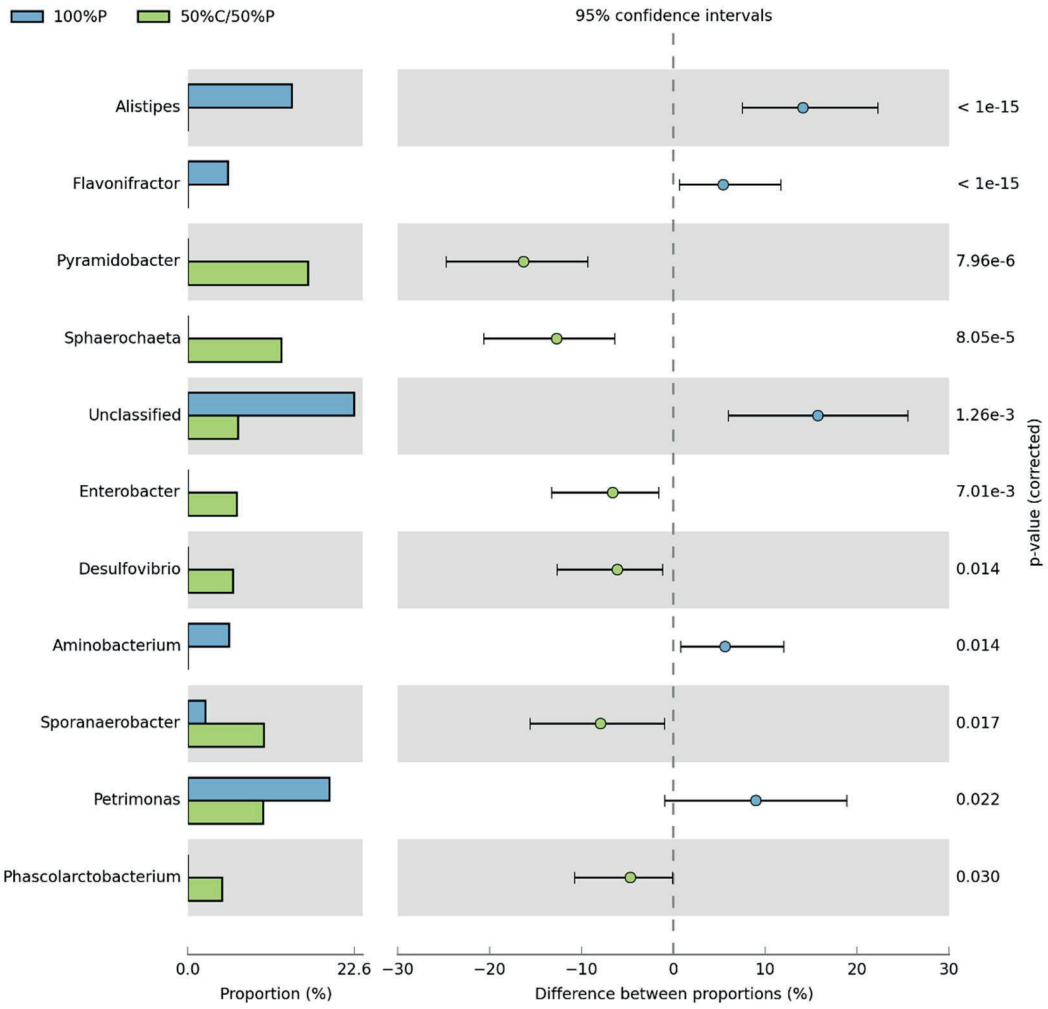

Figure S6.2b. Fisher's exact test for comparison of bacterial communities' relative abundance at genus level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

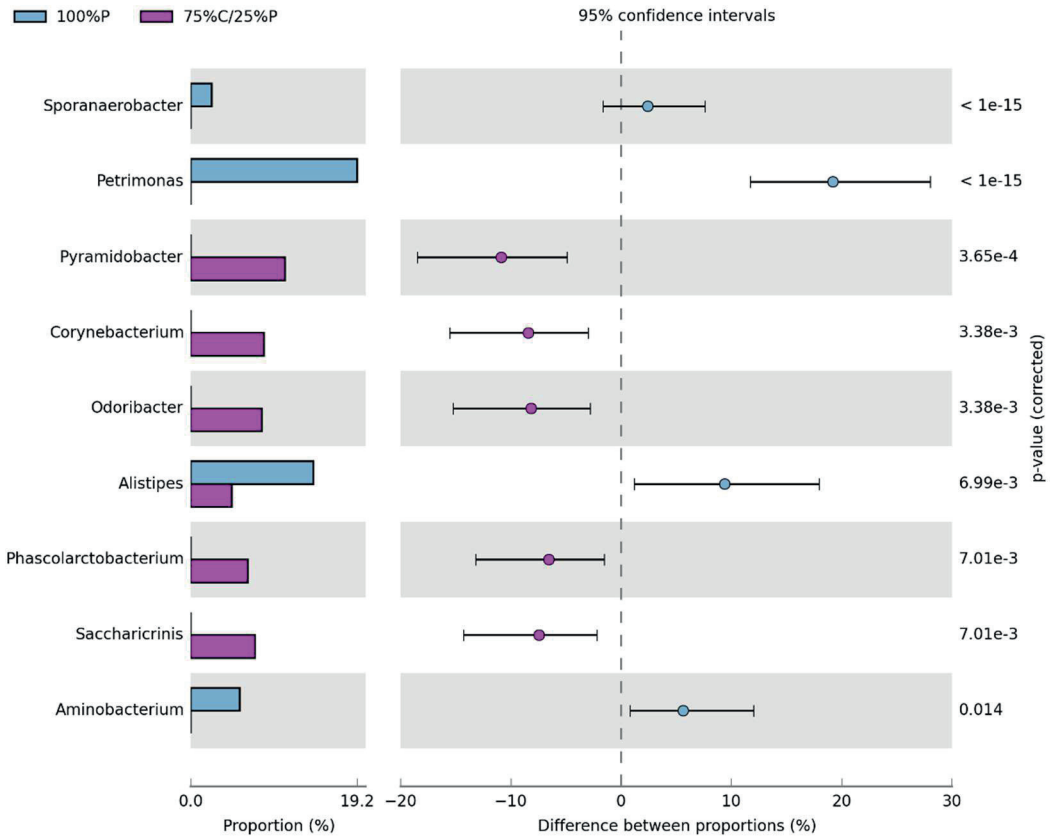

Figure S6.2c. Fisher's exact test for comparison of bacterial communities' relative abundance at genus level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

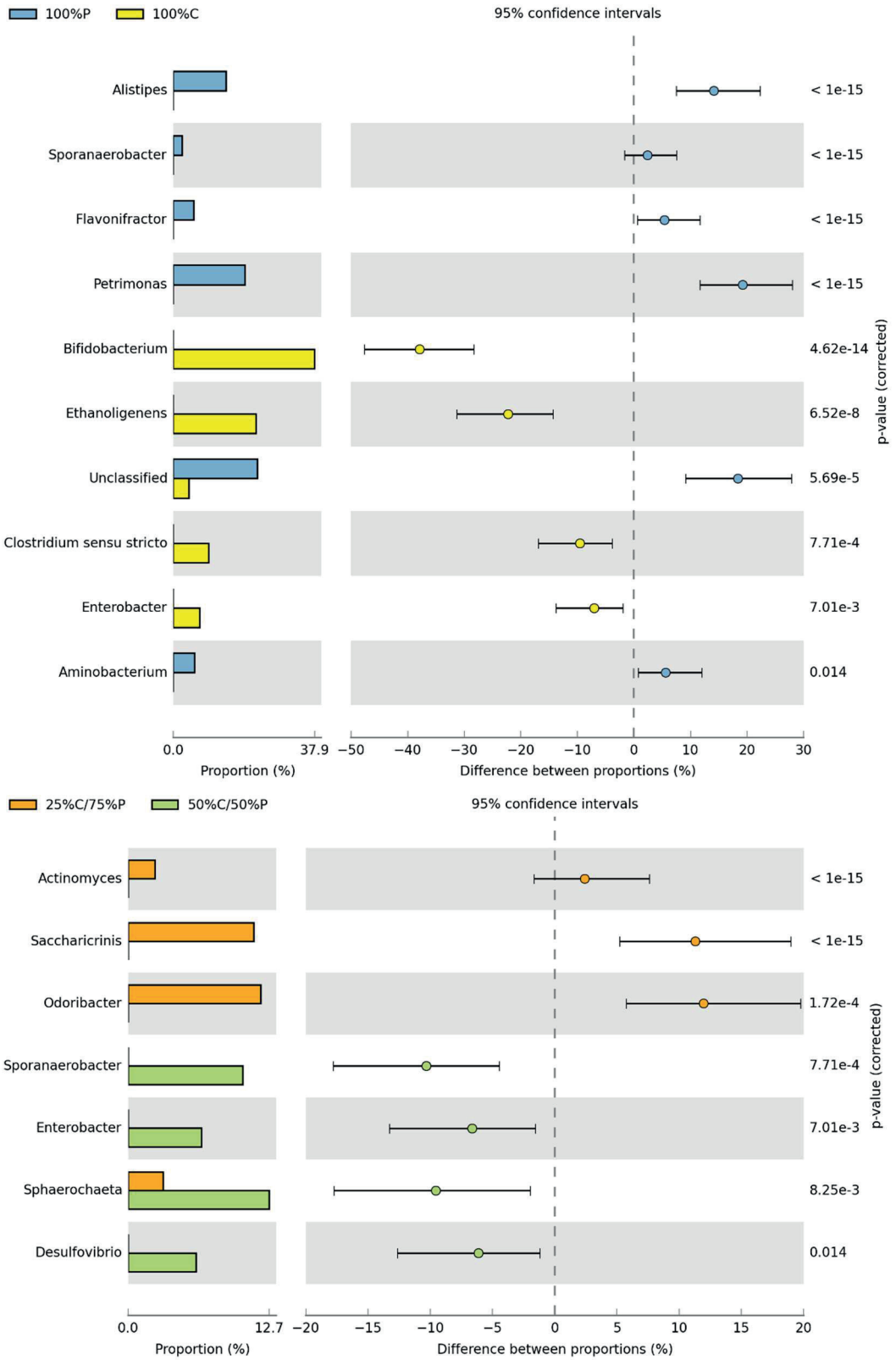

Figure S6.2d. Fisher's exact test for comparison of bacterial communities' relative abundance at genus level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

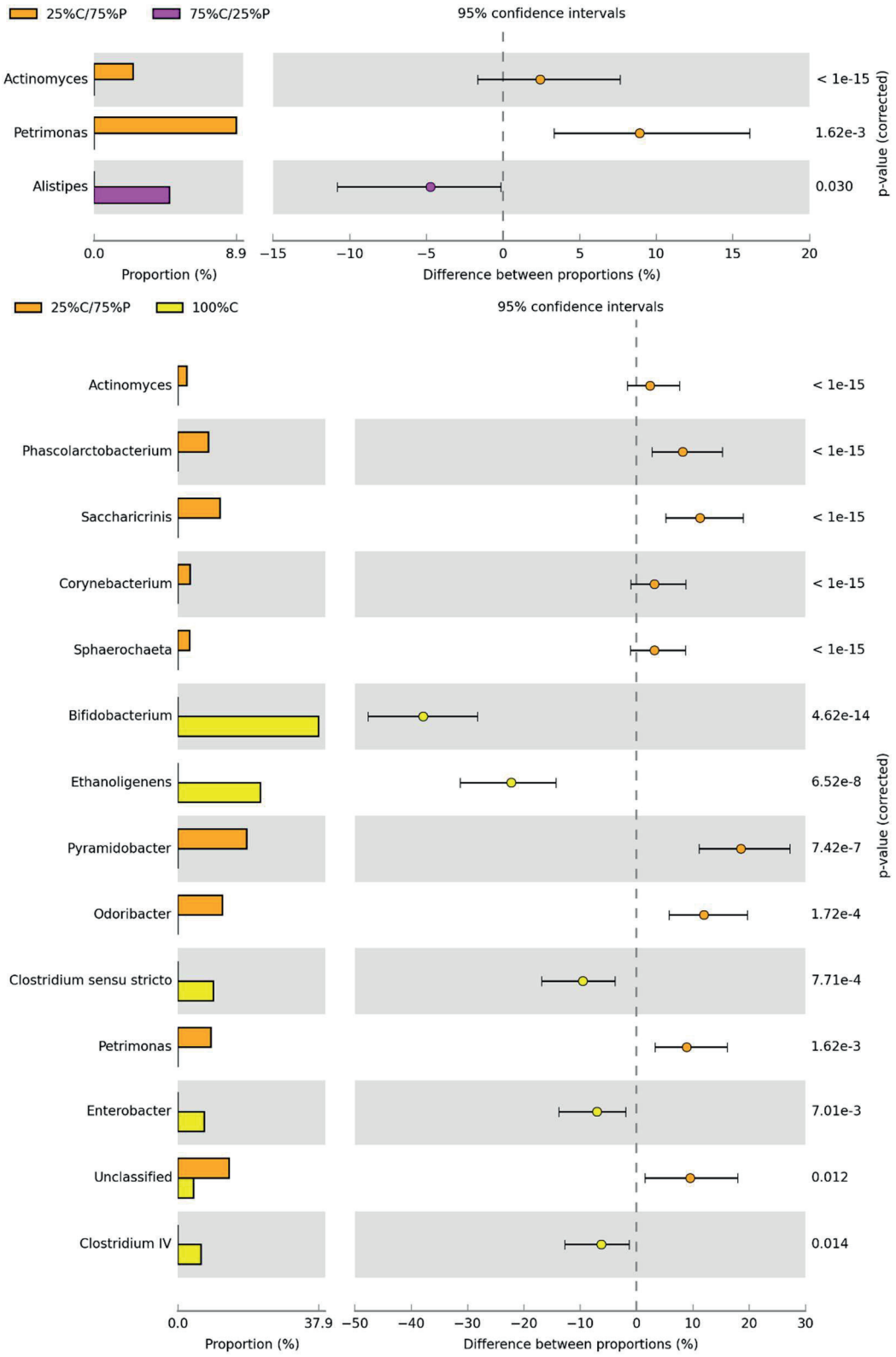

Figure S6.2e. Fisher's exact test for comparison of bacterial communities' relative abundance at genus level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

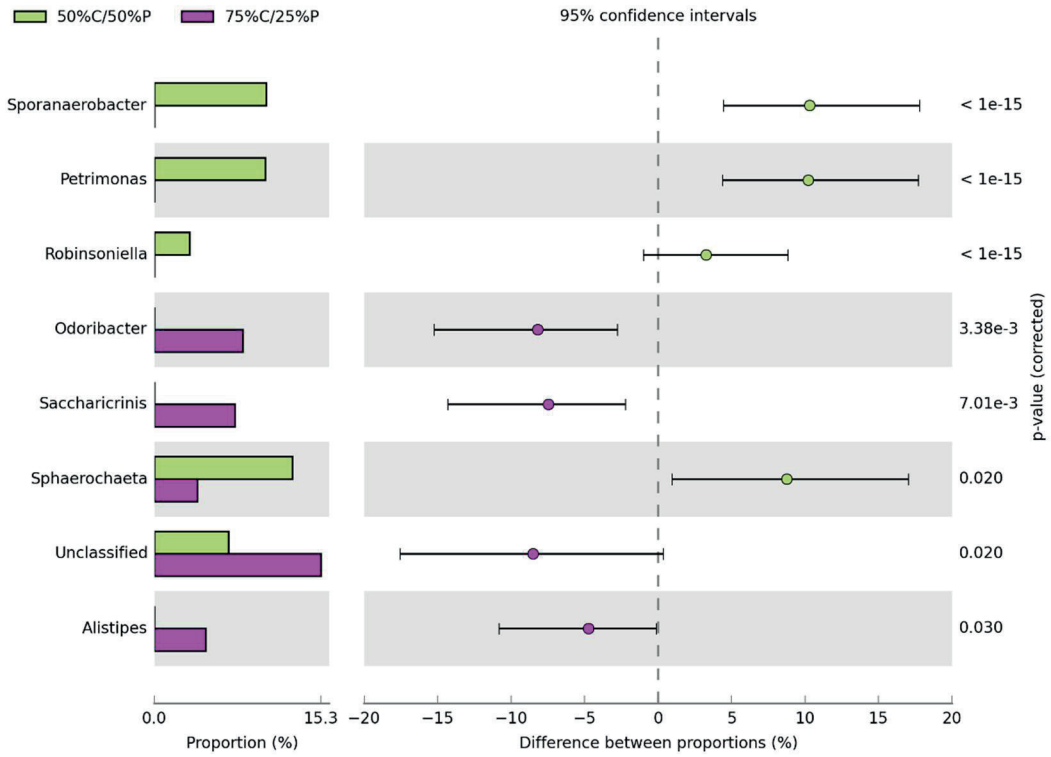

Figure S6.2f. Fisher's exact test for comparison of bacterial communities' relative abundance at genus level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

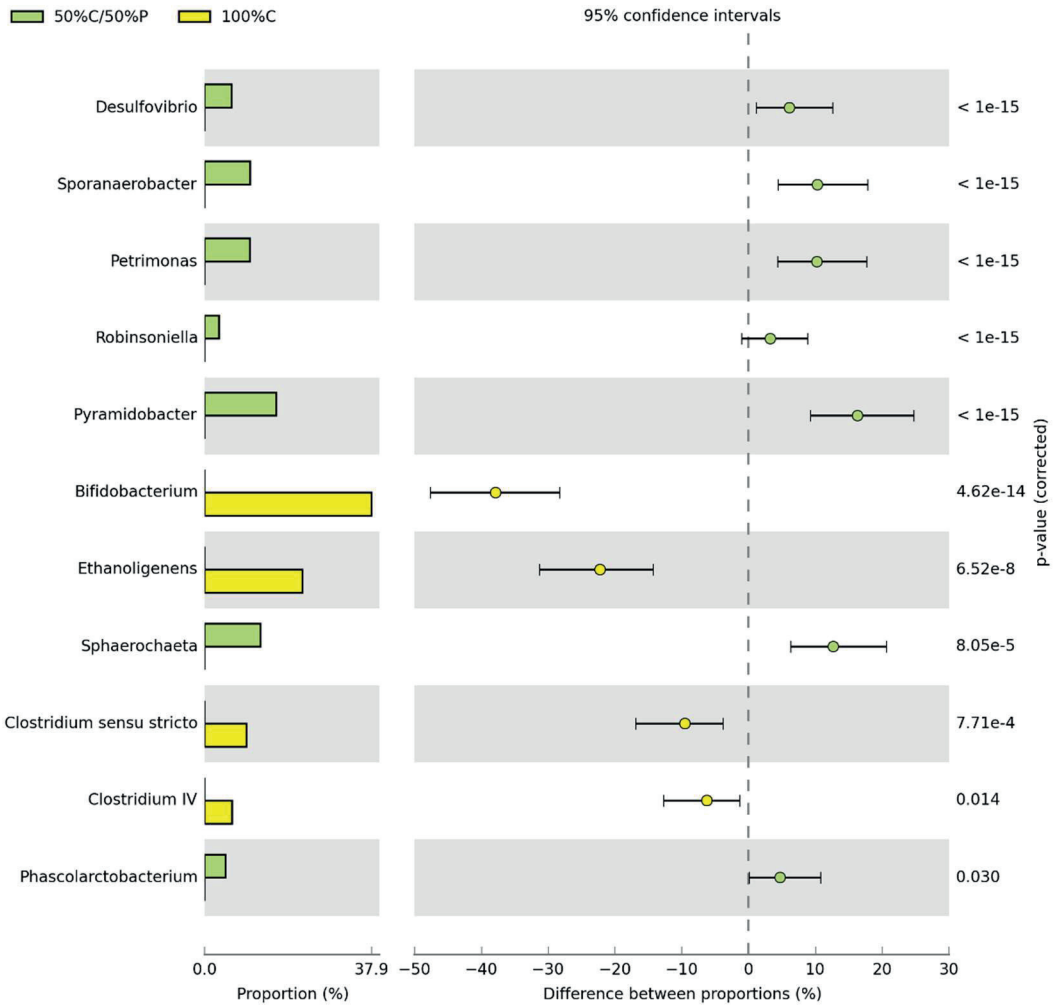

Figure S6.2g. Fisher's exact test for comparison of bacterial communities' relative abundance at genus level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

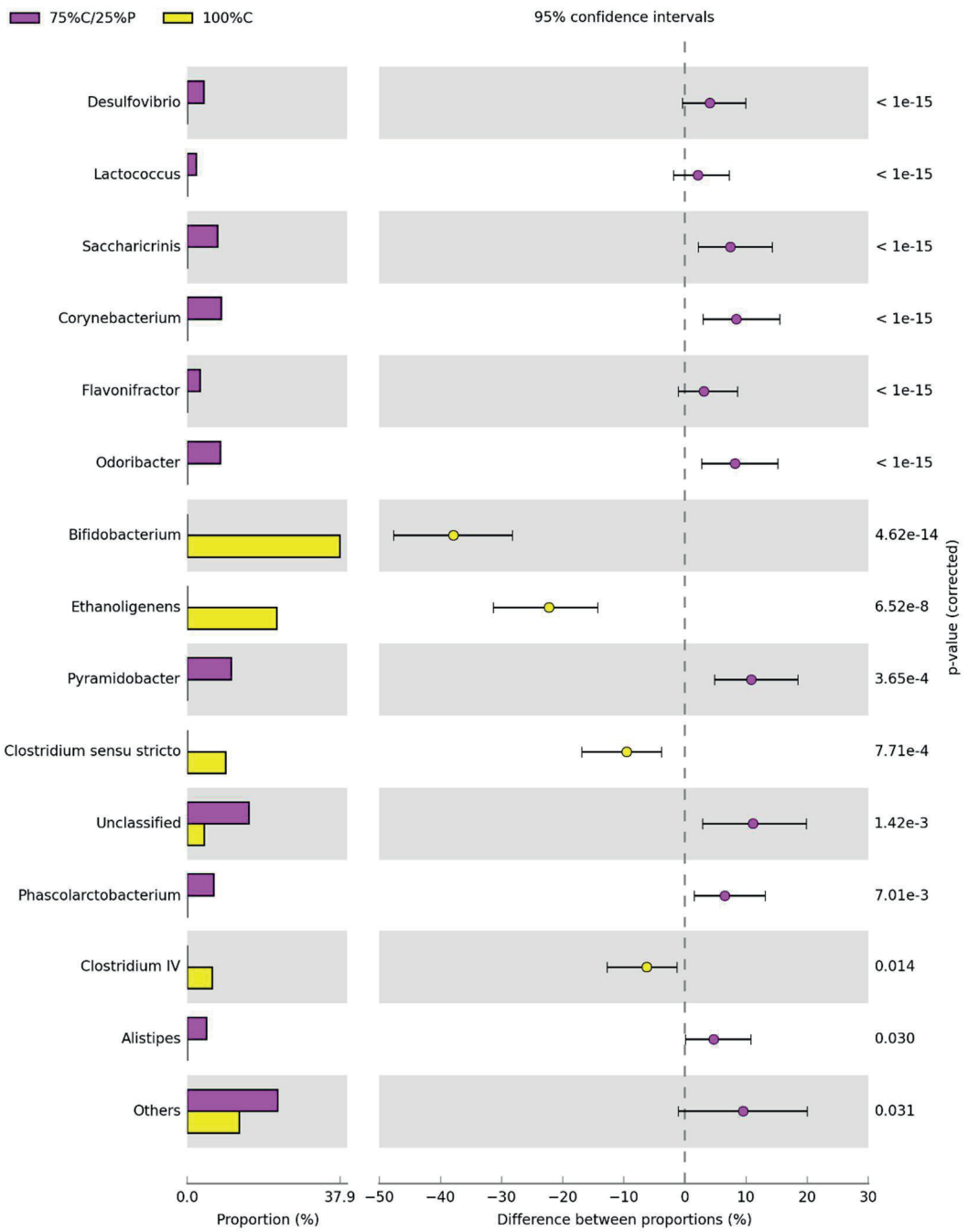

Figure S6.2h. Fisher's exact test for comparison of bacterial communities' relative abundance at genus level parameters for the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}.

List of abbreviations

C	Carbohydrate
CA	Carboxylic Acid
CoA	Coenzyme A
COD	Chemical Oxygen Demand
EA	Electron Acceptor
ED	Electron Donor
EMP	Embden-Meyerhof pathway
ETF	Electron Transfer Flavoprotein
ETFH	Reduced Electron Transfer Flavoprotein
FAB	Fatty acid biosynthesis
FabG	3-ketoacyl-CoA reductase
Fd_{ox}	Oxidized Ferredoxin
Fd_{red}	Reduced Ferredoxin
HAc	Acetic acid
HBt	Butyric acid
HPr	Propionic acid
HRT	Hydraulic Retention Time
HV	Valeric acid
LCFA	Long Chain Fatty Acid
MCCA	Medium Chain Carboxylic Acid
mcl-PHA	Medium Chain Length Polyhydroxyalkanoate
MMC	Mixed Microbial Cultures
OFMSW	Organic Fraction of Municipal Solid Waste
OLRs	Specific Organic Loading Rate
OLRv	Volumetric Organic Loading Rate
P	Protein
PHA	Polyhydroxyalkanoate
PhaA	β-ketothiolase
PhaB	NADPH-dependent reductase
PhaC	PHA synthase
PhaG	3-Hydroxyacyl-ACP-CoA transferase
PhaJ	(R)-enoyl-CoA hydratase/enoyl-CoA hydratase I
PhaZ	PHA depolymerase
PHB	Polyhydroxybutyrate
PHB-co-PHV	Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)
PK	Phosphoketolase pathway
PPP	Pentose Phosphate pathway
RBO	Reverse β-oxidation
SBR	Sequencing Batch Reactor

SCCA	Short Chain Carboxylic Acid
SCFA	Short Chain Fatty Acid
scl-PHA	Short Chain Length Polyhydroxyalkanoate
SRT	Sludge Retention Time
VFA	Volatile Fatty Acid
VS	Volatile Solids
VSS	Volatile Suspended Solids
WAS	Waste Activated Sludge
WWTP	Wastewater Treatment Plant
Yobs	Observed Growth Yield
3HB	3-Hydroxybutyrate
3HHx	3-Hydroxyhexanoate
3H2MB	3-Hydroxy-2-methylbutyrate
3H2MV	3-Hydroxy-2-methylvalerate
3HV	3-Hydroxyvalerate

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of 12 using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S4.10. Shannon index and p values for t-test statistical comparison of the bacterial diversity under the effect of the different influent carbon-to-nitrogen ratios tested (12 and 25) at an OLRv of approximately 16 g COD L^{-1} d^{-1} and at an OLRs of approximately 3.5 g COD g^{-1} VSS d^{-1} using food-grade sugar as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S5.1. Effluent composition (mg O_2 L^{-1}) in the different conditions tested in the reactor when using whey protein isolate as sole carbon source. ND: Non-Detectable.

Table S5.2. ANOVA test and Tukey's multiple comparisons test for comparing the resultant process effectiveness parameters at the different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S5.3. t-test for comparing the resultant process effectiveness parameters at the different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S5.4. ANOVA test and Tukey's multiple comparisons test for comparing the effluent composition obtained at the different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S5.5. t-test for comparing the effluent composition obtained at the different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S5.6. Shannon index and p values for t-test statistical comparison of the bacterial diversity under the effect of the different OLRv tested (6.5, 12.5 and 18.5 g COD L^{-1} d^{-1}) at an OLRs of approximately 1.9 g COD g^{-1} VSS d^{-1} using whey protein isolate as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S5.7. Shannon index and p values for t-test statistical comparison of the bacterial diversity under the effect of the different OLRs tested (1.9 and 3.3 g COD g^{-1} VSS d^{-1}) at an OLRv of approximately 18.5 g COD L^{-1} d^{-1} using whey protein isolate as sole carbon source. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S6.1. Effluent composition (% COD) yielded for the different substrate organic compositions tested at an OLRv of 6 g COD L^{-1} d^{-1} and an OLRs of 2 g COD g^{-1} VSS d^{-1}.

Table S6.2. ANOVA test and Tukey's multiple comparisons test for comparing the resultant acidogenic fermentation effectiveness parameters for the different influent

organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.

Table S6.3a-b. ANOVA test and Tukey's multiple comparisons test for the different effluent organic components proportion (%COD) obtained with the different influent organic compositions tested (100%P, 25%C/75%P, 50%C/50%P, 75%C/25%P and 100%C) at an OLRv around 6 g COD L^{-1} d^{-1}) and an OLRs around 2 g COD g^{-1} VSS d^{-1}. P-values highlighted in orange indicate no significant difference and p-values highlighted in green denote significant difference.